Front. Agric. China 2009, 3(3): 279–283
DOI 10.1007/s11703-009-0060-8

RESEARCH ARTICLE

Jingyan SUN, Weiming LI, Hanshuang ZHANG, Junli ZHAO, Xiaolin YIN, Li'an WANG

Somatic embryogenesis and plant regeneration in glandless upland cotton (*Gossypium hirsutum* L.)

© Higher Education Press and Springer-Verlag 2009

Abstract Glandless upland cotton has an important economic value. Embryogenic calli and regenerated plants were obtained from the hypocotyl explants of glandless upland cotton seedlings, cultivar Jisheng1. The results indicated that somatic embryogenesis was significantly influenced by the types of auxin and cytokinin. 2, 4-D was advantageous to induce cotton callus, but embryogenic callus could not be obtained on the 2, 4-D medium. Embryogenic calli were also not obtained on the MSB sold medium with the combination of IBA and BA. However, embryogenic calli were induced when the hypocotyl explants were cultured on the IBA and KT medium. More than 31% of the hypocotyl segments produced embryogenic calli when the MSB medium was supplemented with 1.0 mg·L^{-1} IBA and 0.5 mg·L^{-1} KT. Embryogenic calli with somatic embryos could be observed within three months. Somatic embryo germination and maturation occurred on the hormone-free MSB medium with 1.0 g·L^{-1} Gln and 0.5 g·L^{-1} Asn. A number of regenerated plants could be obtained in six months. In the present study, a simple and efficient system was established to induce a number of embryogenic calli and regenerate plantlets from hypocotyl explants.

Keywords cotton (*Gossypium hirsutum* L.), glandless, somatic embryogenesis, plant regeneration

Received January 9, 2009; accepted March 29, 2009

Jingyan SUN, Weiming LI (✉), Hanshuang ZHANG, Junli ZHAO, Xiaolin YIN
Institute of Cotton, Hebei Academy of Agriculture and Forestry Sciences, Shijiazhuang 050051, China
E-mail: li_wyman@yahoo.com

Li'an WANG
College of Life Science, Hebei Normal University, Shijiazhuang 050016, China

1 Introduction

Cotton is an important commercial crop, playing a vital role in the economic, political and social affairs of the world. 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, little genetic diversity exists for further improvement. However, gene transformation techniques have provided a molecular approach which is to widen the genetic diversity of crop plants through the stable expression of foreign genes obtained from divergent sources, including bacteria. The introduction of foreign genes into cotton through either agrobacterium or abiotic transformation involves the development of an efficient regeneration system from the transformed host tissues. Regeneration through somatic embryogenesis is preferred over organogenesis because of the probable single-cell origin of the somatic embryos, thus reducing the chimeric transformation events. However, highly efficient *in vitro* techniques required for the regeneration of large numbers of cotton plantlets are limited when compared to other major commercial crops. Somatic embryogenesis in cotton (*Gossypium klotzchianum*) was first reported in 1979, although complete plants could not be regenerated (Price and Smith, 1979). Subsequently, plantlet regeneration was obtained from a 2-year-old callus culture of *G. hirsutum* var. Coker 310 through somatic embryogenesis (Davidonis and Hamilton, 1983). Regenerated plantlets were also obtained through somatic embryogenesis in Coker312 (Chen et al., 1987). Since then, plantlet regeneration via somatic embryogenesis has rapidly developed and the regeneration system has been established in some labs. However, these procedures involve a lengthy culture period and are difficult to repeat, and cotton regeneration via somatic embryogenesis is highly genotype-specific. Despite the fact that glandless upland cotton has great economic value, there are still few reports so far on tissue culture of glandless upland cotton (Li et al., 1996; Cai et al., 1997; Li et al., 2008). However,

calli were obtained from glandless upland cotton 89-30 hypocotyl explants. Also, embryogenic calli were obtained by several subcultures after one year (Li et al., 1996). Subsequently, a high frequency tissue regeneration strain Jisheng1 was obtained from 89-30 by regeneration and field breeding recurrent selection (Li et al., 2008). However, these procedures involved a complex process, a lengthy culture period, high cost and repetition difficulty, and hence it did not facilitate cotton genetic engineering which is the basis of regeneration via somatic embryogenesis.

The objective of this study was to develop a glandless upland cotton regeneration system via somatic embryogenesis, which can be utilized for genetic transformation and create variations. We report a simple and efficient method of inducing embryogenic callus and development of somatic embryos and plantlets within 5–6 months. This model is based on the variety Jisheng1 of previous studies.

2 Materials and methods

2.1 Materials

The seeds of glandless upland cotton variety Jisheng1 were provided by the Institute of Cotton, Hebei Academy of Agriculture and Forestry Sciences, Shijiazhuang, China. Delinted seeds were surface sterilized with 0.1% $HgCl_2$ for 30 min. Then the surface-sterilized seeds were thoroughly washed with sterile distilled water and soaked overnight for softening seed coats. The seeds were germinated on 1/5 strength MS medium.

2.2 Methods

2.2.1 Induction of embryogenic callus

Hypocotyl explants, 4–6 mm in length and excised from 5- to 7-day-old seedlings, were used for inducing callus. In order to evaluate the effect of the hormones on the embryogenic callus induction, hypocotyl segments were inoculated onto the MSB (MS salts with B_5 vitamins, 30% glucose and 0.6% agar, pH 5.8) solid medium supplemented with the combination of different types and concentrations of hormones. 120 hypocotyl segments were raised for each treatment. The calli derived from hypocotyl segments in each treatment were subcultured on new media for 30 d. The number of hypocotyl segments producing callus, the weight of induced callus and the color and texture of induced callus were recorded after 30 d of culture. The number of hypocotyl segments producing embryogenic callus were recorded after 90 d of culture. The induction rate of callus (IRC) was the percentage of hypocotyl segments producing callus in total 120 hypocotyl segments after 30 d of culture. The weight of induced callus was the total weight of 120 hypocotyl segments after 30 d of culture. The induction rate of embryogenic callus (IREC) was the percentage of hypocotyl segments producing embryogenic callus in total 120 hypocotyl segments after 90 d of culture.

The treatments of hormone combinations and measures for inducting embryogenic callus were the following:

Treatment 1: Hypocotyl segments were cultured on the MSB solid medium supplemented with 0.1 $mg \cdot L^{-1}$ 2, 4-D, 0.1 $mg \cdot L^{-1}$ IAA and 0.1 $mg \cdot L^{-1}$ KT to induce callus for one month. Then the calli were subcultured on the same medium for another two months.

Treatment 2: Hypocotyl segments were cultured on the MSB solid medium supplemented with 0.1 $mg \cdot L^{-1}$ 2, 4-D, 0.1 $mg \cdot L^{-1}$ IAA and 0.1 $mg \cdot L^{-1}$ KT to induce callus for one month. Then the calli were subcultured on the MSB solid medium supplemented with 0.05 $mg \cdot L^{-1}$ 2, 4-D, 0.1 $mg \cdot L^{-1}$ IAA and 0.1 $mg \cdot L^{-1}$ KT in the second month. Finally, the calli were subcultured on the MSB solid medium supplemented with 0.1 $mg \cdot L^{-1}$ IAA and 0.1 $mg \cdot L^{-1}$ KT in the third month.

Treatment 3: Hypocotyl segments were cultured on the MSB solid medium supplemented with 0.5 $mg \cdot L^{-1}$ IBA and 0.5 $mg \cdot L^{-1}$ BA to induce callus for one month. Then the calli were subcultured on the same medium for another two months.

Treatment 4: Hypocotyl segments were cultured on the MSB solid medium supplemented with 0.5 $mg \cdot L^{-1}$ IBA and 0.5 $mg \cdot L^{-1}$ KT to induce callus for one month. Then the calli were subcultured on the same medium for another two months.

Treatment 5: Hypocotyl segments were cultured on the MSB solid medium supplemented with 0.1 $mg \cdot L^{-1}$ 2, 4-D, 0.1 $mg \cdot L^{-1}$ IAA and 0.1 $mg \cdot L^{-1}$ KT to induce callus for one month. Then the calli were subcultured on the MSB solid medium supplemented with 0.5 $mg \cdot L^{-1}$ IBA and 0.5 $mg \cdot L^{-1}$ KT for another two months.

Optimized concentrations of hormones in treatment 4 to induce a large number of embryogenic calli were:

MSB1: MSB + 1.0 $mg \cdot L^{-1}$ IBA + 0.5 $mg \cdot L^{-1}$ KT,
MSB2: MSB + 1.0 $mg \cdot L^{-1}$ IBA + 0.1 $mg \cdot L^{-1}$ KT,
MSB3: MSB + 0.5 $mg \cdot L^{-1}$ IBA + 0.5 $mg \cdot L^{-1}$ KT,
MSB4: MSB + 0.5 $mg \cdot L^{-1}$ IBA + 0.1 $mg \cdot L^{-1}$ KT.

2.2.2 Proliferation of embryogenic callus

Embryogenic calli were proliferated on the MSB solid medium supplemented with 1.0 $mg \cdot L^{-1}$ IBA and 0.5 $mg \cdot L^{-1}$ KT by subculturing ever 4 weeks. As the embryogenic callus proliferated, the subcultured embryogenic calli could develop into somatic embryos at any time.

2.2.3 Maturation of somatic embryos and acclimatization of plantlets

The effects of Gln and Asn on the germination and maturation of somatic embryos were studied. Somatic

embryo germination media supplemented with different concentrations of Gln and Asn were as follows:

Medium1: MSB,

Medium2: MSB + 0.5 $g \cdot L^{-1}$ Gln + 0.5 $g \cdot L^{-1}$ Asn,

Medium3: MSB + 0.5 $g \cdot L^{-1}$ Gln + 1.0 $g \cdot L^{-1}$ Asn,

Medium4: MSB + 1.0 $g \cdot L^{-1}$ Gln + 0.5 $g \cdot L^{-1}$ Asn,

Medium5: MSB + 1.0 $g \cdot L^{-1}$ Gln + 1.0 $g \cdot L^{-1}$ Asn.

When leaves appeared on the somatic embryos, they were transferred onto the MSB solid medium supplemented with 3.0 $mg \cdot L^{-1}$ IBA for roots induction. Then the plantlets with well-developed roots and shoots were transferred to soil and cultured for 2–3 weeks at room temperature. Finally, they were transplanted into field.

2.2.4 Culture conditions

Each culture was maintained at 28±2°C under a 16/8-h (day/night) photoperiod with light supplied at an intensity of 2000 lx.

2.2.5 Statistical analysis

All analyses were performed with DPS statistical software, Duncan's new multiple range test was used to test significance.

3 Results

3.1 Induction of embryogenic callus

2, 4-D + IAA + KT combination is commonly used to induce callus in cotton. By inoculating the hypocotyl segments on the MSB solid medium supplemented with 0.1 $mg \cdot L^{-1}$ 2, 4-D, 0.1 $mg \cdot L^{-1}$ IAA and 0.1 $mg \cdot L^{-1}$ KT, calli were rapidly induced and developed after one week of culture. After one month, calli grew on the whole segments and the induction rate of callus reached 100%, but the calli were soft and unfriable (Fig. 1(a)). After two subcultures on the same medium, embryogenic callus could not be observed. After subculturing the calli on the gradually decreasing concentrations of 2, 4-D medium for two months, no embryogenic callus was observed.

IBA and BA are popular hormones used to induce callus. When hypocotyl segments were cultured on the MSB medium supplemented with 0.5 $mg \cdot L^{-1}$ IBA and 0.5 $mg \cdot L^{-1}$ BA for one month, dark hard compact calli were induced (Fig. 1(b)), but no embryogenic callus was observed after subculturing on the same medium even after another two months.

The IBA + KT combination is a seldom used combination for inducing callus in cotton. Hypocotyl segments inoculated on the MSB medium supplemented with 0.5 $mg \cdot L^{-1}$ IBA and 0.5 $mg \cdot L^{-1}$ KT formed calli only at the cortex of hypocotyl segments (Fig. 1(c)), and IRC was 55.8% (Table 1). However, after three months of culture, a large number of yellow friable embryogenic calli were obtained (Fig. 1(d)), and IREC reached 31.7% (Table 1), and then somatic embryos germinated to different step embryos (Fig. 1(e)).

Because the callus induction rate and the weight of callus at IBA + KT combination were much lower than that of 2, 4-D + IAA + KT, the calli which were induced on the medium supplemented with 2, 4-D, IAA and KT were transferred onto the medium supplemented with

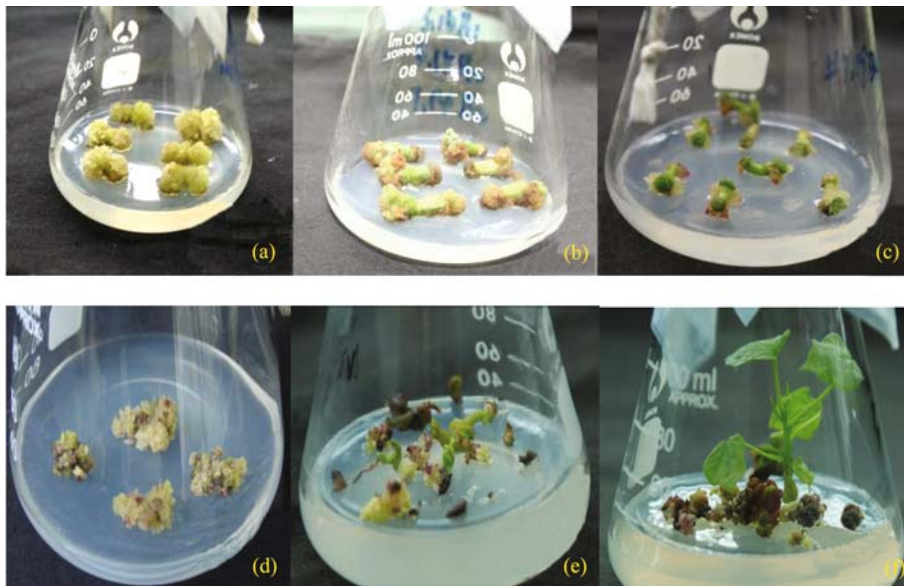

Fig. 1 Somatic embryogenesis of the glandless upland cotton

Note: (a)–(f) represent callus induced on the medium with the combination of 2,4-D, IAA and KT, callus induced on the medium with the combination of IBA and BA, callus induced on the medium with the combination of IBA and KT, embryogenic callus, the germination of somatic embryos and regenerated plants of Jisheng1, respectively.

Table 1 Effects of hormone combinations and measures on the induction of embryogenic callus

treatment	NHSPC	IRC/%	weight of callus/g	NHSPEC	IREC/%	color and texture of callus
1	120a	100a	53.8a	0b	0b	white, soft unfriable
2	120a	100a	53.5a	0b	0b	white, soft unfriable
3	100b	83.3b	28.2b	0b	0b	dark, hard compact
4	67c	55.8c	13.5c	38a	31.7a	yellowish, friable
5	120a	100a	53.7a	0b	0b	white, hard unfriable

Note: NHSPC, NHSPEC, IRC and IREC represent No. of hypocotyl segments producing callus, No. of hypocotyl segments producing embryogenic callus, the induction rate of callus, and the induction rate of embryogenic callus, respectively. Means followed by different letters in a column represent significance at 0.01 probability level according to SSR.

0.5 $mg \cdot L^{-1}$ IBA and 0.5 $mg \cdot L^{-1}$ KT for another two months, but no yellowish friable callus was observed.

3.2 Optimizing the concentrations of hormones

To optimize the embryogenic callus induction medium for Jisheng1, four combinations of IBA and KT in basal MSB solid medium were evaluated to determine the best hormone concentrations. When the hypocotyl segments were cultured on the MSB solid medium supplemented with 1.0 $mg \cdot L^{-1}$ or 0.5 $mg \cdot L^{-1}$ IBA, yellow friable calli were induced. This kind of callus could develop to different steps of embryos. Low concentration of KT could increase the induction effect. The induction rate of callus and embryogenic callus increased continuously as the concentration of KT increased, when the concentration of IBA was a constant (Fig. 2). As a result, a large number of embryogenic calli were induced from the Jisheng1 hypocotyl segments which were cultured on the MSB1 solid medium after three months of culture.

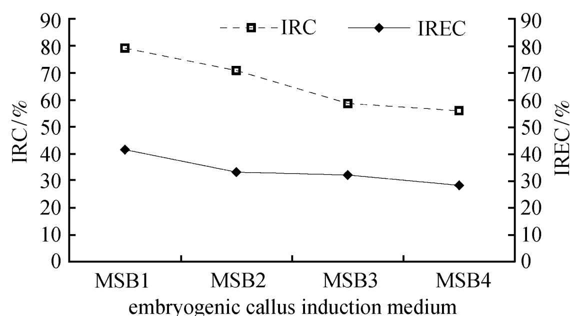

Fig. 2 Effect of the concentrations of the IBA and KT on the induction rate of callus and embryogenic callus

3.3 Somatic embryo germination and plantlet generation

After being transferred onto hormone-free MSB solid medium, embryogenic calli induced from Jisheng1 hypocotyl segments began to differentiate. Somatic embryos developed from globular embryo, heart-shaped embryo, torpedo-shaped embryo, cotyledonary embryo to plantlets. 1.0 $g \cdot L^{-1}$ Gln and 0.5 $g \cdot L^{-1}$ Asn facilitated embryo germination and maturation (Table 2). A number of regenerated plants were obtained in six months (Fig. 1(f)).

Table 2 The effects of the concentrations of glutamine and asparagine on the germination of embryo

treatment	Gln/($g \cdot L^{-1}$)	Asn/($g \cdot L^{-1}$)	embryo conversion/%
medium 1	0	0	6.3d
medium 2	0.5	0.5	11.8c
medium 3	0.5	1.0	22.6b
medium 4	1.0	0.5	38.5a
medium 5	1.0	1.0	37.9a

Note: Means followed by different letters in a column represent significance at 0.01 probability level according to SSR.

4 Discussion

Although embryogenic calli were obtained from hypocotyl segments after several transfers onto different media, the problems associated with a prolonged culture period and complex culture process remained unsolved. The long-term subculture brought the appearance of chromosomal aberrations in tissue callus and the decrease of embryogenic capability of callus (Sharma and Kumar, 1994). We attempted to find a rapid and efficient protocol of the embryogenic callus induction and plant regeneration from Jisheng1 hypocotyl segments.

Our results indicated that the kind of hormone was an important factor affecting the induction of embryogenic callus from Jisheng1 hypocotyl segments (Wang et al., 2004). Calli containing most of non-embryogenic calli were induced on the 2, 4-D containing medium and few somatic embryos were observed during a prolonged culture period. It is known that 2, 4-D is disadvantageous to somatic embryogenesis (Zhang et al. 1991). On the 2, 4-D containing medium, calli quickly grew unrestrained and overly proliferated, which was disadvantageous to the embryogenic callus differentiation. However, when the concentration of 2, 4-D was gradually decreased in the medium, embryogenic calli were observed after several subcultures on the medium (Yu et al., 2004). Other researchers have found that embryogenic callus were also obtained after the calli which were induced on the 2, 4-D containing medium were transferred onto the IAA and KT

containing media (Guo et al., 1996; Cai et al., 1997). These investigators obtained embryogenic callus by changing the kinds and concentrations of hormone in the medium. However, we did not obtain the embryogenic callus after the calli induced on the 2, 4-D containing medium were transferred onto the medium with decreasing 2, 4-D concentration and onto the IBA and KT medium for two months of subcultures.

Embryogenic calli and non-embryogenic calli form from different parts of explants. Embryogenic callus are derived from the cortex cells and non-embryogenic callus are derived from the vascular bundle cells and marrow cells. It means embryogenic callus are formed from the parts facilitating de-differentiation while the non-embryogenic callus are formed from the parts which do not facilitate de-differentiation (Zhang and Guo, 1996). 2, 4-D is the most effective hormone causing tissues to de-differentiate. It is advantageous to induce callus, but it is disadvantageous to somatic embryogenesis (Dong et al., 1993; Zhang et al., 1993; Wang et al., 2002). We presume that a large number of non-embryogenic calli were induced from the vascular bundle cells and marrow cells which were difficult to de-differentiate and a small number of embryogenic calli were induced from the cortex cells, when hypocotyl segments were cultured on the 2, 4-D containing medium. The rapid proliferation of non-embryogenic callus inhibited the proliferation of embryogenic callus. It was necessary to adjust the kind of hormone in the medium to inhibit the proliferation of non-embryogenic callus and to advance the proliferation of embryogenic callus. After adjusting the kind of hormone and selective subculture, embryogenic calli were obtained (Wu et al., 1988). However, when cultured on the IBA and KT containing media for three months without adjusting the concentration and kind of hormone, the Jisheng1 hypocotyl segments produced embryogenic calli and somatic embryos. It was presumed the vascular bundle cells and marrow cells did not de-differentiate and the cortex cells produced embryogenic callus on the IBA and KT containing medium (Li et al., 2006). The mechanism of the IBA and KT containing medium on which the hypocotyl segments could rapidly and efficiently produce embryogenic callus will be further studied.

A large number of embryogenic calli were induced after glandless upland cotton Jisheng1 hypocotyl segments were cultured on the MSB solid medium supplemented with 1.0 $mg \cdot L^{-1}$ IBA and 0.5 $mg \cdot L^{-1}$ KT for three months. Somatic embryos were obtained when the embryogenic calli were transferred onto the MSB solid medium supplemented with 1.0 $g \cdot L^{-1}$ Gln and 0.5 $g \cdot L^{-1}$ Asn. In the present study, we demonstrate a simple and efficient system to induce a number of embryogenic calli and regenerate plantlets from hypocotyl explants within 6 months of culture.

Acknowledgements This research was supported by the Natural Science Foundation of Hebei Province, China (No. C2007001054).

References

Cai X N, Wu J Y, She J M (1997). Embryogenic callus induction and plant regeneration in upland cotton. Jiangsu Journal of Agricultural Sciences, 13(4): 225–230 (in Chinese)

Chen Z X, Li S J, Trolinder N L, Goodin J R (1987). Some characteristics of somatic embryogenesis and plant regeneration in cotton cell suspension culture. Sci Agric Sin, 20(5): 6–11 (in Chinese)

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

Dong H Z, Jiao G L, Chen Z X (1993). The effect of 2, 4-D and KT on callus initiation and somatic embryogenesis in cotton culture. Acta Agriculturac Boreali-Sinica, 8(3): 87–91 (in Chinese)

Guo S G, Li S J, Hu Y X (1996). Tissue culture and plant regeneration of elite cotton species. Journal of Henan University, 26(4): 77–80 (in Chinese)

Li G D, Xiao J L, Luo X L (2006). Somatic embryogenesis and plant regeneration of calli derived from cotton (*Gossypium hirsutum* L.) depending on their quality. Journal of Shanxi Agricultural Sciences, 34(1): 29–31 (in Chinese)

Li J L, Zhang H S, Gao P, Wang H B (1996). Embryogenesis and plant regeneration from somatic cells culture of glandless upland cotton. Cotton Science, 8(4): 223 (in Chinese)

Li W M, Zhang H S, Zhao J L, Sun J Y (2008). Breeding and simple-rapid regeneration protocol for Jisheng1 with glandless trait and high-frequency somatic embryo production ability. Cotton Science, 20 (Suppl): 95

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

Sharma A, Kumar A (1994). Somatic embryogenic and plant regeneration from leaf-derived cell suspension of a mature tree: *Thevetia peruviana* L. Plant Cell Rep, 14: 171–174

Wang L Q, Liu F, Zhou Y (2002). Direct somatic embryogenesis and plant regeneration from cotton tissue culture. Cotton Science, 14(6): 340–343 (in Chinese)

Wang L Q, Wang M, Shi H R (2004). Hormones regulation on cotton somatic embryogenesis. Letters in Biotechnology, 65(4): 577–579 (in Chinese)

Wu J Y, Chen W P, Yin S Z (1988). Effect of exogenous and successive selection on the ability of embryogenesis from long-term culture of *G. davidsonii* Kell. Jiangsu Journal of Aricultural Sciences, 4(2): 9–15 (in Chinese)

Yu Y, Liu C L, Ma Z Y (2004). Embryogenic callus induction and plant regeneration from “CCRI 24”. Acta Botanica Boreali-Occidentalia Science, 24(2): 306–310 (in Chinese)

Zhang B H, Guo T L (1996). Studies on cytology and histology of cotton tissue culture. Journal of Hubei Agricultural College, 16(1): 58–66 (in Chinese)

Zhang X L, Sun J Z, Liu J L (1991). Somatic embryogenesis and plant regeneration in upland cotton. Journal of Genetics and Genomics, 18 (5): 461–467 (in Chinese)

Zhang X L, Zhang J M, Yao M J (1993). Studies on somatic cell culture and its application in cotton. Journal of Huazhong Agricultural University, 12(5): 421–426 (in Chinese)