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High frequency plant regeneration from protoplasts in cotton *via* somatic embryogenesis

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Abstract

A highly reproducible system for efficient plant regeneration from protoplast *via* somatic embryogenesis was developed in cotton (*Gossypium hirsutum* L.) cultivar ZDM-3. Embryogenic callus, somatic embryos and suspension culture cells were used as explants. Callus-forming frequency (82.86 %) was obtained in protoplast cultures from suspension culture cells in KM_8P medium with 0.45 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.93 μM kinetin (KIN), 1.5 % glucose and 1.5 % maltose. Protocolonies formed in two months with plating efficiency of 14 %. However, the callus-forming efficiencies from other two explants were low. The calli from protoplast culture were transferred to somatic embryo induction medium and 12.7 % of normal plantlets were obtained on medium contained 3 % maltose or 1 % of each sucrose + maltose + glucose, 2.46 μM indole-3-butyric acid (IBA) and 0.93 μM KIN. Over 100 plantlets were obtained from protoplasts derived from three explants. The regenerated plants were transferred to the soil and the highest survival rate (95 %) was observed in transplanting *via* a new method.

Additional key words: *ex vitro* transplantation, *Gossypium hirsutum* L., plant growth regulation, sugar combinations.

Introduction

Plant regeneration from protoplasts has proved to be a very useful technique for crop genetic improvement programs, genetic transformation and somatic hybridization. There were some reports on the cotton regeneration from protoplast culture (El-Shihy and Evans 1986, Chen *et al*. 1989, Lü *et al*. 1999, Peeters *et al*. 1994, She *et al*. 1989, 1991). Recently, fertile plants regenerated from protoplast of six explants in *Gossypium hirsutum* L. cv. Coker 201 had been reported by Sun *et al*. (2005b), but the protoplast division and plating efficiency was low. Sun *et al*. (2005a) firstly described the plants obtained from protoplasts in wild cotton *G. klotzschianum,* but the frequency of plant regeneration was also low. Therefore, an effective and simple protocol for high frequency plant regeneration in cotton from protoplasts is still in need.

Cotton regeneration *via* somatic embryogenesis is highly genotype-dependent (Mishra *et al*. 2003). Most lines developed from the *G. hirsutum* cv. Coker 312 (Trolinder and Xhixian1989) were able to regenerate, and cv. YZ1 exhibited even higher regeneration potential than cvs. Coker 312 and Coker 201 and maintained it after long-term cultures (Jin *et al*. 2006). An upland cotton cv. ZDM-3 is a cotton cultivar with outstanding agronomic and economic characters. In addition, ZDM-3 exhibits much higher regeneration potential in somatic culture than Coker 201, although the subculture period was longer. The present paper describes techniques for high frequency plant regeneration from protoplasts isolated from *G. hirsutum* L. cv. ZDM-3 that might be useful for somatic hybridization and genetic transformation in cotton.

Materials and methods

Plants and growth conditions: Embryogenic callus was induced from 10 mm long hypocotyl sections from sterilized plantlets of upland cotton *G. hirsutum* L. cv. ZDM-3 on MSB medium [MS mineral salts (Murashige

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; IBA - indole-3-butyric acid; KIN - kinetin; NAA - α-naphthaleneacetic acid.
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and Skoog 1962) plus B_5 vitamins (Gamborg *et al*. 1968)] supplemented with 4.92 μM indole-3-butyric acid (IBA), 0.465 μM kinetin (KIN), 0.555 mM myo-inositol, 0.25 % (m/v) *Phytagel*, 3 % (m/v) glucose. Embryogenic callus subcultured on MSB medium supplemented with 2.46 μM IBA, 0.465 μM KIN, 0.555 mM myo-inositol, 6.8 mM glutamine, 3.8 mM asparagine, and 0.25 % *Phytagel*, 3.0 % glucose. With callus subculturing, many somatic embryos are formed and used for protoplast isolation.

Embryogenic suspension cultures, embryogenic callus and immature somatic embryos were prepared for protoplast isolation. The procedure of establishing suspension cultures has been described by Sun *et al*. (2003).

Callus induction and protoplast culture were carried out at temperature of 28 °C and 14-h photoperiod with irradiance of 50 - 60 μmol m^{-2} s^{-1} provided by daylight fluorescent lamps.

Protoplast isolation and culture: The process of protoplast isolation and protoplast viability control has been explained by Sun *et al*. (2004, 2005a). Enzyme solution for protoplast isolation was 3 % (m/v) cellulase (*Onozuka R-10*), 1.5 % (m/v) pectinase and 0.5 % (m/v) hemicellulase dissolved in cell and protoplast washing solution (CPW9) (Srinivasan *et al*. 1986).

Purified protoplasts were suspended and cultured in KM_8P liquid medium in 60 mm diameter plates at 2×10^5 cell cm^{-3}. The modified KM_8P medium was supplemented with three combinations of plant growth regulators (PGR) that is 0.45 μM 2,4-dichlorophenoxyacetic acid (2,4-D) + 0.93 μM KIN, 5.37 μM α-naphthaleneacetic acid (NAA) + 0.93 μM KIN and 2.69 μM NAA + 0.23 μM 2,4-D + 0.46 μM KIN+ 2.28 μM zeatin (ZT) (Table 1). Different sugars were used in the KM_8P medium (Fig. 1) namely, 3.0 % glucose (G) or maltose (M) or sucrose (S) or 1.5 % maltose + 1.5 % glucose (MG) or 1.5 % maltose + 1.5 % sucrose (SM) or 1.5 % glucose + 1.5 % sucrose (SG), or 1.0 % maltose + 1.0 % sucrose + 1.0 % glucose (SMG).

Protoplasts culture in the KM_8P medium was a thin liquid layer culture according to Sun *et al*. (2005a). After callus formed in protoplast culture, the "proto-calli" were transferred to somatic embryo induction medium (MSB medium having 18.791 mM KNO_3, 6.8 mM glutamine, 3.8 mM asparagine, 0.555 mM myo-inositol, 0.25 % *Phytagel*, 3 % glucose without NH_4NO_3). After two months, plating efficiency and callus-forming frequency were counted. The plating efficiency was the frequency of protoplasts that continued to divide and form cell group related to the total number of protoplasts at the same visual field of microscope. Callus-forming frequency was described as frequency of plates of callus-forming to all plates of protoplast culture.

Somatic embryos (globular, hearted-, torpedo-shaped embryos) easily formed from embryogenic calli after 3 - 4 weeks when proto-calli were transferred to somatic embryo induction medium. These embryos were collected from embryogenic calli and subcultured every three weeks on the same medium supplemented with different combinations of sugars (Table 3) for germination and development into plantlets. After two months on MSB medium with different sugars, the frequencies of conversion of somatic embryos into normal and abnormal plantlets and abnormal embryos were calculated.

In the study, 10 cultures were raised for each treatment, and all treatments were repeated more than three times. The data mean ± SD (standard deviation) was analyzed by Duncan's multiple range tests using the SAS program.

Transplantation of regenerated plants: Four methods were developed for transferring the regenerated plants and survival frequencys were counted after 7 d:

1) Direct transplantation: The regenerated plants with well developed roots and 4 - 5 true leaves on the MSB medium were directly transferred to the soil.

2) Grafting 1: Plantlets with two true leaves of local cultivar (*G. hirsutum* cv. ZDM-3) were planted in soil as the rootstocks and the regenerated plantlets were used as the scions. The scion was taken out from flask and its stem was cut into cuniform. The cuniform side of scion was inserted into the cleaved stock stem then bound with *Parafilm*. The grafted plants were cultured in the greenhouse (22 - 28 °C，80 % humidity) for 4 weeks before they were transplanted into soil.

3) Grafting 2: The buds of the regenerated plantlets were grafted into the sterile plants grown in the flasks from seeds. When the grafted plants had 4 to 6 true leaves, they were directly transplanted into the soil as in the first method.

4) Grafting 3: The protocol was almost same as Grafting 1 but the plantlets at the cotyledon stage in soil plots were used as rootstocks. Before grafting, the terminal bud of the rootstock was cutoff and its stem was split between two cotyledons. The regenerated plants in the flasks were cut to cuniform and grafted onto the rootstock with cotyledons then bound with *Parafilm* as well.

Results and discussion

The present study was focused on improving protoplast division, plating efficiency, promoting somatic embryos conversion to plantlets and their survival after transplantation. The three steps were crucial in protoplast culture of cotton, which was improved simultaneously.

The three PGRs combinations and seven sugar combinations had a significant effect on protoplast division and proto-callus formation (Tables 1, 2). The frequencies of cell division in protoplast culture media with 2,4-D + KIN supplemented with seven different combinations of sugars were significantly higher than that in the media with NAA + KIN. For 2,4-D + KIN combination, the frequency of division reached 55 % in average in the medium supplemented with sucrose +

maltose + glucose and the frequency of callus formation was over 70 % in the media supplemented with glucose and maltose + glucose. The effects of NAA + 2,4-D + KIN + ZT to protoplast division and callus formation were medial between that of 2,4-D + KIN and NAA + KIN. The combination of PGRs, NAA + KIN was effective in promoting callus formation in protoplasts from different explants of *G. hirsutum* L. cv. Coker 201 (Sun *et al*. 2005b) while NAA + KIN in the media with different sugars was not as much effective as 2,4-D + KIN in case of ZDM-3. Different sugars supplemented in media had different effects on protoplast culture. In the media with maltose, glucose and maltose+glucose supplemented with 2,4-D + KIN and NAA + 2,4-D + KIN + ZT, the frequency of protoplast division and plating efficiency were significantly enhanced.

Table 1. Influence of different combinations of PGRs and sugars on frequency of protoplast division [%]. Protoplasts were isolated from immature somatic embryos (G - glucose, M - maltose, S - sucrose). Means ± SE, $n = 4$.

	2,4-D+KIN	NAA+KIN	NAA+2,4-D+KIN+ZT
G	45 ± 6.1	34 ± 4.2	46 ± 5.5
M	44 ± 4.2	34 ± 4.2	44 ± 4.2
M+G	47 ± 2.7	42 ± 4.5	46 ± 4.2
S	33 ± 5.7	27 ± 5.7	33 ± 4.5
S+G	52 ± 5.7	43 ± 5.7	42 ± 5.7
S+M	48 ± 5.1	38 ± 5.1	46 ± 3.7
S+M+G	55 ± 7.1	45 ± 3.7	48 ± 2.4

Table 2. Influence of different combinations of PGRs and sugars sources on frequency of callus formation [%]. Protoplasts were isolated from immature somatic embryos (G - glucose, M - maltose, S - sucrose). Means ± SE, $n = 4$.

	2,4-D+KIN	NAA+KIN	NAA+2,4-D+KIN+ZT
G	70 ± 15.9	48 ± 13.0	60 ± 15.8
M	52 ± 13.0	32 ± 8.4	64 ± 11.4
M+G	78 ± 13.0	44 ± 5.5	68 ± 8.4
S	40 ± 7.1	38 ± 8.4	34 ± 8.9
S+G	40 ± 7.1	32 ± 8.4	40 ± 7.1
S+M	52 ± 8.4	46 ± 5.5	50 ± 7.1
S+M+G	56 ± 11.4	48 ± 8.4	52 ± 8.4

The frequencies of callus formation in medium with glucose and maltose + glucose were about 60 % in average with three PGRs, and 70 and 78 % in the media supplemented with 2,4-D + KIN. The effects of six sugar combinations to protoplast division were not significantly different each other, but markedly higher than that of sucrose. Among the three PGRs and seven sugar combinations, the best was medium with 2,4-D + KIN and maltose + glucose and this medium was selected for protoplast culture.

From 6 to 12.8 % of the plating efficiency of cotton protoplast was reported in cv. Coker lines (Chen *et al*. 1989, Peeters *et al*. 1994, Sun *et a*l. 2005b), while by this culture system, the plating efficiencies of the protoplast culture were 14.0, 11.43, and 10.43 % for the explants of suspension culture cells, immature somatic embryos and embryogenic callus, respectively (Table 3) and the frequency of plates of callus-forming reached over 80 % in average. The frequency of plating and callus formation in protoplast culture isolated from suspension culture were significantly higher than those isolated from embryogenic callus.

Table 3. Effects of explants on callus formation and plating efficiency. Protoplasts were cultured in KM_8 medium at the density of 2 ~10×10^5cells cm^{-3} supplemented with 0.45 μM 2,4-D + 0.93 μM KIN and 1.5 % glucose + 1.5 % maltose. Means ± SE, $n = 4$. Means followed by the same letters within a column do not differ significantly at $P < 0.05$ according to Duncan's multiple range test.

Explants	Callus formation [%]	Plating efficiency [%]
Embryogenic callus	64.29 ± 9.76b	10.43 ± 2.94b
Somatic embryos	68.57 ± 10.69b	11.43 ± 2.64ab
Suspension culture	82.86 ± 7.56a	14.00 ± 2.71a

Table 4. Effects of sugars (G - glucose, M - maltose, S - sucrose) on germination of somatic embryos. Medium was supplemented with 2.46 μM IBA + 0.93 μM KIN. Means ± SE, $n = 3$. Means followed by the same letters within a column do not differ significantly at $P < 0.05$ according to Duncan's multiple range test.

	Abnormal embryos	Abnormal plantlets	Normal plantlets
G	62.7 ± 5.03ab	27.3 ± 5.03 a	8.0 ± 2.00c
M	54.7 ± 4.62b	28.7 ± 6.43 a	12.7 ± 3.06a
M+G	57.3 ± 6.11b	28.0 ± 4.00 a	11.3 ± 1.15ab
S	68.7 ± 3.06a	28.0 ± 5.29 a	2.0 ± 2.00d
S+G	54.7 ± 5.03b	32.0 ± 5.29 a	8.0 ± 0.00c
S+M	61.3 ± 6.43ab	28.0 ± 6.00 a	9.3 ± 1.15bc
S+M+G	55.3 ± 7.57b	28.7 ± 4.16 a	12.7 ± 1.15a

The light yellow and white calli from protoplast culture were observed in the plates in 6 - 7 weeks of culture (Fig. 1*B*). When proto-callus was incubated on embryo induction medium with different sugars, light green somatic embryos appeared on the periphery of the protocallus in two weeks (Fig. 1*C*). These somatic embryos were inoculated on the media supplemented with seven sugar treatments for embryo maturity and germination (Table 4). In the medium with sucrose, the frequencies of abnormal embryos and plantlets were high, and the frequency of normal plantlets was lowest among

seven sugar combination, the maltose or sucrose + maltose + glucose were better for somatic embryo germination (Table 4). When the cotyledonary embryos were developed from the somatic embryo, the elongated plumular axis appeared (Fig. 1*D*) and normal plantlets formed afterward. In the media with maltose or sucrose + maltose + glucose, the frequency of normal plantlets was high (12.7 %) while that in the media supplemented with sucrose was only 2 %. However, most of the somatic embryos developed into abnormal plantlets and abnormal embryos (Fig. 1*E*). Many of abnormal plantlets could convert into normal plantlet with new leaves and roots (Fig. 1*F*). The regenerated plantlets were subcultured in the media with maltose for further development (Fig. 1*G*). Then normal regenerated plants were transferred to the root inducing medium to produce roots for 3 - 4 weeks (Fig. 1*H*). The overall regeneration frequency including both abnormal and normal plantlets was 40 %.

It is a key step in cotton tissue culture to transplant the regenerated plants from flasks to the soil. Four methods were employed for transplanting the regenerated plants. The direct transplantation method was simplest among the four methods but survival was lowest (15 %). Grafting 1 was also simple and easy to handle and the survival increased to 26.7 %. Grafting 2 was complicated but the survival ability was the highest (> 95 %; Fig. 1*I*). Grafting 3 was also very easily handled and the survival ability was much higher than that in Grafting 1 (> 90 %; Fig. 1*J*). Over

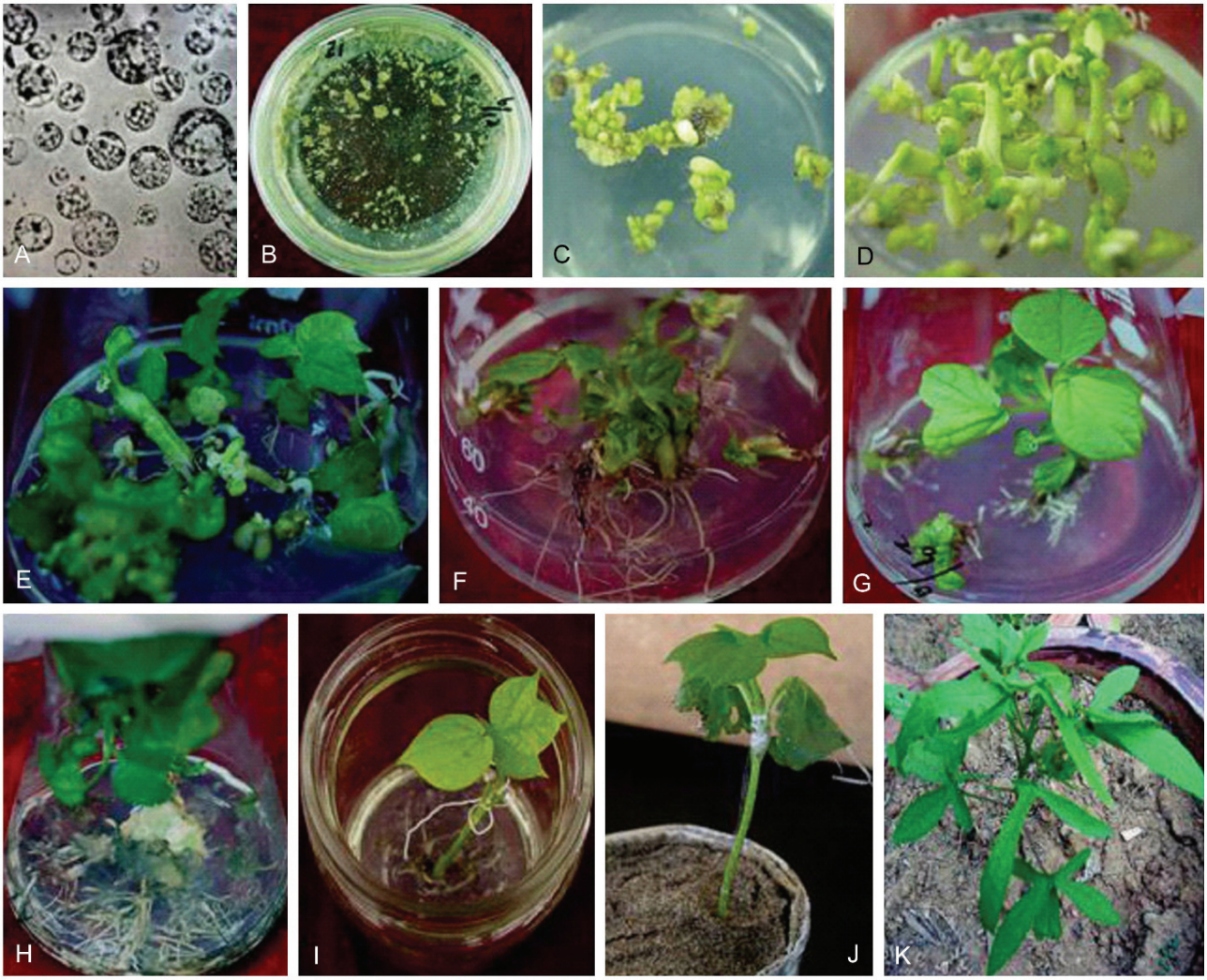

Fig. 1. Plant regeneration from protoplast culture *via* somatic embryogenesis in *G. hirsutum* cv. ZDM-3. *A* - Purified protoplasts isolated from immature somatic embryos of ZDM-3; *B* - proto-calli formed from protoplasts; *C* - Directed somatic embryogenesis from proto-calli on solid medium; *D* - High frequency of somatic embryo maturation and development; *E* - High frequency of somatic embryos germination and plantlets regeneration; *F* - Abnormal plantlets developed normally as well roots and leaves; *G* - Regenerated plant further development; *H* - Regenerated plant with well roots in the rooting medium prepared for directly transplanting; *I* - Normal plant *via* grafting 2 in the flask prepared for transferring to the soil; *J* - Normal plant *via* grafting 3 in the pot; *K* - Plant of ZDM-3 grown in the soil in the greenhouse.

100 plantlets were regenerated from the protoplast-derived calli from three explants and more than 20 normal plants were transplanted successfully in the soil (Fig. 1*K*).

Table 5. Survival after grafting and transplantation *via* four methods.

Methods	Grafting [%]	Transplantation [%]
Direct		15.0
Grafting 1	46.7	26.7
Grafting 2	100.0	95.0
Grafting 3	93.8	93.8

In conclusion, the present investigation reports an efficient and easy-to-handle protocol for high frequency plant regeneration from protoplasts in cotton (*G. hirsutum* L. cv. ZDM-3) via somatic embryogenesis through improvement of three key steps: protoplast division and plating efficiency, somatic embryo conversion to plantlets, and survival after *ex vitro* transplantation. This simple protocol here described would facilitate the development of genetic transformation via protoplast and protoplast fusion (Sun *et al*. 2004, 2005c). This has been employed in our laboratory in somatic hybridization between cultivated and wild species which might develop new materials for cotton breeding.

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