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BRIEF COMMUNICATION

Plant regeneration from *Gossypium davidsonii* protoplasts *via* somatic embryogenesis

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Abstract

Protoplasts isolated from wild cotton *Gossypium davidsonii* were cultured in KM_8P medium supplemented with different phytohormones. The most effective combination was 0.45 μM 2,4-dichlorophenoxyacetic acid, 2.68 μM α-naphthaleneacetic acid and 0.93 μM kinetin and the division percentage at the 8th day was 30.78 ± 3.04 %. The density of protoplasts at 2 - 10 × 10^5 cm^{-3} was suitable for protoplast division and calli formation, with a division percentage of 32.21 ± 3.64 % and a plating efficiency of 9.12 ± 2.61 % at the 40th day. The optimal osmotic potential was achieved using 0.5 M glucose or 0.1 M glucose plus 0.5 M mannitol. Protoplasts were cultured in three ways, a double-layer culture system, with liquid over solid medium was proved to be the best way. Embryo induction was further increased by addition of 0.14 μM gibberellic acid.

Additional key words: cotton, 2,4-dichlorophenoxyacetic acid, gibberellic acid, kinetin, α-naphthaleneacetic acid, protoplast culture.

There are about 45 wild species in *Gossypium* which are regarded as a large reservoir of desirable traits potentially transferable to cultivars, such as resistance to green-sickness, blight, tolerance to drought, and cytoplasm male sterility (CMS). Somatic hybridization might be an ideal way to realize hybridization between different species. Therefore, work was carried out on cotton somatic cell fusion (Sun *et al.* 2004). An effective protoplast culture system is necessary for obtaining somatic hybrids *via* cell fusion. It was reported that the combination of hormones and osmotic potential is the sticking points to many plants (Kao and Seguin-Swartz 1987, Kyozuka *et al.* 1987, Jumin and Nito 1996, Hassanein and Soltan 2000). Until recently, plant regeneration from protoplasts was reported in upland cotton by several researchers (Firrozabady and DeBoer 1986, Chen *et al*. 1989, Peeters *et al.* 1994, Sun *et al.* 2005), and the effect of nitrogen, the combination of hormones, the source of explants and the density of protoplasts on the protoplast regeneration in upland cotton has been evaluated (Bhojwani *et al*. 1977, Saka *et al*. 1987, Chen *et al.* 1989, Sun *et al*. 2005). Unfortunately, only mini-cells-groups or calli were derived from protoplast in some wild cotton species (Finer and Smith 1982, Lu and Xia 1991).

Gossypium davidsonii, a diploid wild cotton, is a potential candidate for somatic hybridization for possessing much great values in genetic and breeding use. To establish an effective system of protoplast regeneration is of great significance. However, there were few references for successful protoplast culture of *G. davidsonii*. Lu *et al*. (1991) reported that mini-cells-groups had derived from protoplast of embryogenic calli and suspension cultures of *G. davidsonii*, but no plant was obtained. This paper describes an effective and simple system for protoplast culture of *G. davidsonii*, and plantlets were recovered *via* suspension culture of callus derived from protoplast.

Embryogenic callus induction and suspension culture establishing were according to Sun *et al.* (2004) and Aydin *et al*. (2004). Embryogenic callus was induced

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Abbreviations: CPW - cell and protoplast washing solution (Frearson *et al.* 1973); 2,4-D - 2,4-dichlorophenoxyacetic acid; FDA - fluorescein diacetate; GA_3 - gibberellic acid; IBA - indole-3-butyric acid; Kn - kinetin; MES - 2-(N-morpholino)ethane sulfonic acid; MSB - MS medium (Murashige and Skoog 1962) and B_5 (Gamborg *et al.* 1968) vitamins; NAA - α-naphthaleneacetic acid.

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from 5- to 7-mm-long hypocotyl sections of *G. davidsonii* on MSB medium [MS inorganic salts (Murashige and Skoog 1962) plus B_5 vitamins (Gamborg *et al.* 1968)] supplemented with 0.90 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 2.32 μM kinetin (Kn), 0.55 mM myo-inositol, 0.25 % (m/v) *Phytagel* and 3 % (m/v) glucose. Embryogenic callus was subcultured on MSB medium supplemented with 2.46 μM indole-3-butyric acid (IBA), 0.70 μM Kn, 0.55 mM myo-inositol, 6.8 mM glutamine, 3.8 mM asparagine, 0.25 % (m/v) *Phytagel*, and 3 % (m/v) glucose. Four weeks after subculture, fresh, compact, gray-green embryogenic calli were transferred into 40 cm^3 liquid MSB medium containing 0.55 mM myo-inositol, 1.36 mM glutamine, 0.76 mM asparagine and 3 % (m/v) glucose in 100 cm^3 Erlenmeyer flasks. The culture flasks were incubated in the dark on a rotary shaker (110 rpm). The suspensions were subcultured once a week. Before protoplast isolation, the suspensions were transferred to liquid MSB medium supplemented with 0.23 μM 2,4-D and 0.93 μM Kn. Three to seven days later, the fresh suspensions were collected for protoplast isolation.

About 1 g of suspension cultures was mixed with 3 cm^3 filter-sterilized enzyme solution in 60 mm diameter plate. The enzyme solution was 2 % (m/v) cellulase *Onozuka R-10* (*Yakult Honsha Co. Ltd.*, Tokyo, Japan), 1.5 % (m/v) pectinase (*Serva*, Heidelberg, Germany), and 2 % (m/v) hemicellulase (*Sigma*, St. Louis, USA) dissolved in CPW9M solution [10 mM $CaCl_2 . 2 H_2O$, 0.2 mM KH_2PO_4, 1.0 mM KNO_3, 1.0 mM $MgSO_4 . 7 H_2O$, 0.1 μM $CuSO_4 . 5 H_2O$, 10 μM KI, 15.37 μM MES, 9 % (m/v) mannitol, pH 5.8]. The mixture was incubated on a shaker (40 rpm) at 28 - 30 °C in the dark for about 14 - 16 h. For purification of protoplasts, the protoplast-enzyme mixture was firstly passed through double layers stainless steel sieves (100 μm and 38.5 μm) and then centrifuged in CPW9M at 80 *g* for 5 min, the protoplasts were collected at the bottom of the centrifuge tube, and then resuspended by 1.5 cm^3 CPW9M. The resuspended cultures were gently added to the top of 3 cm^3 CPW25S (replace 9 % mannitol by 25 % sucrose in CPW9M) in another tube, and the floating protoplasts were collected from the solution interface after centrifugating at 80 *g* for 7 min. The collected protoplasts were transferred to a new tube and washed by CPW9M first and then KM_8P medium at 80 *g* for 5 min. The viability was determined by staining with FDA (fluorescein diacetate) under fluorescent microscope (*Olympus AHBS3*, Tokyo, Japan). 0.5 cm^3 purified protoplast with the density of 2×10^6 cm^{-3} was pipetted into a 1.5 cm^3 centrifuge tube, FDA solution (2 mg cm^{-3} in acetone) was added to a final concentration of 0.01 % (m/v). The mixture was incubated at 25 °C for 5 min. Viable protoplasts emitted green light under the fluorescent light. Viability was accounted as the mean percentage of fluorescent protoplasts to the total protoplasts at the same visual field of microscope of ten visual fields.

Purified protoplasts were suspended in KM_8P medium at various densities. The KM_8P medium was supplemented with different combinations of hormones (see Table 1). Protoplasts were cultured in three ways: thin liquid medium, agarose embedded culture and double-layer culture with liquid over solid medium. For thin liquid culure, 4 cm^3 of protoplast suspensions at the density of 5×10^5 cm^{-3} were added to a 60 mm diameter plate. After 20 d, about 2 cm^3 fresh liquid medium was added to the cultures to promote cells dividing. Effect of agarose embedded culture was also investigated in this research. Purified protoplasts were resuspended in $2 \times KM_8P$ medium containing 0.4 M glucose, supplemented with 0.45 μM 2,4-D, 5.37 μM NAA, 1.86 μM Kn to a density of 2×10^5 cm^{-3}. Meanwhile, 1.2 % (m/v) low melting point agarose was prepared for embedding the protoplasts. The protoplast suspensions were mixed with an equal volume of agarose medium to a final density of 10^5 cm^{-3} of protoplasts, 2 cm^3 protoplasts suspension-agarose mixture were added to a 60 mm diameter plate and shaped a thin layer. After solidifying for 5 min at room temperature, all plates were sealed with parafilm and incubated at 28 ± 1 °C in darkness for 20 d, and then 2 cm^3 fresh KM_8P liquid medium containing 0.2 M glucose, supplemended with 0.23 μM 2,4-D, 2.68 μM NAA, 0.93 μM Kn was added to the cultures. As for double-layer culture, about 5 cm^3 melted solid MSB medium were infused into the plate, after solidification, 4 cm^3 protoplasts-KM_8P medium mixtures at the density

Table 1. Effect of combination of hormones on protoplast division at the 8th day and plating efficiency at the 40th day. Means ± SE, $n = 5$. Significant differences were marked by different letters (a, b, c at $P \leq 0.05$ or A, B, C at $P \leq 0.01$) according to LSD test. [a]- the ratio of number of protoplasts dividing to the total number of protoplasts at the same visual field of microscope; [b]- the ratio of number of protoplasts that continued to divide and form cell group related to the total number of protoplasts at the same visual field of microscope. No response was found on medium without hormones.

Combination of hormones [μM]	Division[a] [%]	Plating efficiency[b] [%]	Cells-group formation	Calli formation	Plant regeneration
0.23 2,4-D + 2.68 NAA + 0.93 Kn (H_1)	30.78 ± 3.04aA	9.12 ± 2.61aA	+	+	+
2.46 IBA + 0.93 Kn (H_2)	25.74 ± 3.98bB	0cC	+	-	-
0.45 2,4-D + 0.93 Kn (H_3)	28.31 ± 3.80abAB	0cC	+	-	-
5.37 NAA + 0.93 Kn (H_4)	21.73 ± 2.81cC	1.45 ± 0.68bB	+	+	-
5.71 IAA + 0.93 Kn (H_5)	14.45 ± 3.33dD	0cC	-	-	-

Fig. 1. Protoplast culture and pant regeneration of *G. davidsonii*. *A* - calli formation from protoplast in plate; *B* - calli transferred to solid medium and proliferation; *C* - embryos induction on solid medium; *D* and *E* - germination of abnormal embryo; *F* - normal plant development after grafting; *G* - plant transferred to soil.

of 5 × 10^5 cm^{-3} were added to the plate. All plates were sealed with parafilm and incubated at 28 ± 1 °C in darkness. Twenty days later, 2 cm^3 fresh liquid medium with half amount of mannitol was gradually added to the cultures to accelerate cells dividing and microcalli formation.

As far as the calli (1 - 5 mm in size) were formed, they were transferred to solid MSB medium supplemented with 0.04 μM 2,4-D, 0.46 μM Kn, 6.8 mM glutamine, 3.8 mM asparagine, 0.25 % (m/v) *Phytagel*, 3 % (m/v) glucose for proliferation. Subculture was made at 20-d intervals. The effects of glucose, maltose and sucrose as sugar sources and GA_3 on embryo induction and development have been tested. Regenerated plants were transferred to half strength MS basal medium containing 3 % (m/v) glucose and solidified with 0.27 % (m/v) *Phytagel*, then the developed shoots were grafted and transferred to the soil as described by our previous report (Jin *et al*. 2005).

Ten plates were used for each treatment and the experiment was repeated for five times. Cell division and plating efficiency was calculated on the basis of the percentage of protoplasts that initiated division and continued to form cell groups.

About 4 × 10^6 protoplasts were obtained from 1 g suspension cultures. FDA staining showed that the viability of the isolated protoplast was 80 - 95 %. First cell division was observed in 3 - 7 d, the second division was observed in 5 - 9 d, and after fresh liquid medium was added to the plate for 7 - 10 d, the protoplast developed to 20-cells-groups, and the plating efficiency obtained after 40 d of protoplast culture was from 0.8 to 12 %, the formation of calli was observed in 45 - 55 d (Fig. 1*A*).

Table 2. Effect of protoplast density [×10^5 cells cm^{-3}] on protoplast division at the 8th day and plating efficiency at the 40th day. Means ± SE, n = 5. Significant differences were marked by different letters (a, b, c at $P \leq 0.05$ or A, B, C at $P \leq 0.01$) according to LSD test. [a] - the time that 10 % protoplasts divided at the same visual field of microscope; [b] - the time when the high division percentage persisted; [c] - the ratio of number of protoplasts dividing to the total number of protoplasts at the same visual field of microscope; [d] - the ratio of number of protoplasts that continued to divide and form cell group related to the total number of protoplasts at the same visual field of microscope.

Density	First division[a] [d]	High division[b] [d]	Division[c] [%]	Plating efficiency[d] [%]
0.1 - 2	3	5	15.11 ± 2.51cC	1.56 ± 0.56cC
2.0 - 10	3	5	32.21 ± 3.64aA	9.86 ± 1.87aA
10.0 - 50	5	7	24.18 ± 2.42bB	5.44 ± 1.12bB
>50	5	>8	14.19 ± 2.16cC	0dD

Among the 5 combination of hormones tested (Table 1), the protoplast can initiate division and obtain cells-groups in all media, but no division can be observed in the control medium in 8 d. However, callus could be observed in H_1 and H_4 medium, but most cells-groups went brown and died in 2 - 3 months in other media. H_1 was effective to accelerate protoplast division, and the division percentage at the 8th day was 30.78 ± 3.04 %, and the plating efficiency at the 40th day reached 9.12 ± 2.61 %. The culture density also greatly influenced protoplast division and calli formation (Table 2). When

the density was lower than 10^4 cm^{-3}, the protoplasts can not initiate division in 8 d, as well as the viability gradually decreased, and the protoplast congregated. When the density was more than 5×10^6 cm^{-3}, the protoplast could initiate division and form cells-groups, and the division persists for more than 8 d, but most of the cells-groups went brown in 2 - 3 weeks and then died. The density at 2 - 10×10^5 cm^{-3} is suitable for protoplast division and calli formation, and the division percentage at the 8th day is 32.21 ± 3.64 %, the plating efficiency at the 40th day reached 9.86 ± 1.87 %. In this study, three methods were used to regulate osmotic potential (Table 3). 0.5 M glucose and 0.1 M glucose plus 0.5 M mannitol as sugar source and osmoticum have no significant difference in protoplast division and plating efficiency, and are suitable for protoplast culture, but 0.1 M glucose plus 0.45 M sucrose is not so efficient. We also tested the effect of culture methods on protoplast division and plant regeneration (Table 4). The protoplast division percentage of thin liquid culture method is just parallel to that of the agarose embedded culture method, but with a lower plating efficiency. Solid-liquid double layers culture method was showed to be the most efficient method for *G. davidsonii* protoplast culture. The division of this culture method reached 30.76 ± 3.07 %, and the plating efficiency was 9.69 ± 2.24 %.

Table 3. Effect of osmoticum on protoplast division at the 8th day and plating efficiency at the 40th day. Means ± SE, $n = 5$. Significant differences were marked by different letters (a, b, c at $P \leq 0.05$ or A, B, C at $P \leq 0.01$) according to LSD test. G - glucose, M - mannitol, S - sucrose. For other details see Table 2.

Sugar [M]	First division[a] [d]	High division[b] [d]	Division[c] [%]	Plating efficiency[d] [%]
0.5 G	3	5	31.82 ± 4.55aA	8.98 ± 1.23aA
0.1 G+0.5 M	3	5	30.09 ± 4.87aA	9.35 ± 2.32aA
0.1 G+0.5 S	5	6	16.78 ± 4.09bB	0.78 ± 0.09bB

After 6 - 8 weeks cultured in KM_8P medium, the light yellow or white calli can be observed in the plates (Fig. 1*A*). Upon subculturing to MSB solid medium containing 2.460 μM IBA, 0.698 μM Kn and 3 % (m/v) glucose, the calli grew to light green, light yellow and green-yellow (Fig. 1*B*). When the minicalli developed to large masses, they were transferred to different medium for embryo induction. Glucose, sucrose and maltose as sugar source and GA_3 have been tested, among which the maltose was the best to induce embryo, but glucose plus GA_3 had significant effect on embryo induction (Fig. 1*C*). Among the embryos observed, most of them were abnormal, they can root and elongate with strong stem, but most of them can not germinate (Fig. 1*E*), and some embryos died when they grew to 5 cm long. Only a few abnormal embryos can germinate and form plantlets when they were subcultured for more than 1 month (Fig. 1*D*). The shoot of plantlet was grafted and transferred to soil for further development (Fig. 1*F*,*G*).

Table 4. Effect of culture method on protoplast division at the 8th day and plating efficiency at the 40th day. Means ± SE, $n = 5$. Significant differences were marked by different letters (a, b, c at $P \leq 0.05$ or A, B, C at $P \leq 0.01$) according to LSD test. L - thin liquid culture, A - agarose embedded culture, SL - solid-liquid double layers culture. For other details see Table 2.

Sugar [M]	First division[a] [d]	High division[b] [d]	Division[c] [%]	Plating efficiency[d] [%]
L	3	7	26.78 ± 3.74bB	5.48 ± 1.74bB
A	5	5	25.45 ± 2.58bB	6.33 ± 1.52bB
SL	3	5	30.76 ± 3.07aA	9.69 ± 2.24aA

The process of somatic embryogenesis and plant regeneration from protoplast is complex. Protoplasts have to come through cell wall regeneration, cell division, microcalli and calli formation, embryogenesis and plant regeneration. Also protoplast culture is influenced by many factors, such as explants, culture medium, osmotic pressure, culture density, culture method and the combination of hormones (Kao and Michayluk 1975, Kyozuka *et al.* 1987, Vazquez-Tello *et al.* 1995, Fellner and Lebeda 1998, Sun *et al.* 2005). Previously report revealed that KM_8P medium is applicable to cotton protoplast culture (Sun *et al.* 2005). In this study, KM_8P medium supplementing with IAA, NAA and IBA can initiate cell division, but the effect is not so well as 2,4-D. 2,4-D is effective for protoplast division, but fewer microcalli were obtained in the medium only supplemented with 2,4-D and Kn. NAA was necessary to induce sustained divisions and microcalli formation.

In this paper, we systematically studied the factors affecting plant regeneration from protoplast in wild cotton *G. davidsonii via* somatic embryogenesis. An effective protoplast culture system for *G. davidsonii* had been displayed, and morphologically normal plants were obtained.

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