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Plant regeneration from somatic embryogenic suspension cultures of cotton (*Gossypium hirsutum* L.)

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ABSTRACT

Maintainable, highly embryogenic suspension cultures of cotton (*Gossypium hirsutum* L. cv. 'Coker 310') have been obtained. Callus cultures were initiated from cotyledonary tissues from aseptically-germinated seedlings. To establish the suspension cultures, callus tissue was placed in a liquid medium containing either 0.5 mg/l picloram or 0.1 mg/l 2,4-dichlorophenoxyacetic acid. For proliferation of the embryogenic suspension, 5 mg/l of 2,4-dichlorophenoxyacetic acid was used. Embryo development took place when the embryogenic tissue was transferred to an auxin-free liquid medium containing 15 mM glutamine. Early embryo development was fairly synchronous and large numbers of somatic embryos were produced. Regenerated plants were fertile and smaller than seed-derived plants.

Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid, NAA = 1-naphthaleneacetic acid, IAA = indole-3-acetic acid

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) has been difficult to manipulate in tissue culture systems until recently. Davidonis and Hamilton (1983) first described somatic embryogenesis and plant regeneration in cotton callus cultures. Embryogenic callus arose spontaneously in 2-year old cotyledon-derived calli and, although plants were regenerated, the embryos appeared abnormal. Since that first report, there have been numerous reports on cotton regeneration; these include induction of embryogenesis from leaf and petiole tissues (Gawel *et al.* 1986), development of a liquid suspension culture (Trolinder and Goodin 1987), optimization of initiation of embryogenesis (Trolinder and Goodin 1988a), embryo development (Trolinder and Goodin 1988b), characterization of embryogenesis (Shoemaker *et al.* 1986), and transformation and regeneration (Umbeck *et al.* 1987; Firoozabady *et al.* 1987). None of these reports describe controlled, high frequency embryo proliferation and development. In all previously published reports on cotton regeneration, the embryos and embryogenic tissues were in various stages of organization on various media and attempts to establish and maintain early-staged embryogenic cultures were not made.

This report describes a method for initiation, proliferation, and development of somatic embryos in suspension cultures of cotton. Proliferating liquid cultures consisted of clumps of early-staged embryogenic tissues with little contamination from nonembryogenic tissues. Subculture at a low inoculum density was beneficial for continued maintenance of liquid cultures. Due to the high medium-to-tissue contact in this liquid culture system, media effects were rapid and embryo development could be better controlled than with a solid-support system.

MATERIALS AND METHODS

Initiation of Callus Cultures: Seeds of cotton (*Gossypium hirsutum* L. cv. "Coker 310") were surface-sterilized for 20 min in a 20% commercial bleach solution (1.05% sodium hypochlorite), washed 5 times with sterile water, and placed on a medium containing Murashige and Skoog (MS) salts (Murashige and Skoog 1962), B5 vitamins (Gamborg *et al.* 1968), 3% sucrose, and 0.8% Nobel agar (pH 5.7). One week following germination at 31°C, cotyledonary tissue was excised for culture. All callus and suspension cultures were maintained under a 16:8 hour light:dark photoperiod with a light intensity of 30 $\mu Em^{-2}s^{-1}$. Cotyledon pieces (3 x 3 mm) were placed on induction media as described by Rangan *et al.* (1984). Their procedure utilized initiation and weekly subculture on a medium containing MS salts, B5 vitamins, 2 mg/l NAA, 1 mg/l kinetin, 3% glucose, and 0.8% agar (pH 5.7). Callus cultures were maintained at 31°C. After 4 weekly subcultures, calli were transferred to the induction medium with 3% sucrose in place of glucose (Rangan *et al.* 1984).

Suspension Culture Induction and Proliferation: After callus cultures were maintained on the sucrose-containing medium for one month, 250 mg of tissue were placed in 50 ml of suspension culture induction medium. This induction medium contained MS salts, B5 vitamins, 2% sucrose, and either 0.1 mg/l 2,4-D or 0.5 mg/l picloram. Cultures were agitated at 150 rpm at 28°C.

Large clumps of embryogenic tissue, which were first seen 4-8 weeks following initiation of suspension cultures, were transferred to the embryo proliferation medium. The embryo proliferation medium was the same as that used for induction except 5 mg/l 2,4-D was used as the auxin. Proliferating suspension cultures were subcultured weekly to monthly depending on subculture inoculum density. Weekly subculture was necessary if 25 ml of an older culture were removed and replenished with 25 ml of fresh medium. A monthly subculture or longer was warranted if a single clump (< 500 μm) of early-staged embryogenic tissue was used to inoculate 50 ml of fresh medium.

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Embryo Development and Germination: For embryo development, embryogenic tissues were washed with auxin-free proliferation medium and transferred to either solid or liquid media. The solid medium was the same as that used for seed germination. Washed, embryogenic suspension culture tissue was pipetted onto the solid medium, the tissue was allowed to settle, and the liquid medium was decanted and discarded. For embryo development in a liquid medium, the embryogenic tissue was resuspended in a hormone-free medium containing 15 mM glutamine. The hormone-free liquid medium was replenished weekly for 3 weeks. Mature embryos, which were obtained in 4 weeks on the solid medium and 2 weeks in liquid medium, were then placed on germination medium.

The germination medium was a modification of the medium used by Davidonis and Hamilton (1983) and contained modified MS salts (no NH_4NO_3 and 2X KNO_3), B5 vitamins, 1% sucrose, and 0.2% Gelrite (pH 5.7). After root and shoot elongation, the plantlets were transferred to pots containing a 1:1:1 mixture of vermiculite, perlite, and peat, and covered with beakers. Plantlets were gradually exposed to ambient humidity over a 2 week period and placed in the greenhouse.

RESULTS AND DISCUSSION

Initiation of Callus Cultures: Callus induction from cotyledonary tissue was initially slow on the medium containing NAA and kinetin. Although Rangan *et al.* (1984) reported decreased browning during cotton callus induction with weekly subcultures, browning of the tissue and the medium was not diminished in this present study. Calli were highly variable in color and texture. Typical colors of callus were green, yellow, white, brown, and red. Some cultures were rough-surfaced and friable while others were smooth-surfaced and hard. Calli, which were yellow to cream-colored and appeared friable, were visually selected and subcultured. This callus type has been also reported in the literature as the precursor to embryogenic callus in cotton (Gawel *et al.* 1986; Shoemaker *et al.* 1986; Firoozabady *et al.* 1987). In contrast to previous reports, organized structures resembling embryos were not seen in or on calli prior to the induction of suspension cultures in this study.

Suspension Culture Initiation: Embryogenic tissues were first seen in suspension cultures 4-8 weeks after inoculation. Maintenance of the embryogenic suspension in the initiation medium was difficult. The embryogenic tissue formed large clumps of globular and later staged embryos and was slow-growing. The large clumps of embryos had to be transferred individually with forceps or a spatula, the medium darkened between subcultures, and the embryos underwent proliferation and development in the same medium. This type of suspension culture is similar to that obtained by Trolinder and Goodin (1987) with some important distinctions. In that report, a hormone-free medium was used to establish and maintain embryogenic suspension cultures of the cotton cultivar Coker 312. In the present report, low auxin levels (0.1 mg/l 2,4-D or 0.5 mg/l picloram) were utilized to initiate embryogenic suspensions.

Suspension Culture Proliferation: Following transfer of embryogenic tissues to the proliferation medium containing 5 mg/l 2,4-D, the growth characteristics of the culture changed (Figure 1). Embryogenic tissues were reduced in size and proliferated as preglobular clumps of tissue (Figure 2). Growth was more rapid (doubling time of 4-7 days), the embryogenic tissue became yellow, and the medium did not darken between subcultures.

In contrast to the initial suspension cultures, subculture of liquid cultures maintained in the medium containing 5 mg/l 2,4-D was relatively simple. Due to the small clump size, the cultures could be transferred with a wide-mouth pipet. Subculture regime was dependent on the amount of tissue used to inoculate a new flask. Weekly subcultures were necessary if the cultures were divided; that is, 25 ml of medium and tissue were removed from a flask and replaced with fresh medium. If suspension cultures were divided, only those flasks containing the highest quality of embryogenic tissue were selected for subculture. Prolific, high quality embryogenic tissues of cotton consisted of small clumps of dense, highly cytoplasmic cells containing small plastids (Figure 2) and were characteristically yellow-brown in color when viewed with an inverted microscope. With selective subculture, the suspension cultures remained highly embryogenic and reversion to a nonembryogenic pattern of growth was not observed.

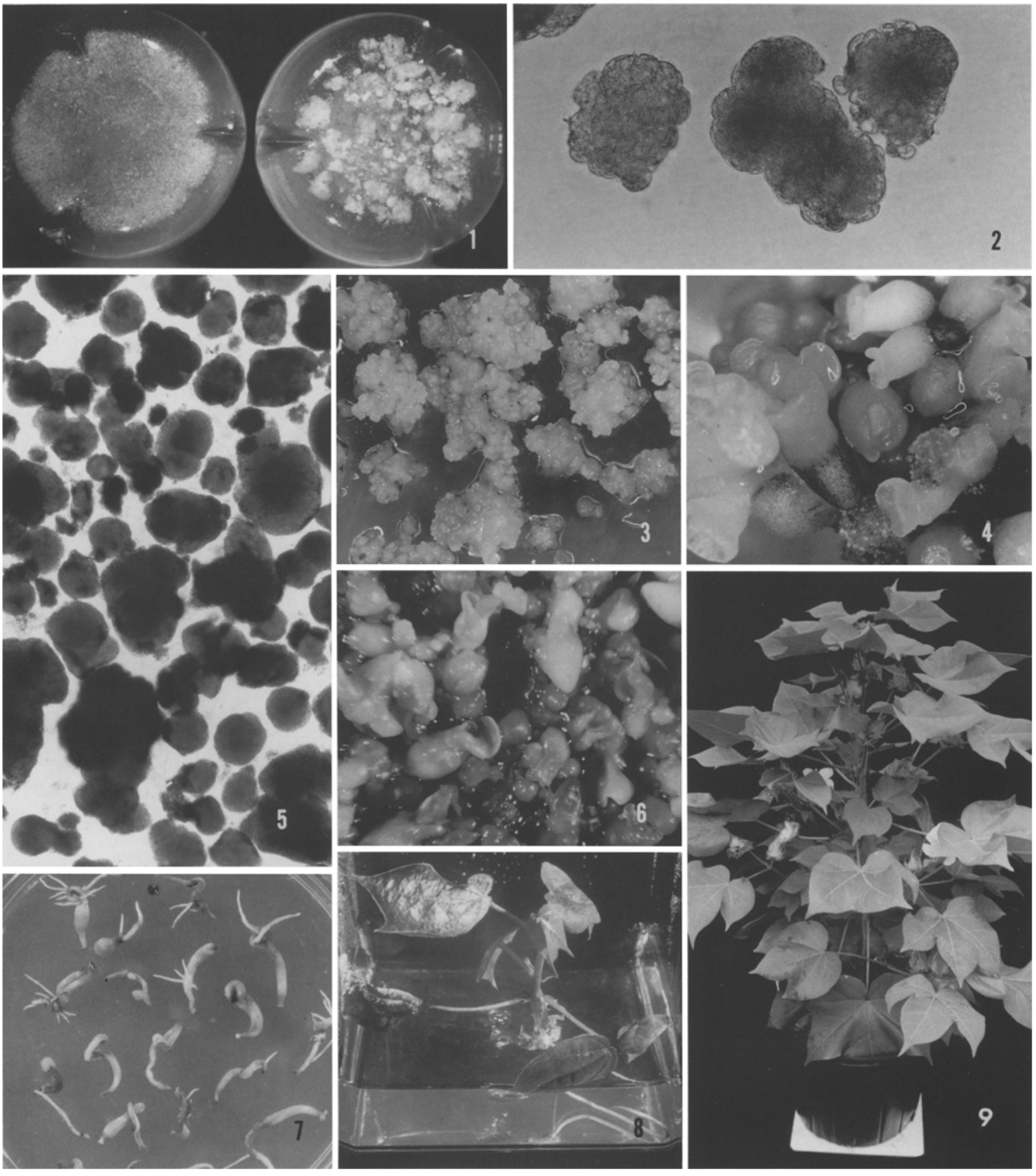
A longer subculture period of one month to six weeks was necessary if a single piece (< 500 μm) of embryogenic tissue was transferred to 50 ml of fresh proliferation medium. This low inoculum subculture gave rise to a very prolific embryogenic suspension. The use of a low inoculum for maintenance of embryogenic suspension cultures has been previously reported for *G. klotzschianum* (Finer *et al.* 1987) and *Glycine max* (Finer and Nagasawa 1988). A critical inoculum density for *G. hirsutum* embryogenic suspension culture tissue (Trolinder and Goodin 1987) was not observed in this present system.

Somatic Embryo Development and Germination: The response of embryogenic tissue following transfer to a hormone-free medium depended on the use of either a solid or liquid medium. When a hormone-free, agar-solidified medium was used for development, embryogenic calli were invariably formed (Figure 3). These embryogenic calli gave rise to both developing somatic embryos and additional embryogenic callus (Figure 4) after 4 weeks of culture. This callus could be maintained with subculture on a hormone-free medium. Embryogenic callus of cotton is apparently habituated as Trolinder and Goodin (1987) suggest. Although large amounts of embryos and embryogenic tissue were obtained, the difficulty with this solid support system was that embryo development was largely sporadic and uncontrolled.

Development of cotton somatic embryos in a hormone-free liquid medium was faster than on a solid medium and under more strict control. In a solid-support system, calli or embryogenic tissues are only in partial contact with the medium. A liquid system is faster growing and more responsive to media changes due to the high medium-to-tissue contact.

Glutamine was advantageous for somatic embryo development in liquid culture. If glutamine was added to the proliferation medium, the embryogenic tissue would enlarge and the medium would darken. It seems that glutamine "pushes" embryo development in a liquid medium while sufficient 2,4-D levels keep the tissue in a proliferative state. When both glutamine and 2,4-D are available, or if sufficiently low auxin levels are used (as in the suspension initiation medium) in liquid culture, embryogenic tissues are pushed both towards proliferation and development.

For somatic embryo development in a liquid medium, embryos were placed in a hormone-free liquid medium containing 15 mM glutamine. Early embryo development was fairly synchronous (Figure 5) and large numbers of embryos were produced (Figure 6). Removal and replacement of the old culture medium with fresh medium after one week appeared to promote embryo development. This enhancement of development could be due to either replenishing depleted nutrients in the medium or removing auxin, which would otherwise keep the tissue in a proliferative state. A source of auxin in these embryogenic suspensions could be either a 2,4-D carry-over from proliferative suspensions or IAA overproduction by habituated embryogenic tissues. Mature embryos, which were formed in liquid medium within 2 weeks and on solid medium within 4 weeks, were transferred



Figures 1-9. Somatic embryogenesis and plant regeneration in cotton. Fig 1. Embryogenic suspension cultures of cotton. Flask on left contains small, early-staged clumps of tissue maintained using 5 mg/l 2,4-D. Flask on right contains large clumps of tissue maintained using 0.5 mg/l picloram (X 0.6). Fig 2. Proliferating, early-staged suspension culture tissue maintained using 5 mg/l 2,4-D (X 150). Fig 3. Embryogenic callus on hormone-free solidified medium (X 5). Fig 4. Embryogenic callus containing developing somatic embryos (X 9.5). Fig 5. Early development of embryos in suspension culture (X 32). Fig 6. Large numbers of mature somatic embryos from suspension culture (X 7.6). Fig 7. Germinating somatic embryos on germination medium (X 0.8). Fig 8. Plantlet from somatic embryo (X 0.8). Fig 9. Mature, flowering plant derived from somatic embryo (X 0.1).

to the germination medium.

The cotton somatic embryo germination medium contained 2x MS KNO_3, no NH_4NO_3 (Davidonis and Hamilton 1983), and Gelrite as the solidifying agent. This medium or slight variations of this medium have been used in previous reports for embryo development (Trolinder and Goodin 1987; Umbeck *et al.* 1987). In this present report, embryo development occurred on a basal MS medium and the 2x nitrate medium was utilized specifically for embryo germination. Following placement of the mature embryos on the germination medium, hypocotyl and root elongation were first observed in 3-7 days (Figure 7). Within 2 weeks, the majority of the embryos had undergone some elongation. Cotyledon development in these embryos was reduced, and shoot elongation required an additional 1-2 months on either the germination medium or a basal MS medium. Following the generation of at least 2 true leaves (Figure 8), the plantlets were transferred to the potting mix.

Over 50 plants were regenerated from the suspension culture-derived tissues. Regenerated plants (Figure 9) were fertile (selfed plants produced seed) but smaller than seed-derived plants. All regenerated plants were fertile, even though some embryogenic tissue was maintained in a medium containing 5 mg/l 2,4-D for over one year. Trolinder and Goodin (1987) maintained embryogenic tissue in and on hormone-free media and reported 15% fertility in their regenerants. Most of the infertile plants were derived from a small number of embryogenic callus lines. Firoozabady *et al.* (1987), using a different culture regime, recovered all fertile plants from their embryogenic callus lines. Deleterious effects of extended exposure of cotton embryogenic tissue to 2,4-D have not been seen with the primary regenerants in the present study.

With the growing amount of information on the molecular biology of zygotic embryogenesis in cotton (Chlan *et al.* 1986), the suspension culture system reported here can provide a useful system for comparable studies on somatic embryogenesis. The culture is early-staged and embryo development can be better controlled than with a solid-support system. Proper expression of cotton embryo specific genes (Chlan *et al.* 1986) can be monitored during somatic embryo development in an attempt to normalize somatic embryogenesis (Crouch 1982). Sufficient quantities of embryogenic tissues at different developmental stages can also be produced for basic biochemical studies.

In addition to basic studies on embryogenesis, the embryogenic suspension cultures may be useful for the development of a protoplast-to-whole plant system in cotton. Currently, protoplast systems in cotton have only advanced to the callus stage (Saka *et al.* 1987) and embryogenic suspension cultures have been shown to be a superior source of regenerable protoplasts (Rhodes *et al.* 1988). Although protoplasts are not necessary for transformation (Umbeck *et al.* 1987; Firoozabady *et al.* 1987), they may permit greater efficiencies of transformation and would be useful for somatic hybridization studies.

In summary, the embryogenic suspension culture reported here is early-staged, very prolific, and highly responsive to media changes. Large numbers of embryos, which are capable of forming whole plants, can be easily produced.

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