ESTABLISHMENT OF EMBRYOGENIC SUSPENSION CULTURES OF A WILD RELATIVE OF COTTON (Gossypium klotzschianum Anderss.)

JOHN J. FINER, ANN A. REILLEY, AND ROBERTA H. SMITH

Department of Agronomy, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH 44691 (J. J. F.); Department of Soil and Crop Sciences, Texas A&M University, College Station, Texas 77843 (A. A. R., R. H. S.)

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Summary

Maintainable, highly embryogenic suspension cultures of a wild relative of cotton (Gossypium klotzschianum Anderss.) have been obtained. Callus with no apparent organization was used to establish the liquid culture. Callus growth conditions as well as suspension medium composition were optimized. A visual selection scheme was beneficial for the maintenance of the embryogenic suspension. These liquid cultures have been maintained for over 10 mo. with no loss in embryogenic capacity. The somatic embryos developed after transfer of the embryogenic tissues to a hormone-free liquid medium.

Key words: Gossypium; cotton; embryogenesis; suspension culture.

Introduction

The first report of somatic embryogenesis in Gossypium was that of Price and Smith (14) with G. klotzschianum. Somatic embryos were obtained after placement of callus tissue into a liquid suspension medium containing 0.1 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D) and 15 mM glutamine. An average of 71 embryos were obtained from 2 to 3 g of callus tissue after 3 wk in the suspension culture medium. This short-term liquid system was not maintainable after 3 wk and gave rise to embryos that were abnormal and not capable of plant regeneration.

Within the past 5 yr there have been a number of reports on somatic embryogenesis and plant regeneration of cultivated cotton, G. hirsutum cultures (4,8,16). These reports all utilized a solid medium support system whereby embryos originated and underwent development in or on stationary callus cultures. Although plant regeneration was obtained in each report, the number of somatic embryos obtained from a given amount of tissues was low.

This study was undertaken to provide a basic understanding of somatic embryogenesis in Gossypium. A maintainable, embryogenic suspension culture of G. klotzschianum could help in the establishment of high-frequency embryogenesis systems in other commercially important Gossypium species. High quality, early staged embryogenic liquid cultures could also be used as a source of protoplasts. Protoplast systems of Gossypium have not advanced past the multicellular stage (2,5,6,10) and transformation followed by regeneration has only recently been reported (18). In addition, the physiology and molecular biology of somatic embryogenesis can be studied by controlled manipulations of both media and environment. This information can be utilized along with the growing amount of information dealing with zygotic embryogenesis in cotton (3) to “normalize” and better understand somatic embryogenesis in Gossypium.

Materials and Methods

Seeds of G. klotzschianum Anderss. were surface sterilized in 95% ethanol. After a 30-s dip, seeds were flamed, scarified, and planted on a hormone-free medium (OMS) containing Murashige and Skoog salts (12), Gamborg’s B-5 vitamins (7), 2% sucrose, and solidified with 0.8% agar (pH 5.7). Seeds were germinated at 28°C for 7 d. All culture lighting conditions were 16:8 h light:dark photoperiod with a light intensity of 130 μEm⁻²•s⁻¹ (unless otherwise noted). Hypocotyls were excised, sliced longitudinally, and placed on the callus-induction medium of Smith et al. (17). This medium consists of Murashige and Skoog (12) salts and vitamins, 2 mg/liter indoleacetic acid (IAA), 1 mg/liter kinetin, 3% glucose, and was solidified with 0.8% agar (pH 5.7). One month after callus induction, the callus was transferred to a high cytokinin maintenance medium (17). This medium (Hi2ip) contained Murashige and Skoog (12) salts and vitamins, 1 mg/liter naphthalacetic acid (NAA), 10 mg/liter N⁴-[α-isopentenyl]-adenine (2ip), 3% glucose, and was solidified with 0.8% agar (pH 5.7). Calli were subcultured onto fresh Hi2ip medium every 2 wk.

1 Present address: DNA Plant Technology Corporation, 182 Lewis Road, Watsonville, CA 95076.
Cultures on the Hi2ip medium were initially maintained at 130 \( \mu \text{Em}^{-2} \cdot \text{s}^{-1} \) but this light intensity was later lowered to 30 \( \mu \text{Em}^{-2} \cdot \text{s}^{-1} \) to give higher embryo yields. Some somatic embryos obtained from early experiments were placed back on the Hi2ip medium for callus initiation.

For establishment of suspension cultures, 100 mg of callus tissue were placed in 50 ml of suspension medium in a 125-ml DeLong flask. The suspension medium consisted of Gamborg’s B-5 salts and vitamins \((7)\), 2% sucrose, 0.1 mg/liter 2,4-D and 15 mM glutamine (pH 5.7). Callus grown under varying subculture periods and environmental conditions was placed in this medium to obtain somatic embryos. Embryos were counted 4 wk after initiation of the suspension culture. In later experiments, 2,4-D levels \((0.01, 0.1, 1, 10 \text{ mg/liter})\), length of incubation of tissue in each 2,4-D containing medium \((1, 2, 3, 4 \text{ wk})\), and the effect of washing the developing embryos with hormone-free suspension medium were tested. In these experiments, embryos in the suspension cultures were counted after a 3-wk incubation in a hormone-free medium.

If large numbers of embryos \((>1000/\text{flask})\) were obtained, total embryo counts were made based on three sample counts taken from each flask. If fewer embryos were obtained, the number of embryos were counted from each flask. Three repetitions of each treatment were made and individual embryos as well as total embryos in a clump were counted.

Once conditions were defined for maintaining embryogenic suspensions, the liquid cultures were visually selected for high embryogenic capacity and subcultured weekly to biweekly. Visual selection was based on relative amounts of embryogenic and nonembryogenic tissues when viewed on an inverted microscope. Subculture inoculum varied from 10 mg to 1 g fresh weight into 50 ml of fresh medium. The smaller inoculum required a longer growth period.

Development of the somatic embryos could occur in liquid or on solid media. For development on a solid medium, the liquid cultures were first washed three times with a hormone-free medium. The suspensions were then pipetted onto a solid medium. After the cells had settled, the supernatant was removed with a pipette. The Hi2ip medium was the only solid medium tested for embryo development.

For development in a liquid medium, the 2,4-D-containing medium was simply replaced with a hormone-free medium. Liquid cultures were washed twice the 1st wk and weekly for 3 additional wk with the hormone-free suspension culture medium. For embryo germination, mature somatic embryos were plated on the embryo development medium of Davidsonis and Hamilton \((4)\) with Gelrite in place of agar. Substitution of lactose for sucrose (both at 1%) in this germination medium was also tested.

RESULTS

Early attempts to repeat the work of Price and Smith \((14)\) were unsuccessful. An average of three embryos were produced from 2 to 3 g of tissue under the previously described conditions. When the light intensity received by calli on the Hi2ip medium was decreased to 30 \( \mu \text{Em}^{-2} \cdot \text{s}^{-1} \), there was a resultant sixfold increase in the number of somatic embryos produced. An additional 14-fold increase in somatic embryo production occurred when using somatic-embryo-derived calli rather than hypocotyl-derived callus cultures. An average of 244 embryos could be obtained from 2 to 3 g of tissue inoculated into 50 ml of suspension culture medium. To test the effect of growth stage of the callus on somatic embryo production in suspension culture, calli were taken 1, 2, 3, 4, 5, and 6 wk after subculture on the Hi2ip medium. The results of this experiment in Fig. 1 show that callus taken 2 wk after subculture was optimum for obtaining somatic embryos.

The most significant increase in somatic embryo production resulted from modification of the liquid medium composition rather than manipulation of the calli used to initiate the suspensions. An increase in the 2,4-D levels in the liquid medium from 0.1 mg/liter to 1 or 10 mg/liter resulted in a 26-fold increase in somatic embryo production \((\text{Fig. 2})\). Between \(5 \times 10^4\) and \(6.5 \times 10^4\) embryos were produced in 50 ml medium in a 125-ml flask after either a 3 wk incubation in a liquid medium containing 1 mg/liter 2,4-D or a 2 to 3 wk incubation in a liquid medium containing 10 mg/liter 2,4-D. With these higher 2,4-D levels in liquid culture, the suspensions were comprised primarily of early staged somatic embryos, occurring both singly and in clumps.

Attempts to obtain a maintainable, early stage embryogenic suspension culture using a liquid medium containing 1 mg/liter 2,4-D failed. After 4 to 6 wk, the suspension became clumpy as the embryos underwent early and abnormal development in the 2,4-D-containing medium. Continued maintenance of an early staged liquid embryogenic suspension culture was possible if 10 mg/liter 2,4-D was used in the liquid medium. At this higher 2,4-D level, the suspension contained a mixture of clumps of embryogenic tissue \((1 \text{ mm})\), small proembryos \((>100 \text{ mm})\), and nonembryogenic clumps and cells \((\text{Fig. 3})\). The suspension culture appeared white to light yellow.

![Fig. 1. Effect of callus age on somatic embryo production in suspension culture of G. klotzschianum.](image-url)