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Haploid and Diploid Callus from Cotton Anthers[1]

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ABSTRACT

We have initiated and subcultured large quantities of callus tissue from the anthers of two species of cotton, *Gossypium barbadense* L. and *G. hirsutum* L. A liquid medium was developed and cell suspension cultures were generated from anther-derived callus from *G. hirsutum*. Cytological examination was made only on anther callus from *G. hirsutum* and the preponderance of the tissue was diploid ($2n=4x=52$). Liquid suspension cultures from anther callus were also found to be primarily diploid. However, 13 cultures that grew more than four times faster than 527 diploid cultures had 83% haploid cells. Therefore, haploid tissue was produced in 2 to 3% of the anthers, and it grew obscurely within the diploid callus. The haploid tissue was identified and selected by its rapid growth rate in liquid culture.

Additional index words: Cell Culture, Chromosome, *Gossypium hirsutum*, Organogenesis, Pollen, Tissue Culture.

THE type of media and conditions for the initiation and maintenance of callus tissue have been characterized for many plant species (Gamborg and Wetter, 1975). In cotton, the presence of gossypol and other polyphenolic compounds have limited the initiation and maintenance of callus tissues to a few successful attempts, mainly on Upland cotton, *Gossypium hirsutum* L. (Davis et al., 1974; Schenk and Hildebrandt, 1972). Recently, however, Sandstedt (1975) demonstrated the production of vigorous callus from both *G. hirsutum* and *G. barbadense* L. These calli were induced from split stem sections of young cotton plants. Some of these calli eventually developed into a habituated state after several subcultures. Hsu and Stewart (1976) found ethylene effective in inducing cell proliferation in the micropylar region of fertilized and unfertilized cotton ovules.

Smith et al. (1977) initiated a rapidly growing callus from hypocotyl sections of *G. arboreum* L. and found that it could be subcultured in high light on a glucose, Murashige and Skoog medium with different auxin-cytokinin concentrations. Price et al. (1977) found that by using the basic culture system developed for *G. arboreum*, callus could be initiated and subcultured from hypocotyl sections of *G. anomalum* Wawr. ex Wawr. & Peyr., *G. armourianum* Kearn., *G. hirsutum* L., *G. klotzschianum* Anderss., and *G. raimondii* Ulbr., with only modifications in phytohormone levels compatible with each species.

We tried unsuccessfully to culture haploid plantlets directly from cotton pollen and ovules. We investigated the possibility of producing callus from anthers and attempting subsequent differentiation. For a number of species, this approach apparently is an alternative to producing plantlets directly from pollen culture (Niizeki and Oono, 1968; Kameya and Hinata 1970; Gresshoff and Doy, 1972a, 1972b). Anther callus in some species was found to be a mixture of n and 2n cells (Sharp et al., 1971; Gupta and Carlson, 1972). This callus could serve as a potential source of haploid cells in cotton in future experimentation on mutant selection and organogensis, if (1) haploid cells were present and (2) the appropriate technique could be developed for the separation of haploid from diploid cells.

This paper describes the conditions necessary for the production and maintenance of callus from the anthers of *G. barbadense* and *G. hirsutum*, and reports chromosome numbers observed in anther-derived callus and in liquid suspension cultures of *G. hirsutum*.

MATERIALS AND METHODS

Research for producing callus from anthers of cultivated cotton was conducted for *G. barbadense* by Katterman and Williams in Tucson, Ariz., and for *G. hirsutum* by Barrow in Las Cruces, N.Mex. A number of media with many modifications in carbohydrate and nitrogen levels and growth regulator combinations were investigated. Only those that gave the best growth are reported here.

Flower buds from meiotic prophase to anthesis stages were collected at both locations. The anthers were aseptically removed and plated on specific culture media. A sample of anthers was taken from each bud and collected in a vial of 95% ethanol and glacial acetic acid (7:3 v:v), stored in a refrigerator 24 hours, smeared in iron propionic carmine stain and observed microscopically for the stage of development. The technique described by Bernardo (1965) was used to dissolve the exine and stain the nuclei of microspores past the tetrad stage. Therefore, we were able to determine the developmental stage of microspores up to the mitotic division of the nucleus.

The *G. barbadense* anthers were plated on LS (Linsmaier and Skoog, 1965) induction medium with 5 mg napthalene acetic acid (NAA)/liter and 1 mg/liter benzyladenine (BA). Callus was induced and in about 3 weeks it was subcultured on LS transfer medium modified by substituting glucose for sucrose and reducing NAA and BA to 2 mg and 0.1 mg/liter, respectively. Callus has been subcultured every 3 to 4 weeks up to 25 times. Callus was grown under 44 μEinsteins sec^{-1} m^{-2} of light energy for an 18-hour day and a 6-hour night at a constant 29 C.

The *G. hirsutum* anthers were plated on a basic DBM-II induction medium described by Gresshoff and Doy (1972b), and supplemented with 5 mg NAA and 1 mg BA/liter. Callus was induced and transferred after 3 weeks to DBM-II transfer medium modified by substituting glucose for sucrose and reducing NAA and BA to 1 mg and 0.1 mg/liter, respectively. One gram of inositol/liter was also added to the basal medium. Callus tissues were grown in the dark at 29 C. These cultures have been maintained more than 20 months by subculturing every 3 to 4 weeks.

A liquid DBM-II transfer medium was made exactly as described above with 10^{-4} *M* 2-chloroethylphosphonic acid (2 CEPA) (Hsu and Stewart, 1976) and dispensed in 50-ml aliquots in 125-ml flasks and autoclaved 15 min at 1 atm. Approximately 5-mm pieces of subcultured callus from *G. hirsutum* were used to inoculate the flasks for liquid suspension cultures. A rapid increase in growth was noted in the suspension cultures between 10 and 18 days as the flasks were agitated on an orbital shaker at 60 rpm for 16 hours at 44 μEinsteins sec^{-1} m^{-2} light energy and 8 hours of dark.

For cytological analysis, the liquid cultures were treated with

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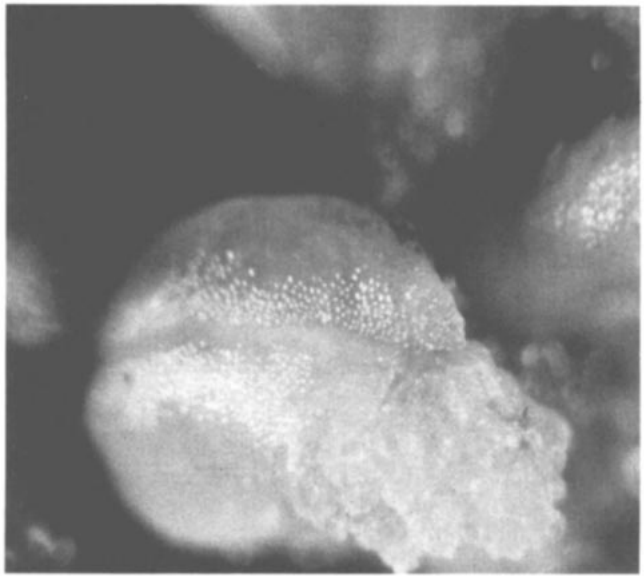

Fig. 1. Callus emerging from within the anther.

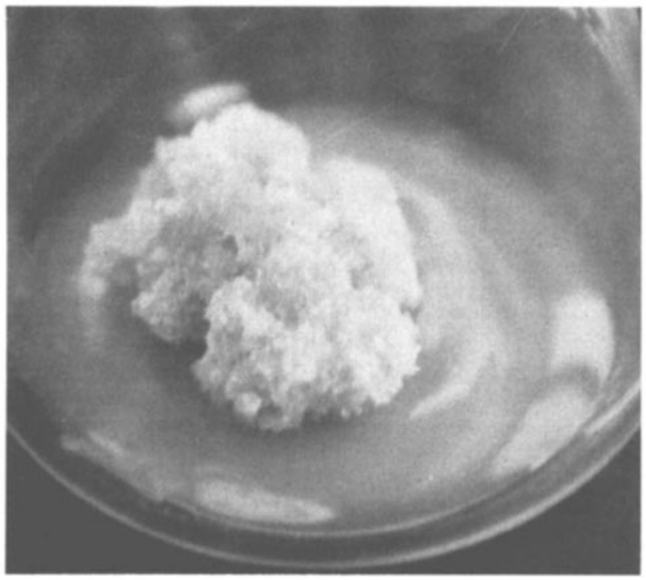

Fig. 2. Cotton anther callus after 3 weeks of culture.

2.5 ml of 1% colchicine giving a final colchicine concentration of 0.05%. After 4½ hours the cells were centrifuged, the liquid was poured off, replaced with 3 parts 95% ethanol and 1 part of glacial acetic acid, and refrigerated over night. The cells were again centrifuged and the liquid was replaced with 6*N* HCL and left at room temperature for 2 hours. The cells were then centrifuged, rinsed twice with distilled H_2O, and treated with Feulgen's reagent from 2 hours to 4 days in a refrigerator. These cells were stained with a 2% modified carbol fuchsin stain (Gamborg and Wetter, 1975) and smeared with maximum pressure. Friable cells from the surface of the callus tissue were collected for cytological examination and placed directly into the killing-fixing solution, without colchicine treatment. Subsequent treatment was the same as for liquid cultures.

RESULTS AND DISCUSSION

The response was similar for the two species of cotton at both locations. Anthers cultured from all sizes of buds produced callus. The optimal bud length for callus production ranged from 4 to 6 mm and from 9 to 10 mm corresponding with meiosis and the first mitotic division of the microspores. Anthers from these buds exhibited variable degrees of anther wall swelling from none to two to three times the original size. Callus emerged from within the anther as shown in Fig. 1, or from the anther wall. Anthers from buds less than 4 mm did not produce callus from within the anther but did from the anther wall. Buds longer than 10 mm produced anthers that exhibited extensive wall swelling and produced callus at a higher rate both from within and without the anther than did anthers from buds 4 to 6 mm in length. The optimal production of callus and haploid cells from cotton anthers occurred at meiosis, which corresponded well with the findings of Greshoff and Doy (1974) and at the first microspore division, similar to the observations of Nitsch and Nitsch (1969).

Barrow (unpublished) has produced callus occasionally from isolated cotton microspores indicating the potentiality of producing haploid callus from pollen. Anther callus grew rapidly and was friable with a light tan color only when the transfer media contained glucose instead of sucrose (Fig. 2). This finding was consistent with those of Sandstedt (1975), Smith et al. (1977), and Price et al. (1977). Average dry weight of the inoculation callus pieces was 0.003 ± 0.001 g, while the average dry weight of callus after 3 weeks of culture was 0.028 ± 0.004g.

The growth of cells from callus pieces transferred to the liquid medium was dependent upon the addition of 10^{-4} *M* 2-CEPA. After 10 days a significant increase in cell number was observed. This continued to 18 days when the rate of growth slowed. Cytological examination of these cells from the liquid cultures showed about 90% of the cells with clear cytoplasm, visible nuclei, and a mitotic index of 0.02 (i.e., number of nuclei from prophase to telophase/total nuclei). Cytological examination of rapidly growing callus masses on agar, showed only 5% of the cells with clear cytoplasm and visible nuclei. Most of the callus consisted of opaque starch accumulating cells with no visible nuclei. Meristematic cells were confined to the surface of the callus or to small sectors within the callus. The mitotic index was 0.016 when only the meristematic type cells were considered in the agar-grown callus masses. Therefore, an 18-fold increase in the frequency of meristematic cells and a higher mitotic index favors liquid cultures over callus for general cytological studies.

After a satisfactory cytological technique was developed, 930 calli were induced from *G. hirsutum* anthers removed from 775 buds. More than 100 of these calli were established in subculture. Cytological examination was made of 56 different anther-derived cell lines. All cell lines were diploid, except for two isolated haploid cells, indicating that most anther callus originated from anther tissue rather than from pollen.

Three hundred liquid cultures were established at random from the 56 cell lines. Cell number increased rapidly in the normally growing cultures from 10 to 18 days after inoculation as described above. Chromosome counts of 36 of these liquid cultures revealed

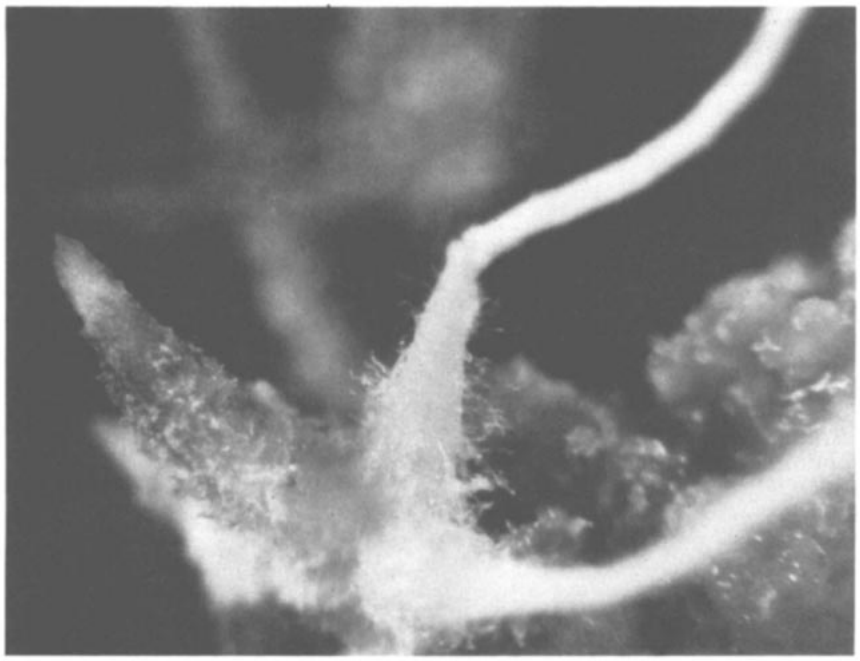

Fig. 3. Root and root hairs differentiating from anther callus.

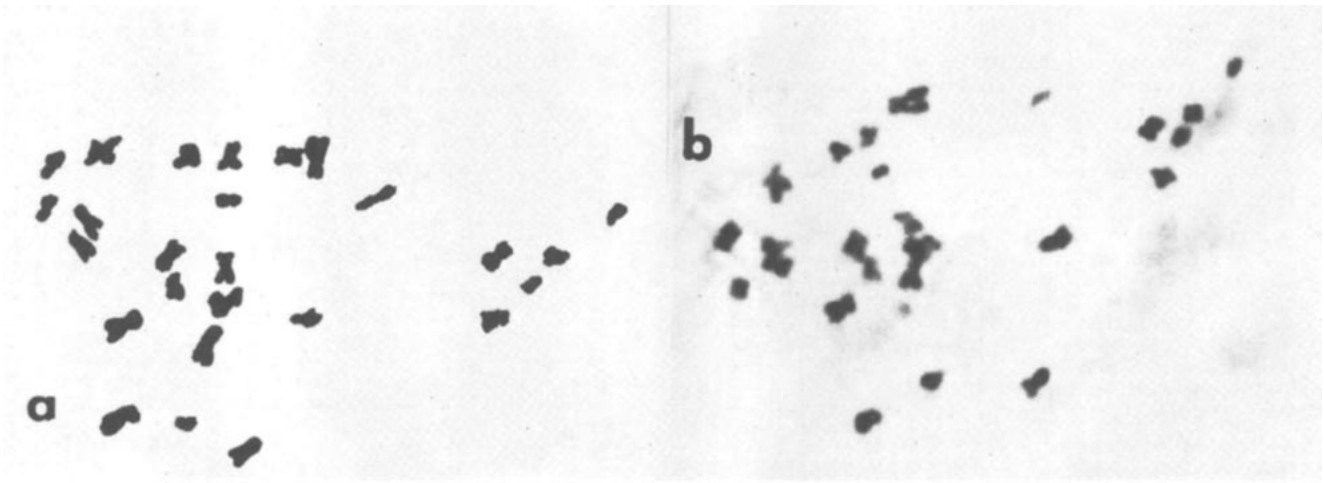

Fig. 4. A haploid cell of *G. hirsutum* derived from anther culture with 26 chromosomes. (a) camera lucida drawing × 1940; (b) photomicrograph × 1940.

that they were diploid. However, nine cultures grew more rapidly than normal, and increased visibly in cell number at 4 days after inoculation. At 10 days these cultures had approximately four times the number of cells by cell count and cell volume measurements as did the normal cultures at 18 days. More than 10,000 dividing cells in the rapidly growing cultures were examined cytologically; 83% of those cells were haploid (Fig. 3).

An additional 240 liquid cultures were grown from different anther calli and four more rapidly growing haploid cultures were found. These data indicate that approximately 2 to 3% of the calli initiated from anthers contained haploid cells derived from pollen. Haploid cells were manifested only in the liquid medium, where they grew more rapidly than diploid cells. Precise quantitative measurements of the frequency of haploid cell production from anthers could not be made because of callus selection methods, mortality of cultures, and variable growth rates.

Attempts to induce organogenesis in this study were not particularly successful. When light energy was increased, from 26 to a range of 56 to 150 μEinsteins sec^{-1} m^{-2}, the callus turned green, xylem-like cells formed, and rarely roots with visible root hairs emerged from the densely-packed cellular regions (Fig. 4). We are now attempting to enhance differentiation. We have also regenerated callus from the liquid suspension cultures, but no organogenesis has occurred in these cultures.

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