Chapter 39

ORGAN AND TISSUE CULTURE OF COTTON

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INTRODUCTION

The use of organ and tissue culture as a research tool is well established, but the use of these techniques on cotton has been relatively limited in the past, considering the importance of the crop. Current trends indicate that past neglects are being rectified. Historically most of the work on cotton was with ovule culture both for developmental and physiological studies on the seed and fiber and for interspecific hybridization. Recent activity, especially in the private sector, has centered on developing systems for organogenesis from unorganized tissues.

The purpose of this chapter is to summarize the work done on cotton organ and tissue culture to this point without detailing the methodologies and media involved. Those interested in specific details are directed to the original references. Since tissue culture in cotton is an area that is now progressing rapidly, this review should be considered only as a starting point for the serious student interested in using the techniques.

EMBRYO CULTURE

The first use of embryo culture in cotton was reported by Skovsted (1935). A weak embryo of *Gossypium davidsonii* x *G. sturtianum* was rescued and cultured on sterile glucose-agar. Beasley (1940) and Weaver (1958) used a similar approach but included White's minerals in the medium to rescue hybrid embryos of diploid and tetraploid cottons. Supplements to White's medium such as coconut milk, tomato-juice extract or casein hydrolysate failed to improve the growth of cotton embryos younger than 27 days postanthesis (DPA) (Lofland, 1950). Embryos older than 27 days grew rapidly on basal medium without supplements. Dure and Jensen (1957) examined the capacity of immature embryos (approx. 37 mg and 64 mg fresh weight) to respond in culture, especially with respect to the presence of indole acetic acid (IAA) or gibberellic acid (GA). The embryos were placed for 10 days on a 2 percent sucrose-agar base containing a modified White's medium (Randolph and Cox, 1943) plus or minus the hormone additive. The

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younger embryos responded very little. In the larger embryos GA promoted cell expansion and inhibited reserve accumulation. Stewart and Hsu (1977b) subcultured embryos, which were obtained through *in ovulo* culture (see below), on a low salt medium that contained a higher level of NH_4+ and NO_3^- than White's medium. Also, vitamin supplements improved results. Subsequently, they found that rescued embryos grew better when the medium was adjusted to pH 7.0 compared to pH 5.5 (Stewart, 1979).

The most successful culture of isolated, immature embryos was reported by Mauney (1961). Although results were highly variable, he found that a high salt medium, and particularly a high osmolarity (circa 10 atm.), was essential for embryo development. He and coworkers (Mauney *et al.*, 1968) analyzed the liquid endosperm of cotton for organic acids and found a high level of what appeared to be malic acid. Subsequently, addition of ammonium or calcium malate (4 mg malate per ml) to the culture medium gave much better survival of heart stage embryos. Sodium malate was inhibitory, and salts of succinate and citrate were inferior to ammonium malate. The optimum osmotic balance also declined to about 8 atmospheres when malate was present.

No additional work on the culture of isolated zygotic embryos of cotton has been reported because *in ovulo* embryo culture (see discussion this chapter) has proven to be a simpler and more successful method for rescuing embryos. However, with the current efforts to obtain somatic embryogenesis from callus or suspension cells (see discussion this chapter), there is increased interest in techniques that will promote development of viable embryos and, subsequently, plants.

OVULE CULTURE

Ovule culture has received the most attention and has been used for a wider range of objectives than any other aspect of *in vitro* culture of cotton. This organ is particularly attractive as an explant for physiological research because it bears both the fibers and the embryo. Accordingly, it has served, and continues to serve, as a model system to study nutrition, phytohormone regulation, differentiation, cellulose synthesis, fertilization and embryogenesis, and interspecific hybridization. These systems are aided by the fact that sterile culture of cotton ovules is relatively easy compared to many crop species because of their size (1-2 mm length), number per ovary (32-40) and ease of excision.

The first reported attempt to culture ovules was by Joshi (1960). Six DPA ovules were excised and cultured on a low salt medium containing casein hydrolysate, vitamins, IAA, and GA. Growth of the ovules was generally abnormal and fibers did not continue to grow. Nevertheless, this method was later used in exploratory work to obtain interspecific hybrids of Asiatic and Upland cottons (Joshi and Pundir, 1966; Pundir, 1972). The original culture method was later elaborated and various ovule growth responses documented (Joshi and Johri,

1972). A few complete but small embryos were obtained in this case. Their observation of polyembryony is interesting in view of the efforts to obtain regeneration from unorganized tissue (see *Regeneration* below).

Rapid progress in the culture of cotton ovules began in the early 1970's and continued through the decade. The research objectives of the period can be placed in two broad categories: (1) culture to determine the physiological and biochemical determinants of fiber and seed growth, and (2) culture to optimize *in ovulo* embryogenesis. The two objectives are not mutually exclusive, and both areas of research have provided a much greater understanding of the physiology of seed and fiber development in cotton.

CULTURE FOR SEED AND FIBER DEVELOPMENT

The first indications of a successful method of ovule culture for fiber development were preliminary reports of Beasley and coworkers (Beasley, 1971; Beasley and Ting, 1971; Beasley *et al.*, 1971). The essential features for success were the use of a high salt medium [a modification of Murashige and Skoog, 1962 medium, (MS)] and use of liquid culture rather than agar solidified medium. The preliminary reports were followed by two classic papers (Beasley and Ting, 1973; 1974) which described the effects of phytohormones on fiber development on fertilized and unfertilized ovules. Independently, reports concerned with fibe development on cultured ovules were published by a Belgium group (DeLange and Eid, 1971; Waterkeyn *et al.*, 1975; Baert *et al.*, 1975). This group also found that MS medium was superior to media with lower mineral concentrations. They examined the effects of auxin and GA and reached the same conclusions as Beasley and Ting (1971), namely, growth of fertilized ovules with their associated fibers was greatly stimulated, if GA were added to the medium. Auxin was much less stimulatory. If the ovules were not fertilized, auxin was a requirement for fiber growth. Beasley and Ting (1973, 1974) found that kinetin slightly inhibited growth of fertilized ovules and fibers but stimulated unfertilized ovule growth. In this latter case the unfertilized ovules did not produce fibers. ABA was inhibitory in either case.

More detailed discussions on the role of phytohormones in fiber development are found in Chapters 23 and 25. Details concerning the culture system and subsequent observations by Beasley and coworkers may be found in three reviews (Beasley, 1974; Beasley *et al.*, 1974; Beasley, 1977b).

The culture system developed by Beasley became the standard procedure from which a number of additional physiological studies were launched. For example, growth of fibers on unfertilized ovules in culture was found to depend not only upon IAA but also upon temperature (Beasley, 1977a). Below 34C many ovules did not produce fibers even though they enlarged. If NH_4^+ were included in the medium, more ovules produced fibers at temperatures below 28-32C than when the cation was absent. Birnbaum *et al.* (1974) showed that boron was essential for normal growth of ovules with their associated fibers. When boron was absent

from the medium, fibers were not produced and the ovules formed extensive callus. The influence of ethylene on cultured ovules was reported by Hsu and Stewart (1976). Ethylene supplied as 2-chloroethylphosphonic acid inhibited normal growth of the ovule but promoted callus growth from the micropylar end of the ovule. A synergistic production of callus from the entire ovule resulted if GA were in the medium, but IAA tended to suppress the callus growth (Stewart and Hsu, 1977a). The tendency for ovules cultured for more than 2-3 weeks to form callus even in the absence of exogenous ethylene was used by Hsu and Stewart (1979) to generate callus cultures of cotton (see below).

The ovule culture system was also used to study the enzymology of fiber development. Dhindsa *et al.* (1975) were able to demonstrate that ovules and fibers grew better when CO_2 was present in the culture environment than when it was absent. Later it was demonstrated that GA stimulated phosphoenolpyruvate carboxylase and other enzymes involved in nonphotosynthetic CO_2 fixation (Dhindsa 1978b). Delmer and coworkers made limited use of the ovule culture system to examine glucan synthesis in fibers (Delmer *et al.*, 1977; Meinert and Delmer, 1977; Maltby *et al.*, 1979). Additional discussion on these reports as they relate to fiber development may be found in Chapter 23.

IN OVULO EMBRYO CULTURE

The second general objective for culturing ovules is to obtain embryogenesis in a controlled environment. The major impetus to develop a suitable culture system has been to overcome the interspecific incompatibility that occurs between many of the *Gossypium* species. The barriers were of major significance since these prevented hybridization of the diploid Asiatic cottons with the tetraploid American cottons. Weaver (1958) showed that the incompatibility resulted from premature degeneration of the endosperm so that the embryo starved. In those hybrid crosses where incompatibility is due to failure of the endosperm to development, one might expect ovule culture to be most successful.

As mentioned earlier, Joshi and Johri (1972) tried to culture ovules on a low salt medium, but they had only marginal success. Eid *et al.* (1973) compared four media with respect to their ability to support ovule and embryo development. The high salt medium of Murashige and Skoog (1962) was superior to three low salt media when ovules aged 5, 10, 15 and 20 DPA were cultured. The authors obtained normal embryos from ovules placed in culture at 10 days postanthesis. Five-day-old ovules also produced embryos that could be subcultured on MS but these grew abnormally. Stewart and Hsu (1977) subsequently reported a method of *in ovulo* embryo culture that had features of both the Beasley-Ting technique for fibers and the technique of Eid *et al.* (1973) for embryos. With their modifications they were able to grow self-pollinated ovules from 2 DPA (zygote stage) to maturity (germination). The basic culture techniques were those elaborated by Beasley (1974), and the medium (BT) contained the phytohormones recommended for optimum fiber growth. An essential ingredient for embryo development was the presence of ammonium ions (NH_4^+). Stewart and Hsu (1977) speculated

that the high KNO_3 of the BT medium was necessary for early embryo growth and that the ammonium was essential for later embryo growth.

Stewart and Hsu (1978) used their ovule culture technique to obtain interspecific hybrids between the Asiatic diploid cottons and the American tetraploid cottons in all possible combinations. In most cases the presence of GA and kinetin in the culture medium was deleterious to recovery of hybrid plants. Consequently, they recommended that only auxin be used when culturing ovules of species other than *G. hirsutum*. Also, they found that the ovules of *G. arboreum* and *G. herbaceum* were prone to callus formation, but that this did not prevent embryogenesis. However, callus growth did cause ovules to sink in the medium, resulting in anoxia of the embryo. A filter paper bridge was constructed and placed in the culture flask to prevent this (Stewart and Hsu, 1978).

The basic *in ovulo* embryo culture technique was subsequently used to make interspecific hybrids between the four cultivated species of cotton and a wide variety of wild *Gossypium* species (Stewart, 1979, 1981). Many of these hybrids had not been obtained by conventional techniques of cross-fertilization.

IN VITRO FERTILIZATION

In vitro fertilization is a technique that is dependent upon an adequate procedure to culture ovules. With a system available to culture cotton ovules from anthesis to maturity, direct fertilization of the ovules should be possible. Stewart (1981) made a preliminary report of successful *in vitro* fertilization, but additional work has not been reported.

TISSUE CULTURE

CALLUS INDUCTION AND CULTURE

Callus formation in cotton was first reported by Beasley (1971) as an outgrowth from the micropylar end of ovules cultured on MS medium. The tissue did not grow well when subcultured. Results reported by Hsu and Stewart (1976) indicated that the callus from the micropylar end of ovules was induced by ethylene. In the presence of ethylene and GA, the entire outer integument of the cultured ovules formed callus (Stewart and Hsu, 1977a). The callus from these cultures, although extensive, was not subcultured.

Schenk and Hildebrandt (1972)[SH] included cotton as one of the many species they used to develop their medium. In that case callus was derived from mesocotyl explants cultured with 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 2 mg/l *p*-chlorophenoxyacetic acid (*p*-CPA), and 0.1 mg/l kinetin (K) with sucrose and the SH salts. Davis *et al.* (1974) were the first to specifically examine the induction of callus from cotton (cotyledon tissue). Since that time numerous reports have appeared concerning the induction and maintenance of callus from various cotton tissues and species (Sandstedt, 1975; Rami and Bhojwani, 1976; Katterman *et al.*, 1977; Smith *et al.*, 1977; Price *et al.*, 1977).

The hormonal regimes and media adjuvants used to induce callus growth varied widely among investigators. In all cases either IAA or a synthetic auxin was used, but the concentration ranged from 0.1 mg/l 2,4-D (Davis *et al.*, 1974) to 17 mg/l NAA (Katterman *et al.*, 1977). In most reports a low level of cytokinin was included in the induction medium. Myoinositol was 100 mg/l in all reports except Davis *et al.* (1974) who reduced the level to 5 mg/l. Most media formulations called for glucose rather than sucrose to decrease the amount of pigment production by the tissue (Sandstedt, 1975; Katterman *et al.*, 1977; Smith *et al.*, 1977). In other efforts to reduce pigment in the cultures, Davis *et al.* (1974) added ascorbic acid, and Katterman *et al.* (1977) added dithiothreitol. Apparently a chemically reducing environment inhibits the formation of the characteristic brown pigmentation that is a common feature of cotton tissue cultures.

In some cases the medium that supported optimum callus induction did not support optimum callus growth on subculture. Price *et al.* (1977) induced callus from six species of *Gossypium* with 2 mg/l IAA and 1 mg/l kinetin, but they found that the best conditions for subculture varied with the species. In most cases the cytokinin, 2iP, and the auxin, NAA, at species specific concentrations gave good callus growth and maintenance over many subcultures. Smith *et al.* (1977) found that NAA and benzyladenine at 2 mg/l and 1 mg/l, respectively, gave good subculture growth after induction with IAA and kinetin.

From the foregoing it is obvious that a standard method for induction and maintenance of cotton callus has not been established. However, only a few criteria seem essential for callus induction. Namely, a high salt medium such as SH or MS and an auxin source such as IAA, NAA or 2,4-D. Cytokinins are beneficial but may or may not be essential. Use of glucose reduces tissue browning, or, if sucrose is used, a reducing agent can be included in the medium for that purpose. Different cotton species have different requirements for optimum hormone levels. In fact, genotypes within *G. hirsutum* respond differently to regimes designed to induce callus growth (Umbeck and Stewart, unpublished data; Fischer, personal communication).

SUSPENSION CELL CULTURE

Culture of cotton cells in suspension has been a natural outgrowth of callus culture and has been of more general use than callus. Davis *et al.*, (1974) first reported suspension cultures of cotton as an appendum to their paper. The successful culture in suspension of cells derived from anthers was accomplished by Barrow *et al.* (1978). Other reports of *G. hirsutum* cell suspension cultures for specific applications will be discussed below. *G. klotzschianum* (Price and Smith, 1979) and *G. arboreum* (Hsu and Stewart, 1979; unpublished data) cells also have been grown in liquid culture. Generally, media that supported actively growing friable callus on agar also supported cell growth in liquid when agitated for aeration.

Cotton suspension cultures have been used effectively for specific purposes. Frear and Swanson (1975) examined the metabolism of cisanilide, an experimen-

tal herbicide. Ruyack *et al.* (1979) developed both callus and suspension cultures from bacterial blight resistant and susceptible cottons. The cultures responded to the pathogen challenge in the same manner as the source plant; thus, they could be used in place of intact plants to study the mechanisms of resistance. The susceptible culture was subsequently used to examine induction of β-galactosidase activity (Mitchell *et al.*, 1980).

Cultures high and low in gossypol production were developed by Heinstein (1981) to examine the biosynthetic reactions leading to gossypol. Rapidly growing light or white cultures had very little gossypol while dark brown cultures were high in gossypol. There was an inverse relation between growth rate and gossypol synthesis.

ANTHER AND MICROSPORE CULTURE

Cell cultures derived from microspores are particularly useful in generating large numbers of haploid plants, if regeneration can be accomplished. A preliminary report by Barrow (1977) was the first indication of success in establishing anther-derived callus cultures from upland cotton. Subsequently, Barrow *et al.* (1978) reported in detail the initiation of callus from anthers of both *G. hirsutum* and *G. barbadense*. Cell suspension cultures were produced from the *G. hirsutum* callus. Most of the cultures were diploid, but approximately 3 percent contained haploid cells. Haploid cells were found to grow more rapidly than diploid cells in culture. Baluch (1979) also reported callus from anthers of *G. hirsutum* and certain interspecific hybrids. He stated that "the callus seemed to be of microspore origin", but no evidence was presented. No one has obtained plant regeneration from anther-derived callus.

Barrow (personal communication) attempted to establish a medium in which cotton microspores would survive. From that effort a number of essential features are evident. Most importantly, the pH must be above 6.5, with 7.0 being optimal. A high level of K^-, such as is found in MS, is beneficial, but NH_4^+ and Ca^+ are toxic and tolerated only at very low concentration. Other constituents frequently used in media are more or less neutral and are tolerated at standard media concentrations. Long term survival of the microspores requires glucose as the carbohydrate source, but ribose may be helpful also. While microspores survive with the medium adjustments described, cell division has not been observed, even under the influence of various auxins and cytokinins (Barrow, personal communication.)

PROTOPLASTS

The potential for using plant protoplasts for somatic cell hybridization and other genetic engineering techniques has stimulated interest in using the technology for crop improvement. Beasley *et al.* (1975) were the first to indicate the possibility of obtaining protoplast from cotton ovules, but their preliminary work was not carried further. A specific effort to obtain cotton protoplasts was reported by Bhojwani *et al.* (1977). The source of material used in that case was a callus

derived from hypocotyl tissue. A mixture of three enzymes with 11 percent mannitol and inorganic salts was used to remove cell walls. Survival of the protoplasts required a temperature above 25C and the presence of ammonium nitrate and calcium chloride in the medium. Cell wall regeneration occurred, but only about 1-2 percent of the protoplasts were competent to form small colonies of cells. Large callus was not obtained. Khasanov and Butenko (1979) succeeded in isolating protoplasts from cotyledons and leaves, but again, only small colonies could be obtained from surviving protoplasts. They reported that galactose aided in the regeneration of cell wall. Finer and Smith (1982) isolated and cultured protoplasts from *G. klotszschianum*, a wild relative of cotton. Small colonies of about 50 cells were obtained from surviving protoplasts, but these did not grow upon subculture.

One of the interesting developments in cotton protoplast work was that reported by Gould and coworkers (Gould and Dugger, 1982; Gould *et al.*, 1983; Gould, personal communication). Protoplasts were isolated from the epidermal layer of ovules on the day of anthesis. Many of these doubtlessly were fiber initials that could be used for additional research into differentiation. They were also able to isolate subprotoplasts from 14 day-old fiber cells of ovules grown in the culture system of Beasley and Ting (1974). Most of these subprotoplasts were anucleate but were capable of wall synthesis, nevertheless. These isolates also hold some potential for nuclear transfer experiments.

The few reports on cotton protoplasts demonstrate that they can be obtained by more or less standard techniques. On the other hand, adequate techniques are yet to be reported for returning the protoplasts to actively growing cell suspension cultures or calli. A number of commercial companies are actively working with cotton cultures, so it is possible that techniques have been developed that are not in the public domain.

REGENERATION OF PLANTS

One goal in most tissue culture research is to induce cells that have proliferated in an unorganized manner to undergo either embryogenesis or organogenesis so that intact plants are obtained. Until recently this had been a seemingly difficult task in cotton. Root regeneration apparently occurs rather frequently (Sandstedt, 1975; Rani and Bhojwani, 1976; Katterman *et al.*, 1977; Smith *et al.*, 1977; Smith and Price, 1978). Price and Smith (1979) reported somatic embryogenesis in suspension cultures of *Gossypium klotzschianum*. Although the cultures were highly embryogenic after recurrent embryogenic callus selection, the embryoids that developed rarely continued development into a complete plant. The first report for efficient and repeatable regeneration of cotton (*G. hirsutum*) plants from callus was that of Davidonis and Hamilton (1983). Their report highlights a significant feature. As in other species, selection of specific types of callus was necessary; namely, repetitive selection of embryogenic callus from non-embryogenic callus. The addition of GA to the medium in order to promote leaf (shoot?) development may also be a factor. Other conditions were those commonly used in

tissue culture. A number of companies involved in genetic engineering have now indicated that their scientists also have regenerated cotton.

The ground work has been laid for the various techniques needed in order to use tissue culture as a tool for cotton improvement. Although a number of difficulties remain, the technology is progressing rapidly, especially in the private sector. A fully established cycle for callus induction (or protoplast production), cell cloning, resistance selection or other genetic modification, followed by plant regeneration holds great promise for cotton. Not only will cotton producers benefit in terms of more efficient crop production, but the genetic engineering concerns will benefit in terms of technology-derived sales.