# INTERSPECIFIC HYBRIDIZATION IN *GOSSYPIUM* USING *IN-OVULO* EMBRYO CULTURE

by

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ABSTRACT

The transfer of caducous bract trait from hexaploid cotton (G. hirsutum L. X G. armourianum Kearney) to commercial cultivars was attempted through a radical breeding procedure of hybridizing the hexaploid with diploids G. herbaceum L. and G. arboreum L. to get an instant tetraploid. Because of the high degree of incompatibility between the hexaploid and diploid in normal breeding procedure, resulting in abortion of bolls 8-10 days post pollination, in-ovulo embryo culture was used. Although this procedure has been successfully used in producing hybrids between G. hirsutum L. and G. arboreum L., the media described in the literature did not support the growth of the hexaploid-diploid hybrid embryos.

Hybrid bolls were removed from the plant between 2 and 9 days after pollination, and cultured in various media for 28 days. Ovules were then dissected aseptically to reveal any embryo development. Embryos were then placed on a medium used to induce germination.

The nitrogen content and hormone regime of a medium developed for the culture of G. hirsutum ovules was modified in an attempt to promote embryo development. G. herbaceum L. X hexaploid hybrid embryo developed in media of relatively high nitrate (55 mM $KNO_3$) and low ammonium (5 and 10 mM $HN_4NO_3$) content. Hybrid embryos developed in media containing 5 X $10^{-7}$ M IAA rather than the 5 x $10^{-6}$ M suggested for the embryo culture of self-pollinated G. hirsutum L.

# INTRODUCTION

Byssinosis or "brown lungs" is a serious problem in the textile industry. Cotton dust entangled with the fibers cause an allergic reaction in the lungs of susceptible people exposed to this dust. Specifically, it has been demonstrated that it is the dust of the pulverized bracts which is one of the main causes of the disease.

A logical approach to eliminating the disease would be to somehow remove the bract trash from the fibers. This could be approached by the installation of specialized filters designed to remove most of the dust from the air in the textile mills. Industry is reluctant to take such measures because of the high cost. To eliminate one of the causes of the disease, a more direct approach can be taken. If the bracts were removed from the plant before harvest or boll opening, they would not be associated with the fibers at all. This idea has provided incentive for the development of a new type of cotton with caducous bracts.

Hexaploid caducous bract cotton was developed by crossing G. hirsutum L. with the wild, lintless diploid species G. armourianum Kearney. The $F_1$ triploid chromosomes were doubled with colchicine (Muramoto 1977). Fertility was a problem at first, but by selecting for fertility this was overcome, and a caducous bract hexaploid cotton population was established.

A backcross program was initiated to transfer the caducous bract trait from the hexaploids to normal commercial tetraploid cultivars. One method was to cross the hexaploid with commercial tetraploid cultivars. The resulting pentaploid was backcrossed to the recurrent commercial cultivars in a series of crosses and selections, eventually resulting in a tetraploid caducous bract cotton. A more direct method was to try and obtain an immediate tetraploid by crossing the hexaploids with diploid A genome species. The problem is that a high degree of incompatibility exists between the A genome species and the caducous bract hexaploids. Hybrid bolls normally abort 8-10 days post pollination. To overcome this problem, the use of in-ovulo embryo culture was attempted to produce hexaploid-diploid hybrids.

In this study, in-ovulo embryo culture technique was selected to try to overcome the incompatibility between the plants varying in chromosomes numbers. Thus, the goal of this research is to find the right chemical combinations and environment which will give a high probability of growing these normally incompatible hybrids.

# LITERATURE REVIEW

## Interspecific Hybridization in *Gossypium*

Cannon (1903) was among the first to pursue a study of an interspecific hybrid of *Gossypium*. The hybrid plants studied were from the cross *G. barbadense* L. X *G. herbaceum* L. He worked with 10 of these hybrids that he believed to be fertile. However, their degree of fertility was really undetermined since most of the flowers were removed for cytological study. Abnormal sex cells were formed in almost all the anthers. The major objective of Cannon's study was to learn whether meiotic divisions in the hybrid were different from either of the parents. He found that in the hybrid, many of the abnormal nuclei in the anthers degenerate in the archesporial cell or some later stage. In every case, this degeneration was before the first nuclear division.

Various solutions were applied on the stigmatic surface of *G. herbaceum* L. by Desai (1927) in an attempt to obtain hybrid seeds from the cross *G. herbaceum* L. X *G. hirsutum* L. Without any pretreatment of the stigma, one boll was set from 200 crosses. Painting the stigma with an extract of the stigma and petals of *G. hirsutum* L. led to 2 successful bolls from 50 crosses. Two bolls were also obtained from 50 crosses when a solution of 2% cane sugar was applied to the stigma. But by covering the stigma with a solution of 1% citric acid and 0.5% cane sugar, Desai (1927) obtained 20 bolls from 100 crosses. It was

mentioned that the solution applied to the stigma must dry completely if success is to be obtained. He also suggested that an excess of fresh pollen must be applied to the stigma. Due to the presence of wilt in the field, all but one of the hybrids died in the seedling stage.

Zaitzev (1927) was successful in obtaining hybrids of G. herbaceum L. X G. hirsutum L. Success was obtained in this case by first stripping off the petals and staminal column of the female. After pollination, the hybrid bolls appeared to develop quite normally although they were most often filled with empty seed. From over 1000 crosses of this type, two hybrids were obtained.

Nakatomi (1931) was successful in obtaining the hybrids G. hirsutum L. X G. herbaceum L. and G. barbadense L. X G. herbaceum L. From 91 crosses on G. hirsutum L. by G. herbaceum L., 2 plants were obtained. Likewise, one barbadense-herbaceum hybrid was obtained from 28 cross pollinations. No hybrids were secured using the diploid as the female parent. Nakatomi found that the hybrids grew very vigorously, developed abnormal pollen grains, and were completely sterile.

Using Asiatic G. arboreum L. as a female parent, Harland (1932) met no success in the cross G. arboreum L. X G. barbadense L. Sound seed were obtained, but they contained only shriveled, minute embryos. In the reciprocal cross, two hybrid plants were produced. Harland found that these hybrids were female sterile, but they did possess some functional pollen. The variety of G. arboreum L. used in this cross

was "Sanguinea" which carries a red leaf pigment. In a backcross G. barbadense L. X (G. arboreum L. X G. barbadense L.), 5 out of 8 of the plants obtained contained the red pigmentation of "Sanguinea." This indicated that the particular arboreum chromosome carrying the gene for red pigment was present in the functional pollen. Upon testing, these 8 backcross plants proved to be fertile on the male and female side. Harland suggests "one or more of the Asiatic chromosomes may be capable of functioning in a New World plant body."

Feng (1935) was successful in obtaining a hybrid of G. herbaceum L. X G. hirsutum L. From 1017 crosses of this type, one hybrid plant was obtained. In the reciprocal cross, 5 hybrids were obtained from 691 crosses. In both crosses, though incompatibility existed, pollen tubes were noted as growing at the base of the style after 24 hours.

Beasley (1940a) also attempted hybridizing American and Asiatic cotton. By staining cut styles at various times after pollination, Beasley found that pollen tubes entered more than half the ovules in both crosses. Hybrid endosperm development began after 4-6 days, but its growth rate was slow compared to self-pollinated endosperm. Beasley tried mixed pollination to help bolls stay on the plant. Using 6-12 grains of pollen from the maternal parent carrying a dominant red leaf gene, and then flooding the stigma with an excess of pollen from the hybrid male parent, hybrids could easily be detected. This mixed pollination accompanied with a miniature seed technique of embryo culture enabled Beasley to produce hybrids from New World X Asiatic cottons.

Beasley (1940b) was also interested in hybridizing other species of Gossypium. A specific, detailed treatment for inducing polyploidy in Gossypium with colchicine is given in this paper. Treating the diploid G. herbaceum L. with colchicine to induce a tetraploid, Beasley found that fiber length was 1.4 times greater, and fiber diameter 1.3 times greater in the tetraploid than in the diploid. In a hexaploid made by doubling the chromosome of an $F_1$ G. hirsutum L. X G. harknessii Brandg. hybrid, 80% viable pollen was noted. Beasley also obtained hexaploids from an $F_1$ of G. hirsutum L. X G. sturtii F. Muell. Although G. sturtii F. Muell. has extremely short fibers, the hexaploid was found to be relatively fertile and had fibers only slightly shorter than G. hirsutum L.

Two synthetic tetraploid species were produced by Brown and Menzel (1950). These were obtained by initially crossing G. hirsutum L. with either G. arboreum L. or G. herbaceum L. The resulting $F_1$ triploid was doubled with colchicine, then crossed with G. harknesii Brandg. Brown and Menzel found that crossing G. arboreum L. and G. harknessii Brandg. directly yielded no sound seed. However, by crossing hexaploids and diploids, these two species could be brought together in a hybrid.

In a later paper, Brown and Menzel (1952) looked at many other hexaploid X diploid, and hexaploid X tetraploid hybrids. Hexaploid hybrids made from crossing G. hirsutum L. with either herbaceum, arboreum, anomalum, or thurberi and then doubling their chromosomes all proved to be highly self-fertile. They found that a G. armourianum Kearney hexaploid was very self-sterile. Crossing any of these hexaploids with

G. hirsutum L. yielded pentaploids. It was found that these pentaploids were all somewhat fertile when backcrossed to G. hirsutum L. This was an indication that this method of crossing might be useful in introducing chromosomes or chromosome parts from wild diploids into G. hirsutum L.

Weaver (1955) looked at a number of interspecific crosses in Gossypium. In the cross G. hirsutum L. X G. raimondii Ulb., Weaver observed that the endosperm started to become cellular after 10-11 days. Sixteen days after pollination, the endosperm of the hybrid appeared completely cellular and normal in development when compared with self-pollinated G. hirsutum L. In the cross G. hirsutum L. X hirsutum-thurberi hexaploid, the embryo did not differentiate properly. Embryo development appeared similar to that of the cross G. arboreum L. X G. hirsutum L. Although embryo differentiation was abnormal, Weaver observed that the growth rate was only slightly slower than that of selfed G. hirsutum L. Other crosses which Weaver made at the time were G. herbaceum L. X G. hirsutum L., G. arboreum L. X G. raimondii Ulb., and G. arboreum L. X G. anomalum Wawr. et Peyr. Only histological observations of the degenerating embryos were made for these particular crosses.

Hybrid embryos from the cross G. hirsutum L. X G. arboreum L. progressed at about the same rate as self-pollinated G. hirsutum L. for the first 10 days after pollination (Weaver 1957). After 10 days, the hybrids' growth was retarded. The hybrid embryos usually reached a maximum length of 0.3 mm at day 15. The endosperm of the hybrid appeared normal for the first 5-6 days. After this time, the endosperm became

cellular and ceased to grow. Weaver's work showed that only 24% of the ovules were actually fertilized by G. arboreum L. One triploid hybrid was produced by single pollination. By using mixed pollination and embryo culture, over 25 hybrids were produced.

In the reciprocal cross G. arboreum L. X G. hirsutum L., Weaver (1958) again observed the growth rate of the hybrid was normal with respect to G. arboreum L. embryos until 10 days post pollination. In this cross, the hybrid embryos never developed good cotyledons. They reached a maximum size approximately 25 days post pollination. For the first 4-5 days, free nuclear divisions occurred in the endosperm. Usually after day 5, many nuclei showed abnormal divisions. After 8 days, the endosperm nuclei were of abnormal size, highly vacuolated, and tended to clump together. Fourteen days after pollination, the endosperm was in the final stages of degeneration. Embryo abortion in this cross appeared to be due to a lack of differentiation, and not the embryo-endosperm antagonism which was observed in the reciprocal cross.

Irvine (1957) studied ovule development of the hybrid G. hirsutum L. X G. klotzschianum Andress. It was noted that the glands which normally develop in self-pollinated Gossypium embryos were absent in this hybrid. The hybrid embryos showed lower concentrations of metabolites than either of the self-pollinated parental embryos. Irvine observed that starch was stored in the integumentary cells as the hybrid embryo degenerates. Hypothesizing as to why the embryo degenerates, Irvine thought that : (1) an essential enzyme was either lacking or

inhibited; or (2) due to the absence of glands, gossypol or its precursors accumulated in the hybrid tissue and caused localized spots of necrosis that spread throughout the embryo.

Joshi and Pundir (1966) also worked with the cross G. arboreum L. X G. hirsutum L. Like Weaver (1958) they found that free nuclear divisions occurred in the endosperm for the first 4 days. After 5-6 days, the endosperm nuclei became abnormally large and dumbbell-shaped. The cytoplasm became highly vacuolated and nuclei tended to clump together. By excising the hybrid embryos at the late globular-heart stage and placing on Heller's (1953) medium supplemented with 1000 ppm casein hydrolysate, 5 ppm gibberellic acid (GA), and 1 ppm kinetin (Kin), miniature seedlings were produced after 36 days in culture.

Pundir (1972) summed up some of the developmental differences which exist in G. arboreum L., G. hirsutum L. and their reciprocal crosses. Seven days after pollination in G. arboreum L., the embryo had reached the globular stage. Wall formation in the endosperm was initiated at the micropylar end 7-8 days post pollination. After 16-18 days, the endosperm was totally cellular. In G. hirsutum L., wall formation in the endosperm was evident at the end of 10 days. The embryo was found to be differentiated even before the endosperm became cellular at the chalazal end. With the cross G. arboreum L. X. G. hirsutum L. free nuclear divisions were noted up to 7-8 days post pollination. Subsequent growth of the endosperm was abnormal. Abnormally large, dumbbell-shaped nuclei filled the embryo sac. Cell wall formation was

never observed in the endosperm. Although globular proembryos were formed within 5 days post pollination, further growth showed abnormalities. In the reciprocal cross, fertilization was much slower. Pundir found that 3-5 days were required between pollination and fertilization. In this cross, cell walls were observed forming in the endosperm 8-10 days after pollination. Globular proembryos form after 6-7 days, but embryo growth beyond the late globular stage was arrested.

Three breeding stocks of hexaploid cotton were released by Muramoto (1975). All were developed by crossing G. hirsutum L. with G. sturtianum F. Muell. and doubling the chromosome number of the $F_1$ hybrid with colchicine. The varieties were described as having strong stalks and short internodes. The plants were considerably more vigorous than either G. hirsutum L. or G. barbadense L. The flowers were very large and had a bright reddish-mauve color. These hexaploids were described as having good cold tolerance, and partial tolerance to leaf perforators. The hexaploid seeds were small, and possessed short, very strong fibers.

All possible crosses between the tetraploids G. hirsutum L., G. barbadense L. and the Old World diploids G. arboreum L., G. herbaceum L. were evaluated by Stewart and Hsu (1978) in an attempt to find the proper phytohormone regimes. The basal medium used was developed by Beasley and Ting (1973) and was supplemented with 15 mM $NH_4Cl$. These workers found that in the cross G. hirsutum L. X G. arboreum L., any addition of GA to the medium proved detrimental to embryo survival.

It was also shown that embryo survival was very dependent on the embryo length.

In an attempt to remove bract trash from the lint, Muramoto (1980) developed hexaploids by doubling the chromosome number of the $F_1$ hybrid G. hirsutum L. X G. armourianum Kearney. The caducous bract trait of G. armourianum Kearney appeared in the hexaploid population. In contrast to Brown and Menzel's (1952) finding that the hirsutum-armourianum hexaploid was sterile, Muramoto found that this hexaploid was relatively fertile after a few years of natural selection in an open-pollinated field. Many selections of the caducous bract hexaploid had fiber exceeding one inch. The average fiber strength of these hexaploids was considerably stronger than the commercial variety Delta Pine 16. Pentaploids have been obtained by backcrossing with G. hirsutum L. Crossing these hexaploids with the Old World diploids G. herbaceum L. or G. arboreum L. leads to embryo abortion. The literature search showed no record of research to hybridize the diploids G. arboreum L. and G. herbaceum L. with the hirsutum-armourianum hybrid. This current course of study is an attempt to hybridize the hexaploid and Old World diploids through the use of in-ovulo embryo culture.

## Techniques of Embryo Culture

There are four major techniques being used to raise embryos to viable seedlings in vitro. These are: (1) true embryo culture; (2) miniature seed technique; (3) in-ovulo embryo culture; and (4) the nurse endosperm technique. The examples given below are for specific plant systems in which one or more of these techniques are employed.

Laibach (1929) may be considered one of the first to raise viable plants from a relatively incompatible hybrid cross using true embryo culture. Immature hybrid embryos from Linum perenne L. X L. austriacum L. were excised and aseptically placed on cotton soaked with 10-15% cane sugar. After an extended period of time, growth was finally realized. The embryos became white and plump; they were then able to be germinated on moist blotter paper.

Skirm (1942) realized the possibilities of using embryo culture to obtain interspecific hybrids of Lilium and Prunus. From Lilium, Skirm was able to germinate approximately 300 hybrid embryos. From 564 interspecific crosses of Prunus, 414 embryos were obtained and germinated. Skirm stressed that there was an optimum time for removal and culture of these embryos. Once an embryo begins to abort, it is impossible to stop this abortion by culturing. And though a minimum size for the culture of embryos had not been established, the limit was determined by the difficulty of embryo removal and the quality of the nutrient medium.

Overbeck, Conklin and Blakeslee (1942) found that self-pollinated embryos of Datura stramonium L. could be successfully grown on artificial medium. An agar containing medium of 1% dextrose, mineral salts and vitamins described by Tukey (1934), plus non-autoclaved coconut milk was found to allow for in vitro embryo development from heart to the torpedo stage. Small ovules were placed between two glass slides, and after six days, embryos were removed and placed on the sterile nutrient medium. Within six days at 25°C, a 500-fold increase in volume was

noted. Viable seedlings were produced by transferring the embryos after one week, in culture to the same medium minus coconut milk.

Blakeslee and Satina (1944) dissected hybrid embryos of Datura using methods previously described by Overbeck et al. (1942). Although no real mention of the exact medium was made, these workers found that coconut milk could be excluded from the media if filter sterilized malt extract was put in its place. Eleven new Datura hybrids were raised using the embryo culture method. Using the same method, these workers were able to increase germination percentage of certain species which characteristically yield 0.1% germination in nature.

Interspecific hybrids of Datura were also obtained by McLean (1946). Working with D. ceratocaula Ort., abortive embryos formed in every case when the plant was used as a pollen parent with nine other species of Datura. When D. ceratocaula Ort. was used as the female, abortive embryos were found in only three of the nine possible crosses. Using embryo culture, abortion was overcome in eight of the nine crosses involving it as the female. McLean also realized the value of embryo culture in germinating the seeds of D. ceratocaula Ort. Normal greenhouse conditions yielded 0.1% germination. But by culturing embryos, the germination was increased to approximately 70%.

Using a medium developed by Randolph and Cox (1943), supplemented with 2% sucrose and 0.8% agar, Kent and Brink (1947) noted germination of 10-15 day old Hordeum embryos after 2-3 days in culture. Noting that most in vitro embryo culture produced small, spindly seedlings, these

workers were concerned with developing a chemical environment which further promoted an embryonic type of growth. They found that the addition of casein hydrolysate to the medium prevented precocious germination. With casein hydrolysate in the medium, embryos grew appreciably in size before they attempted to germinate.

*In vitro* embryo culture has also been successfully employed in a number of other plant systems. Among these are *Portulaca oleraceae* L. (White 1934), *Prunus persica* Stokes, and *P. avium* L. (Tukey 1933, 1934); *Lactuca canadensis* L., *Chrysanthemum leucanthemum* L., *Lycopersicon esculentum* P. Miller, *Nicotiana tabacum* L. (LaRue 1936); and *Zizania aquatica* L. (LaRue and Avery 1938).

Culturing plant ovules is another way to grow embryos *in vitro*. The technique is quite similar to true embryo culture, but in this case the embryo is left intact inside the developing seed. It is hoped that embryonic development continues after the placement of the ovule into the nutrient medium. When the embryo is of mature size, it is removed from the medium and placed in an environment suitable for germination.

Maheshwari (1958) was successful in using ovule culture to rear *Papaver somniferum* L. proembryos into seedlings. Upon opening the capsule, the ovules were directly placed in Nitsch's (1951) medium, supplemented with vitamins. The embryos developed to maturity from the two-cell stage inside the ovule and proceeded to germinate after 23 days in culture. In nature, ovules mature 18 days after fertilization.

*In-ovulo* embryo culture was used by Kapoor (1959) to successfully grow two day post-pollinated *Zephyranthes* embryos to maturity.

In nature, the time between pollination and ovule maturation is 16 days. Using Nitsch's (1951) medium supplemented with sucrose and 25% coconut milk, mature embryos were formed after 12 days in culture. These embryos germinated after 20 days and developed into apparently normal seedlings.

Beasley and Ting (1973) developed a system for growing Gossypium hirsutum L. ovules for two to four days post anthesis. These workers were primarily concerned with the effects of phytohormones on fiber development. However, their method of culture is now a standard for in-ovulo embryo culture of cotton.

Eid, De Langhe and Waterkeyn (1973) were looking for a medium which would support the growth of G. hirsutum L. ovules. They tested and modified media of White (1943), Nitsch (1951), Heller (1953), and Murashige and Skoog (1962) in an attempt to find the right chemical environment. Liquid and agar media were tried, as well as maintaining cultures on stationary or rotary shaker. Three-day-old ovules showed an intense proliferation of the integuments and a degeneration of the endosperm. From ovules cultured 10 days post anthesis, the heart stage was obtained after 50 days on White's (1943) medium. These workers found that in ovules 10, 15, and 20 days old, balanced growth was obtained on media lacking plant growth regulators. Eid et al. (1973) stated that, "The use of ovule culture to grow cotton embryos is simple and gives reproducible results in contrast to pure embryo culture."

Stewart and Hsu (1977) found that the addition of 15 mM $NH_4NO_3$ to the medium developed by Beasley and Ting (1973) greatly enhanced the

ability of self-pollinated G. hirsutum L. ovules to survive. After an initial 8-10 week culture period in this medium, the embryos were dissected from their ovules and placed on a medium to promote germination. This later medium allowed for cotyledon expansion and root growth. When the seedlings were large enough, they were transferred to sterile soil and placed in the greenhouse.

Culturing weak but mature embryos has come to be known as the miniature seed technique. The technique was employed by Beasley (1940a) in an attempt to rear hybrids between G. hirsutum L. X. G. arboreum L. Hybrid bolls were allowed to develop to maturity on the plant. After the boll matures, embryos from the shrunken hybrid seed were dissected and aseptically placed on White's (1934) medium. Beasley found that it was much easier to produce hybrid seedlings by this miniature seed technique than with normal seed germination procedures.

One other type of embryo culture is the nurse endosperm technique. A thorough, detailed description of this technique is offered by Williams and De Lautour (1980). Basically, a hybrid embryo is excised from its maternal tissue and inserted in place of a self-pollinated embryo. The hybrid embryo now lies in contact with normal "nurse" endosperm. This nurse endosperm is frequently from the maternal parent of the hybrid cross. The hybrid embryo/nurse endosperm is then placed on nutrient medium and allowed to incubate.

Zeibur and Brink (1951) were among the first to experiment with nurse endosperm. Working with self-pollinated Hordeum vulgare L., they

found no stimulative response to either coconut milk or malt extract when these were included in the medium. Onc percent casein hydrolysate in the medium proved to be inhibitory to the embryos. But by surrounding the young embryos with developing Hordeum endosperm, embryonic growth was continued and was faster than the growth of embryos without the nurse endosperm. Seedlings were ready to be transferred to soil at an earlier date using this technique.

Using the nurse endosperm technique, Kruse (1974) was able to secure hybrids of Hordeum vulgare L. crossed with Triticum, Agropyron, and Secale. In each case, the nurse endosperm was from the maternal parent. Kruse found that hybrid barley embryos as small as 0.2 mm could be successfully cultured this way. Best results were obtained by using endosperm 2-5 days older than the embryos and by using embryos greater than 1.0 mm in length. In all, 28 plants out of 40 cultured embryos grew to maturity in the Hordeum X. Triticum cross. The cross involving Hordeum and Agropyron yielded 83 plants out of 167 cultured embryos. And 2 plants were obtained when Hordeum was crossed with Secale out of a total of 169 embryos cultured.

De Lautour, Jones and Ross (1978) were able to produce 4 mature hybrids from the cross Lotus pedunculatus Cav. X L. tenuis Waldst. et Kit. using the nurse endosperm technique. The endosperm in these successful cultures came from Lotus pedunculatus Cav. in three cases and Ulex europaeus L. in one case. This represents 10.5% of the embryos cultured. The success using this technique was much higher than the

0.1% success realized by Bent (1962) using pure embryo culture with these same species.

Williams and De Lautour (1980) successfully used the nurse endosperm technique to create 6 new interspecific hybrids in the genera Trifolium and Ornithopus. The hybrid embryos were dissected at approximately two weeks post pollination and placed in contact with nurse endosperm which was 8-10 days old. Although the highest percentage of successful cultures for any of the crosses was 8.3%, the workers stated that this was much greater success than was realized with normal breeding procedures or pure embryo culture.

## Factors Associated with Incompatible Crosses

Embryo abortion in Medicago sativa L. was studied by Brink and Copper (1939). They reported self-pollinated ovules 48-72 hours post-pollination typically showed highly vacuolated cells adjacent to the chalazel end of the seed. There appeared to be extensive meristematic activity of the inner integument near the chalazel end of the ovule. In most cases of embryo abortion, the endosperm failed to grow at the same rate as the surrounding maternal tissue. This type of breakdown was termed as somatoplastic sterility. They found that essentially the maternal tissues divide much more rapidly than the endosperm. The nutrient supply shifted more toward the inner integuments and eveutually the embryo starved.

Somatoplastic sterility was also found to be a cause of seed failure in different interspecific crosses of Nicotinia (Cooper and

Brink 1940). The nucellus and endosperm were the two tissues observed to be responsible for seed failure. In the cross N. rustica L. X N. glutinosa L., these workers found that the nucellus increased greatly in size after fertilization. Opposed to the single layer of cells of the nucellus found in self-pollinated Nicotinia, it was observed that the nucellus became meristematic at about the time the endosperm reached the 8-cell stage. The integumentary cells failed to differentiate into conducting elements in the hybrid ovules. This led to the eventual closure of the endosperm's nutrient supply.

These same factors associated with seed failure appeared in another Nicotinia hybrid study (Brink and Cooper 1941). The differences in seed failure between the cross N. rustica L. X N. glutinosa L. and N. rustica L. X N. tabacum L. were quantitative with respect to retarded endosperm growth, hyperplasia of the nucellus, and the failure of integumentary cells to differentiate into conducting elements. It appeared that the cross N. rustica L. X. N. glutinosa L. was much more incompatible than the cross N. rustica L. X N. tabacum L. Brink and Cooper looked at the percentages of seeds having the endosperm cut off by an overgrowth of the nucellus. This was essentially zero for self-pollinated N. rustica L. Twenth-two percent of the seed from the cross N. rustica L. X N. tabacum L. showed the characteristic overgrowth of the nucellus. This overgrowth was 76% of all seed examined in the cross N. rustica L. X N. glutinosa L.

Working with a 4n X 2n Datura stramonium L. cross, Sansome, Satina and Blakeslee (1942) found disintegration of the embryo sac when

the proembryo was 4-6 celled. Fertilization was found to occur with high frequency approximately 3 days after pollination. After six rounds of division of the endosperm, degeneration of the embryo began. This was found to be 5-6 days post pollination. Accompanying the first visible signs of endosperm breakdown, Sansome et al. noted an enlargement of the endothelial cells. These cells frequently became meristematic and were sometimes found to completely fill the embryo sac.

Fertilization was found to occur more rapidly in the cross Elymus virgenicus L. X Agropyron repens (L.) Beauv. than when E. virginicus L. was self-pollinated (Beaudry 1951). In the hybrid cross, fertilization usually occurred after 18 hours. Twenty-four to 36 hours were required for fertilization to occur in the selfed controls. The growth rates of hybrid embryo and endosperm were initially higher than the controls. After approximately 48 hours post pollination, abnormal endosperm nuclei were found clustered in the midsection of the developing seed. The endosperm began to disintegrate 60 hours post pollination. At this same time, the proembryos were usually 14-celled. Growth of the hybrid embryo continued for some time after the endosperm breakdown. Beaudry found that the embryos may continue growth even after total endosperm disintegration. The nutrient supply was believed to have come from the nucellus. But when both the endosperm and the nucellus were gone, death of the embryo soon followed.

Buell (1953) studied seed failure in reciprocal crosses of Dianthus chinensis L. and D. plumarius L. Most of the hybrid embryos

disintegrated 9-10 days after pollination in the cross D. chinensis L. X D. plumarius L. The basal cells of the embryo appeared more vacuolated than in self-pollinated D. chinensis L. In contrast to the self-pollinated endosperm of the controls, nearly half of the hybrid ovules studied contained small endosperm nuclei that frequently fused. This was noted 3-4 days post-pollination. The nucellus of self-pollinated Dianthus species enlarge to form the major source of food storage for the mature ovule. This was not found to be the case in the cross D. chinensis L. X D. plumarius L. The nucellus remained the same size and form that it was in at the time of fertilization. In the reciprocal cross, there appeared to be a much greater rate of fusion of endosperm nuclei. Occasionally all of the endosperm nuclei were found to be fused into 3 or 4 multinucleate masses. The division rate of the endosperm appeared much slower than self-pollinated D. plumarius L. Buell suspected that it was this abnormal development of the endosperm which was responsible for the eventual abortion of the hybrid embryo.

Diploid and hexaploid species of Solanum were crossed in an attempt to produce a fertile tetraploid (Beamish 1955). The hexaploid S. demissum Lindl. was crossed with four diploid species and seed development was studied. Similarities in seed failure existed in all 4 of the hexaploid-diploid crosses. The differences were mainly in the speed which embryos degenerated. The first similarity noted was the delay of fertilization caused by slow pollen tube growth. When fertilization did finally occur, the endosperm failed to enlarge and

differentiate in a normal manner. Sooner or later, the endosperm ceased to grow, and finally disintegrated. Usually, Beamish found a hyperplasia of the endothelium. This was accompanied by the ending of cell divisions in the integument. These later similarities, hyperplasia of endothelium and ceasation of cellular division of the integument usually occur after endosperm breakdown. Embryo abortion was usually found to occur within the next few days.

Other interspecific crosses of Solanum were made by Lee and Cooper (1958) to follow the course of seed development which eventually led to seed failure. A slow rate of endosperm growth was the first abnormality observed in the interspecific crosses. In the self-pollinated controls, 40 endosperm cells usually accompany a two-celled proembryo. But in a two-celled proembryo of a hybrid, Lee and Cooper usually found only 6-8 endosperm cells. Endosperm cells lie immediately adjacent to the endothelium at the chalazel and micropyle ends of the self-pollinated controls. This suggested a possible passageway for nutrients from the vascular elements of the integument. This differentiation of the endosperm cells in the hybrid ovule was obviously lacking. Comparing embryo development of S. pinnatisectum Dun. crossed with six other diploid species, Lee and Cooper found that hybrid embryos were all completely degenerated after 25 days post pollination. Self-pollinated embryos of these species were usually at advanced stages of cotyledon development at this age.

An anatomical and biochemical analysis was undertaken to investigate the causes of interspecific incompatibility in the genus Pinus.

McWilliam (1959) hypothesized that it was chemical differences which caused incompatibility and seed failure in a hybrid between Austrian (P. nigra L.) and red pine (P. resinosa L.). Five sugars were identified in the ovules of both species. Sucrose was found to be the major sugar present. All others were found to be equal in concentration in both species. Though the same 15 amino acids were found in both Austrian and red pine, differences in concentrations existed. Austrian pine ovules contained 5 times the amount of arginine found in red pine. Concentrations of aspartic acid, alanine, glutamic acid, and lysine were also different. To McWilliam, it appeared that it was chemical differences in the ovular tissues, through which the foreign pollen tubes must pass, which was the primary cause of incompatibility. In 70% of the ovules studied, either no germination, or slow ineffective pollen tube growth was noted.

Cotyledonary cell discoloration and distortion was noted by Phillips and Reid (1975) 10-15 days after anthesis in the cross G. hirsutum L. X G. klotzschianum Andress. Single cells and later clusters of cells in the cotyledons of the embryo became opaque and contracted. Thirteen days after fertilization, all the cotyledonary cells were observed to be necrotic. The maternal tissues of the hybrid ovule developed normally. Typically, embryos collapsed and dissintegrated at this stage. Occasionally though, Phillips and Reid observed that the radicles continued to develop normally although the cotyledons were completely gone.

Phillips (1977) observed that interspecific incompatibility in certain Gossypium species to be due to genetic embryo or seedling-lethal traits. Hybrids from the cross G. hirsutum L. X G. klotzschianum Andress. were embryo-lethal at 17-19 days post fertilization. A G. barbadense L. X G. klotzschianum Andress. hybrid was seedling-lethal at the 1-2 leaf stage. Phillips observed that the lethality of these hybrids was temperature-dependent. The macroscopic leaf and cotyledon lesions, necrotic cells, and degenerate mitochondria could be prevented by growing the hybrid at elevated temperatures. The hybrids could be prevented from dying by keeping seedlings in a growth chamber at 40°C. Likewise, the embryo lethal from G. hirsutum L. X G. klotzschianum Andress. could be kept alive by excising the embryo and placement on nutrient medium at 40°C.

## Nutrients Found to Stimulate Embryo Growth in Vitro

A simple medium developed by White (1934) consisting of salts, vitamins, and sucrose was used by LaRue and Avery (1938) to culture embryos of Zizania aquatica L. Seedlings were obtained through precocious germination when yeast extract was deleted from the medium. Best results were obtained with embryos that were 0.4 mm or larger in length when cultured. These workers' major concern was comparing cotyledon development in vitro and in vivo. Cotyledonary growth ceased after placement of the embryos in nutrient medium. Shoots and roots did elongate to allow precocious germination into a small seedling.

Zeibur et al. (1950) culturing Hordeum embryos on a medium containing salts, 2% sucrose, and agar found precocious germination within 3-4 days after embryo placement on the medium. They found that the addition of 1% casein hydrolysate to their basal medium prevented precocious germination and allowed for continued embryonic growth. The inhibition of germination was found to be due to the high osmotic pressure of the casein hydrolysate produced by sodium chloride and amino acids.

Using normal G. hirsutum L. embryos on a basal medium recommended by White (1943), Lofland (1950) attempted to culture embryos at three levels of development. Altering the pH of the medium between 5.0 and 8.0 had no effect on embryo development. Likewise, no differences in embryo growth were noted at each level of development when temperature was varied between 20° and 30°C. The addition of tomato extract, coconut milk, casein hydrolysate, sodium nucleate, or an extract made from ovules of developing bolls did not seem to stimulate growth of the embryos. Supplementing the basal medium with 0.5% malt extract did seem to favor embryo development. Embryos cultured from 27-day-old ovules showed signs of activity after 2-3 days on the medium. When cultured at this level of development, embryos usually produced normal seedlings. Culturing embryos from 20-day-old ovules allowed fewer embryos to develop, and a much higher percentage of abnormal forms produced. Small, spindly seedlings may be produced, but these typically do not survive. Lofland observed that, as a general rule, growth was very slow or there was no growth at all from embryos cultured from 15-day-old ovules.

Knowing that precocious germination of Hordeum vulgare L., embryos were suppressed by casein hydrolysate, Zeibur and Brink (1951) attempted to improve in vitro embryonic growth. In contrast to Datura embryos, coconut milk or malt extract did not seem to stimulate growth of barley embryos. Improved growth was obtained by surrounding the excised embryos with Hordeum vulgare L. endosperm before placement into the nutrient medium. Using this technique, seedlings were able to be transferred to soil at an earlier date than those grown in culture without endosperm.

Joshi (1960) varied many nutrients in an attempt to improve the in vitro growth of cotton ovules. The age of ovules used in his work was 6 days post pollination. Using his basal medium supplemented with 500 ppm casein hydrolysate, embryos reached the dicotyledonous stage after 81 days in culture. Increasing the casein hydrolyate concentration to 2000 ppm, 75-80 days were required to reach this same stage of development. Joshi observed that the addition of 5 ppm GA or 1 ppm Kin to the basal medium did not significantly improve embryo growth. Maximum growth of the cotton embryos was observed when 5 ppm GA was added to the basal medium in combination with 1000 ppm casein hydrolysate. All media used in this work contained 0.7% agar. Cultures were held at 23-25$^{o}$C in the light and at a pH of 5.5.

Mauney (1961) observed that the deletion of agar from a nutrient medium resulted in the death of G. hirsutum L. embryos. However, embryos placed in the surface moisture on an agar medium developed more

rapidly than those placed on a dry agar surface. In his experiments, 90°F and partial light appeared to be the optimum for embryo growth and development. Embryos which were cultured in darkness developed chlorophyll slowly when transferred to light. By increasing the level of calcium to $3 \times 10^{-3}$ M, Mauney observed that the growth rate of the embryos increased substantially. The embryos developed a healthier, deeper green appearance. The plant hormones indole acetic acid (IAA), GA, and Kin were observed to be inhibitory, even at low concentrations. Most importantly, the osmotic pressure of the medium was critical. The osmotic pressure of the expressed ovular sap was found to decrease as the seed matures. Sodium chloride was added to the medium at the level corresponding to the osmotic value of the ovular sap at the initial culture period. The level of NaCl was decreased in media used to subculture the embryos at a later stage of development.

The effects of sodium, ammonium, and calcium salts of citrate, malate, and succinate were analyzed for their effects on the *in vitro* development of self-pollinated *G. hirsutum* L. embryos (Mauney, Chappell and Ward 1967). Embryo growth was evaluated as an increase in embryo length by means of an ocular micrometer. Color and morphological conformations were also noted. The basal medium used was the same as described by Mauney (1961). The addition of sodium citrate, malate, or succinate at 0.01 mg/ml appeared to stimulate growth slightly. Higher concentrations of these salts proved to be inhibitory. Calcium and ammonium malate stimulated growth even at concentrations up to 4 mg/ml

Beasley and Ting (1973) tested the effects of plant hormones and different forms of nitrogen in the *in vitro* fiber development of *G. hirsutum* L. A high salt and sucrose medium such as the one described by Marashige and Skoog (1962) was found necessary to support expansion and fiber elongation. Fifty millimolar total nitrogen was set as the control for further testing. Varying the $NH_4NO_3$:$KNO_3$ ratio by increasing $KNO_3$ and proportionately decreasing $NH_4NO_3$ to keep total nitrogen at 50 mM, these workers observed an increase in both ovule and fiber growth. Callusing and browning of the ovules was less with this treatment also. Kinetin and abscissic acid (ABA) essentially stopped fiber production. When fiber production was low, there was typically an increase in browning of the ovules and the medium. Beasley and Ting noted that fiber development increased with increasing GA levels to $5 \times 10^{-6}$ M. They also remarked that the predisposition of the parent plant greatly affects the capacity of ovules to grow *in vitro*. The response of the ovule to various hormone or nitrogen treatments was also affected by the predisposition of the parent plant.

A balance between embryo and endosperm growth was believed necessary for successful culture of cotton ovules (Eid et al. 1973). These workers believed that the young proembryos were sensitive to physical conditions of the medium. Ovules 5, 10, 15 and 20 days post-anthesis were used for this study. Coconut milk or GA seemed to improve embryo growth in 5-day-old ovules. Ten-day-old ovules were not stimulated by the addition of these substances to any of the basal media used.

The results showed that ovules 10, 15 and 20 days post anthesis exhibited balanced growth in basal media lacking plant hormones. However, hormones were necessary to support the growth of 5-day post-anthesis ovules.

Stewart and Hsu (1978) did extensive work testing different hormone regimes on the embryo development in vitro of the cross between G. hirsutum L. X G. arboreum L. Ovules were harvested and cultured 2-4 days post anthesis. Like Beasley and Ting (1973), these workers found that GA promoted fiber production. Fiber growth did help keep the ovules afloat on the liquid medium. But the addition of GA to the basal medium always decreased the yield of viable embryos. Experimental results were evaluated in three ways. These were: (1) percentage of ovules with embryos; (2) percent germinated embryos; and (3) ungerminated embryo size. The length of ungerminated embryos was not affected by the addition of IAA to the basal medium. Kinetin, on the other hand, reduced the length of ungerminated embryos when added to the basal medium. This point was crucial since a direct correlation was found between embryo size and survivability at the time when embryos were placed on germination medium.

## MATERIALS AND METHODS

The basic medium used and modified for evaluation of embryo growth was developed by Beasley and Ting (1973). The chemical constituents and concentrations as described by them are listed in Table I of the Appendix. All other media and stock solutions are also listed in table form in the Appendix.

One modification of this medium from the original formulation was the carbon source. Glucose and fructose were never used in this study. They were replaced exclusively by sucrose. The modified medium used as the control in this study is shown in Table II of the Appendix.

Two basic modifications of the nitrogen content of this medium were used. First, the ratio of reduced:non-reduced nitrogen was altered from the original formulation. Secondly, casein hydrolysate was added to the medium at various concentrations.

A stock solution of $KNO_3$ was prepared by dissolving 50 grams of $KNO_3$ into 1 liter of distilled deionized water (DDW). Likewise, the $NH_4NO_3$ stock solution had a concentration of 48 g/L. The milliliters of $KNO_3$ stock used in various amounts of medium having known non-reduced nitrogen are shown in Table III of the Appendix. Table IV of the Appendix shows the amounts of $NH_4NO_3$ stock used to make various amounts of medium at known reduced nitrogen levels.

Stock solutions for the major and minor salts differed from those described originally by Beasley and Ting (1973). One stock for

the major elements and one other for the minor elements was found to be sufficient. This also proved to save time preparing the medium. Stocks for major and minor salts are shown in Tables V and VI, respectively. When media with altered levels of $KNO_3$ or $NH_4NO_3$ were tested, a separate major stock solution was prepared. This was identical to the stock shown in Table V of the Appendix except that $KNO_3$ and $NH_4NO_3$ were left out.

Plant hormones were dissolved in DDW and stored as stock solutions in the refrigerator. Indole acetic acid (IAA) stock solution was 1 mg/ml. Gibberellic acid (GA) and kinetion (Kin.) were both stored as 0.2 mg/ml stocks. Hormones were added to the medium prior to autoclaving.

Casein hydrolysate and myo-inositol were weighed and used fresh with each batch of medium. Both compounds were dissolved separately in small quantities of DDW. Fresh stock solutions were prepared if different concentrations were to be tested in the same batch of medium. Both compounds were added to the medium prior to autoclaving.

(Fe/EDTA) was kept as a stock solution under refrigeration. The stock was prepared by thoroughly dissolving 2.5 g of $FeSO_4 \cdot 7H_2O$ in 400 ml DDW. Next, 3.36 g of NaEDTA was dissolved in the solution. The stock was then brought to a boil on a heating/stirring machine. The heat was then turned off, and the solution was stirred for 30 minutes. The stock was then brought to a final volume of 450 ml.

Medium used for the purpose of inducing germination was the same as described by Stewart and Hsu (1977). No variation of this medium

was attempted except that 2, 4, or 6% sucrose was used. The concentrations of chemicals found in this medium are shown in Table VII of the Appendix.

Another germination medium used in this study was developed by Norstog (1973). One modification was that 1 gram of filter-sterilized malic acid was added to each liter of medium. The chemical constituents and directions to make this medium are listed in Table VIII of the Appendix.

All dissection and embryo evaluation was carried out under aseptic conditions. A positive air flow hood equipped with an ultraviolet light was used for all culturing in this study.

All media, either for inducing germination or for in-ovulo culture, were sterilized by the autoclave. Media were autoclaved at 121°C and 14-15 lbs/in$^2$ for a period of 20 minutes. Plant hormones and all other chemicals except malic acid were sterilized by this method.

Malic acid was sterilized by passing the solution through a 0.45 µm millipore filter. This procedure was performed under the hood. The solution was collected in a small sterile vial and frozen until use.

Instruments used for dissection were flamed in 95% ethyl alcohol and allowed to cool a few seconds before use. Glassware and DDW used for dissection were sterilized by the autoclave as described above.

The first few hundred ovules were cultured in 50 ml and 100 ml screw top flasks. These proved to be inadequate due to the small size of the top. Standard 25 x 150 mm glass culture tubes with plastic

Bellco kaput caps were then used. The initial in-ovulo culture as well as germination attempts were carried out in these tubes.

Ovules were initially cultured in a dark growth chamber at 30 ± 2°C. The initial culture period was 28 days. When germination was attempted, embryos dissected were then transferred to a germination medium and placed in a lighted growth chamber. Photoperiod was 16 hours light and 8 hours dark. Temperature was held at approximately 27°C.

Embryos which germinated were removed from the germination medium and carefully placed in moistened peat pellets. This was the first exposure to a non-sterile environment. Peat pellets were placed in 1-gallon glass jars to retain a high relative humidity. After 14-20 days in this jar, the lid was removed for a few hours a day. Twenty-five days were usually required before seedlings were sufficiently large enough to be transferred to the greenhouse.

Bolls containing ovules to be cultured were normally collected from the plant between 10:00 AM and 1:00 PM. These bolls were tagged on the day of pollination. The varieties of male and female parent, as well as the anthesis date, were included on the tag. To avoid any confusion, all ovules cultured in this study were referred to as being a certain number of days "post anthesis." Bolls were cut from the plant at the base of the peduncle and placed in a small ice chest until being brought to the laboratory.

Once in the lab, bolls were stripped of their bracts and calyx. They were then placed in a solution of 10% Purex (1 drop of Tween 20

per 250 ml solution) for 5-10 minutes. Bolls were rinsed twice in changes of sterile DDW and then placed inside a sterile petri dish on several layers of moistened filter paper. Holding the boll by the peduncle, a cross-sectional cut was made at the very top of the ovary. The base of the boll was then cut in the same manner. After standing the boll on its base, incisions were made along each suture. The bolls were then gently pulled apart, leaving the placenta and ovules intact.

Ovules were then removed, one by one, by cutting the funiculus nearest to the placenta. Two to four ovules were carefully placed on the surface of the nutrient medium in each tube. This was accomplished best by first moistening the end of a long thin spatula with nutrient medium. The ovules clung easily to the spatula using this method. Culture tubes were flamed before closure.

The initial in-ovulo culture lasted 28 days. After this time, ovules were removed individually and dissected to reveal whether or not any embryo development had occurred. This procedure was performed aseptically.

A dissecting microscope was placed under the hood, wiped thoroughly with 70% alcohol, and left under U.V. light for 30 minutes. This procedure ensured a clean working surface. Sterilized petri plates and sterile DDW were also placed in the hood.

Ovules were initially observed under low power. Irregular callus or fiber development was noted. The ovules were then gently teased open to reveal whether or not an embryo was present. Necrosis and hyperplasia of the inner integument was noted at this time.

If an embryo was present, it was removed from the ovule and measured. A precise ocular micrometer was used to take measurements of the embryo length and width. Many times embryos were simply discarded due to their small size or watery appearance. Larger embryos were rinsed many times with sterile DDW and then placed on germination medium.

Bolls were collected from three to fifteen days post pollination for histological observations. Ovules were fixed in an 80:15:5 solution of 95% ethanol:glacial acetic acid: formaldehyde. The tissues were fixed for 20 days and were then put through an ethanol-tert-butyl alcohol-toluene dehydrating series. Paraplast infiltration lasted approximately 24 hours.

Sections were microtomed between 11-15 microns, mounted using a warm water bath, and allowed to dry at 38-46$^{o}$C for 24 hours. Slides were normally stained with 1% aqueous safranin and fast green.

No distinction was made between greenhouse and field-grown plants in this study. Crosses were made on all flowers, greenhouse or field-grown, which appeared normal. No differential effects caused by "secondary" flowers as described by Beasley (1974) were considered. Greenhouse temperatures ranged from 27-38$^{o}$C and were normally no lower than 24$^{o}$C in the winter.

All hexaploid plants used in this study were from the 6X hirsutum-armourianum breeding program of Dr. Muramoto. All plants used were plant selections carrying the caducous bract trait. Chromosome

number was not always constant in these plants. Since most plants appeared normal and were fertile, this fact was not considered a major factor in the *in vitro* embryo development.

One unknown variety of *G. herbaceum* L. ($A_1$) was used in this study. Seed was harvested each season to maintain planting seed for the following year. Two varieties of *G. arboreum* L. ($A_2$), "Nanking" and "Sanguineum," were also used as A genome diploid species. Self-pollinated ovules from a prolific *G. davidsonii* Kell. ($D_3$-d) plant were also cultured during this study.

Hexaploid selections were removed from the field in November and transplanted in 5-gallon pots for the winter. A few $A_1$ and $A_2$ plants were also placed in the greenhouse to be used as a flower source. Flowers grew abundantly during the winter to allow for many crosses. When temperatures rose in the spring, the hexaploids were transferred back to the field. Using this method, cross-pollinations could be made approximately 10 months out of the year.

# RESULTS

## In Vitro Culture of Self-Pollinated Ovules and Embryo Germination

### Hexaploids

A total of 626 hexaploid ovules were cultured in vitro on various nutrient media. Ovules exhibited a wide array of responses on any given medium. Some ovules produced only minimal callusing on the ovular surface while others completely filled the culture tube. This wide variation was observed even among ovules from the same hexaploid plant.

Hexaploid ovules were excised from 2, 4, 6, 7 and 8 days post anthesis and cultured on all types of medium. Cumulative results of self-pollinated hexaploids may be seen in Table 1.

Table 1. Cumulative results from 28 day in-ovulo embryo culture of hexaploid ovules.

| Days Post Anthesis | Number of Embryos Cultured | Number of Ovules Cultured | Percent Success |
|---|---|---|---|
| 2 | 3 | 398 | .75 |
| 4 | 1 | 97 | 1.03 |
| 6 | 4 | 83 | 4.82 |
| 7 | 1 | 32 | 3.13 |
| 8 | 1 | 16 | 6.25 |

The nutrient medium developed by Beasley and Ting (1973) for *in-ovulo* culture of *G. hirsutum* L. embryos proved inadequate for supporting embryo growth and development of the hexaploids. No embryos were observed growing in this medium after 28 days in culture.

Deleting GA and Kin for the medium allowed for some embryos to develop. As a whole, less callusing of the ovular surface was noted when these hormones were not present in the culture media. Embryos grew well in media containing $5 \times 10^{-6}$ or $5 \times 10^{-7}$M IAA (Table 1). Two embryos were observed growing in medium completely lacking IAA.

Eight of the ten embryos which developed in culture were found in media containing casein hydrolysate at a concentration of 250 mg/L. This compound appeared to neither inhibit nor stimulate callus production on the ovule surface at concentrations between 100-1000mg/L. Since the majority of hexaploid embryos developed with this compound in the medium, 250mg/L was included in media used for culturing hexaploid X diploid ovules.

Hexaploid embryos grew in a wide range of non-reduced:reduced nitrogen regimes. Half the embryos were observed growing in Stewart and Hsu's (1977) modification of Beasley and Ting's (1973) medium (50mM $KNO_3$ and 15mM $NH_4NO_3$). Other hexaploid embryos were observed growing in Beasley and Ting's (1973) medium containing 35mM $KNO_3$:5mM $NH_4NO_3$, 35mM $KNO_3$:10mM $NH_4NO_3$, 45 mM $KNO_3$:10mM $NH_4NO_3$, and 65mM $KNO_3$: 10mM $NH_4NO_3$.

Embryo size after 28 days in culture was quite variable and appeared to have no real relationship to the nitrogen content of the

medium. This was also true for the day the ovule was cultured post anthesis. Hexaploid embryos ranged in size from 1.29 X .92mm to 5.23 X 1.50mm after 28 days in culture. The average length and width of these embryos was 2.34 ± 1.22 X .98 ± .23mm.

A typical proembryonic mass from a 6-day post-anthesis ovule is shown in Figure 1. Ovules cultured at this date were normally 0.25 - 0.35mm in length and showed extensive fiber elongation.

G. davidsonii Kell.

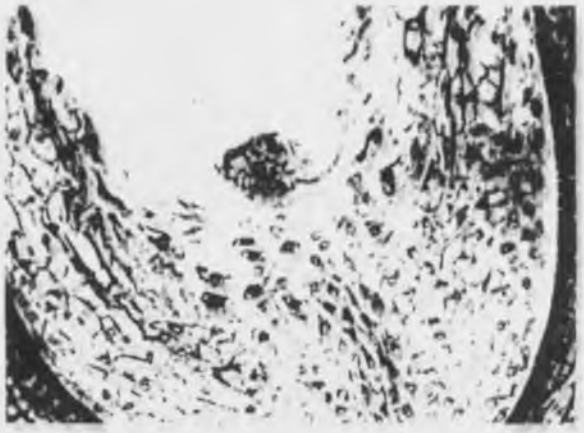
Since the hexaploids used for this study were from the cross G. hirsutum L. X G. armourianum Kearney, and no fertile G. armourianum Kearney plants were available, it was necessary to use G. davidsonii Kell. to learn about specific nutrient requirements for the D genome species. One large G. davidsonii Kell. plant provided ample flowers for testing many nutrient media. The possibility existed that specific nutritional differences might be noted. Since G. armourianum Kearney and G. davidsonii Kell. are related D genome species, knowing what is required for the in vitro embryonic growth of G. davidsonii Kell. might help improve the survivability of hexaploid embryos grown in culture.

In-ovulo embryo culture was carried out on 251 G. davidsonii Kell. ovules. The overall rate of live, differentiated embryos after 28 days in culture was 8.76%. The overall results of in-ovulo embryo culture for G. davidsonii Kell. are shown in Table 2. Embryos grew abundantly when ovules were cultured 2 days post anthesis. Callus growth was minimal and a tough seed coat usually developed during the

Figure 1. Undifferentiated proembryo from CB 31-1 removed from the plant six days post anthesis.

Figure 2. G. davidsonii Kell. embryo after 28-day in-ovulo embryo culture.

Figure 3. Emergence of G. arboreum L. embryos on Norstog (1973) medium.



1

2

3

Table 2. Results from in-ovulo embryo culture of self-pollinated G. davidsonii Kell.

| Days Post Anthesis | Number of Embryos Observed | Number of Ovules Cultured | Percent Success |
|---|---|---|---|
| 2 | 17 | 108 | 15.74 |
| 4 | 2 | 68 | 2.94 |
| 6 | 3 | 75 | 4.00 |

culture period. An embryo dissected from an ovule after 28 days in culture may be seen in Figure 2. A strong radicle is present as well as good cotyledon development.

Stewart and Hsu's (1978) modification of Beasley and Ting's (1973) medium was observed to support embryo development. IAA was the only hormone used in these cultures. This hormone was assumed to be necessary for embryo development when ovules were cultured 2 days post anthesis. No embryos were observed in cultures lacking IAA. Embryos grew optimally in medium containing $5 \times 10^{-7}$ M IAA. The growth and differentiation of embryos was inhibited at IAA concentrations higher than $5 \times 10^{-6}$ M.

IAA concentration in the nutrient medium appeared to influence the length of embryos grown from 2 days post anthesis. Embryos of G. davidsonii Kell. were much more uniform in size than those of the hexaploids. A relationship existed between embryo length and IAA concentration in the medium. An IAA concentration of $5 \times 10^{-7}$ M clearly yielded

larger embryos (Table 3). Large embryos grown in culture have a much better chance of survival when placed on germination medium (Stewart and Hsu 1978). Therefore, $5 \times 10^{-7}$ M IAA was set as a starting point in media used for crosses between hexaploids and A genome diploids.

Table 3. Average lengths of G. davidsonii Kell. embryos grown in culture for 28 days. -- Ovules were cultured 2 days post anthesis in Beasley and Ting's (1973) medium with different IAA concentrations.

| Concentration IAA (M) | Number of Embryos Observed | Number of Ovules Cultured | Average Length (mm) |
|---|---|---|---|
| $2.5 \times 10^{-7}$ | 5 | 26 | 2.11 ± .35 |
| $5.0 \times 10^{-7}$ | 7 | 28 | 2.43 ± .24 |
| $2.5 \times 10^{-6}$ | 2 | 31 | 2.05 ± .05 |
| $5.0 \times 10^{-6}$ | 3 | 23 | 1.34 ± .33 |

G. arboreum L.

Ovules from this species were cultured 2, 5, and 7 days post anthesis. No embryos were observed growing after the 28-day culture period. Ovules from this species generally became necrotic after the first 14 days in culture. A translucent, friable callus sometimes covered the ovule surface. Fibers were very short or completely non-existent. No medium or particular chemical constituent seemed to help embryo growth when cultured at these post-anthesis dates.

Ovules typically exuded a dark substance into the surrounding medium. Whether this was a normal response for this species or a factor

associated with embryo degeneration was not determined. Plating the discolored medium on potato dextrose agar proved that the discoloration was not due to bacterial contamination.

Ten- and 15-day-old ovules could be readily grown on Norstog (1973) medium. Ovules were flame-sterilized, and a small hole was cut through the seed coat near the micropyle end. Ten to 14 days were normally required before the radicle emerged from the seed coat. The G. arboreum L. embryo may be seen emerging from the ovule on Norstog (1973) medium (Figure 3). Once outside the seed coat, embryos grew quickly. Roots developed freely and chlorophyll accumulated rapidly in the cotyledons. Many seedlings were produced in this manner.

G. herbaceum L.

The in vitro response of this species was quite different than that of G. arboreum L. Although these two species are closely related, their nutritional requirements for in vitro embryo growth are definitely not the same. Eighty percent of all G. herbaceum L. ovules cultured produced firm, well-developed embryos after 28 days in culture.

Most ovules were cultured either 3 or 5 days post anthesis. Fiber developed on the ovule surfaces not submerged in the liquid medium. This greatly facilitated keeping the ovules afloat. When callus was removed from the ovule surface, a tough black seed coat was normally present. Very little maternal tissue remained inside the seed coat. The G. herbaceum ovule, and enclosed embryo at the end of the culture period may be seen in Figures 4 and 5. Typically, embryos developed to

Figure 4. G. herbaceum L. ovule after 28-day culture period in modified Beasley and Ting's (1973) medium. -- Embryo may be seen directly below endosperm (en - endosperm).

Figure 5. Dissected G. herbaceum L. ovule showing embryo (center) at heart stage.

Figure 6. G. herbaceum L. embryos after 28 days in culture showing various stages of development.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one of two stages at the end of the culture period. Embryos were either globular (.56 ± .04 X .45 ± .03mm) or were past the heart stage (1.56 ± .60 X .91 ± .32mm) with well-developed cotyledons. Healthy embryos just past the heart stage with various degrees of cotyledon folding may be seen in Figure 6.

Different levels of nitrate and ammonium were tested for the in vitro response of embryos. Volumes of $KNO_3$ and $NH_4NO_3$ stock were added along with Beasley and Ting's (1973) salts to produce media of known nitrogen content. All media used for G. herbaceum L. cultures also contained 250mg/L casein hydrolysate, and 5 X $10^{-7}$ M IAA.

Embryos failed to grow when media contained less than 45mM $KNO_3$. At this level of nitrate, and $NH_4NO_3$ levels of 5, 10, 20, and 30mM, embryos grew abundantly. Ammonium levels above 30mM proved inhibitory to embryo growth. Nitrate levels of 55, and 65mM also produced embryos, although fewer in number. At these higher levels of nitrate, ammonium appeared inhibitory at a lower level. An exception of this is shown in Figure 7. Embryos were growing after a six-week culture in 55:30 and 65:20 media (ratios representative of mM $KNO_3$:$NH_4NO_3$ present in the medium).

The germination medium developed by Stewart and Hsu (1977) for balanced root and shoot growth failed to support the germination of cultured G. herbaceum L. embryos. Three to 4 days after placement of embryos on this medium, a dark brown color appeared in the medium surrounding the radicle. Embryos slowly blackened at the root apex. The

Figure 7. Six-week culture of G. herbaceum L. ovules grown on modified Beasley and Ting's (1973) medium. -- Tube on left contains 55mM $KNO_3$ and 30mM $NH_4NO_3$. (Arrow points to radicle.) The tube on right contains 65mM $KNO_3$ and 20mM $NH_4NO3$.

Figure 8. Integument overgrowth in hexaploid X. G. herbaceum L. ovule. -- Area occupied by the embryo sac was much reduced.

Figure 9. Hexaploid X. G. herbaceum L. ovule fixed 4 days post anthesis. -- Arrow points to fused polar nuclei.

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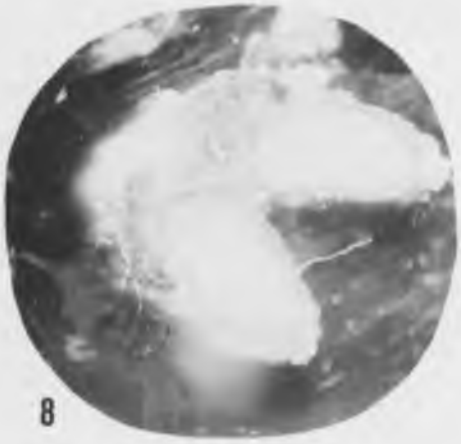

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blackening then spread upward and eventually covered the entire embryo. No chlorophyll development was ever observed in this medium.

Norstog (1973) medium was capable of inducing germination of cultured embryos. Chlorophyll development proceeded rapidly in the cotyledons after 2-3 days. The root apex initially turned a deep red color and failed to expand. After approximately one week in this medium, roots expanded freely. Cotyledons unfolded and the shoot elongated approximately one inch above the medium surface.

## In Vitro Culture of Hybrid Ovules

Hexaploids X G. herbaceum L.

Hundreds of crosses made in the field and greenhouse revealed that hybrid bolls usually abort 8-10 days post anthesis. Following the idea of Skirm (1942) that the culture of hybrid embryos must begin before degeneration takes place, ovules from this cross were cultured 3, 4, 5, and 6 days post anthesis. Table 4 lists the results for the in vitro culture of these hybrid ovules. A very low percentage of success was expected due to the incompatibility of the parents.

Extensive callus formation was observed in most cultures. In series of media where non-reduced nitrogen was tested, a trend in callusing was observed. At any level of nitrate, the lower levels of ammonium (5 and 10mM) produced a firm, crisp callus. Higher levels of ammonium (20, 30, and 40mM) produced a softer more friable callus.

The one embryo found in this cross was cultured in a medium containing Beasley and Ting's (1973) salts with a nitrogen content of

Table 4. Results from in-ovulo embryo culture of hexaploid X G. herbaceum L.

| Days Post Anthesis | Number of Embryos Observed | Number of Ovules Cultured | Percent Success |
|---|---|---|---|
| 3 | 0 | 65 | 0 |
| 4 | 0 | 75 | 0 |
| 5 | 0 | 83 | 0 |
| 6 | 1 | 32 | 3.13 |

55mM $KNO_3$ and 5mM $NH_4NO_3$. The medium also contained IAA at 5 X $10^{-7}$M and 250mg/L casein hydrolysate.

The overgrowth of maternal tissues was extreme for this cross. The characteristic proliferation of the inner integument present in this cross is shown in Figure 8. The area occupied by the embryo sac was much reduced compared to self-pollinated ovules. Ovules were frequently hollow within the inner integument.

The fact that an embryo was found provides ample evidence that fertilization can occur. However, histological examination revealed that fertilization was delayed with respect to self-pollinated hexaploid ovules. A hybrid ovule at 4 days post anthesis is shown in Figure 9. Polar nuclei are fused and the egg cell is missing in the figure. A fertile egg is shown along with fused polar nuclei at 4 days after anthesis in Figure 10. Although the clarity is not revealed in this figure, a sperm nucleus is present and ready to fuse with the polar

Figure 10. Ovule from hexaploid X G. herbaceum L. 4 days post anthesis. -- Arrow points to fused polar nuclei. Fertilized egg is shown to the right of "e."

Figure 11. Unfused polar nuclei (arrow) from a hexaploid X G. herbaceum L. ovule fixed 7 days post anthesis.

Figure 12. Degenerative embryo sac from the cross hexaploid X G. herbaceum L. -- (a) Seven day post-anthesis ovule showing unfused polar nuclei (arrow); (b) Enlargement of polar nuclei.


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12 a

b

nuclei. Unfused polar nuclei may again be seen in Figures 11 and 12. These ovules were removed from the plant and fixed 7 days post anthesis. There was no evidence in any of the prepared slides that nuclear divisions of the endosperm occurred. This would certainly suggest at least one cause for the incompatibility between these two plants.

Hexaploids X G. arboreum L.

The summary of results obtained from the in vitro culture of this cross may be seen in Table 5. Similar to the hexaploid X G. herbaceum L. cultures, the only embryo was cultured 6 days post anthesis. As a whole, these cultures appeared less tolerant to in vitro culture than hexaploid X G. herbaceum L. There was a higher degree of callusing on the ovular surface. Many ovules became necrotic, and turned the medium a dark brown color before the end of the culture period.

Table 5. Results from in-ovulo embryo culture of hexaploid X G. arboreum L.

| Days Post Anthesis | Number of Embryos Observed | Number of Ovules Cultured | Percent Success |
|---|---|---|---|
| 2 | 0 | 32 | 0 |
| 3 | 0 | 100 | 0 |
| 4 | 0 | 82 | 0 |
| 6 | 1 | 48 | 2.08 |

Manipulating the amount of inositol in the nutrient medium helped limit the callusing on the ovular surface. Cultured ovules are

shown at 3 levels of inositol in Beasley and Ting's medium with 250mg/L casein hydrolysate and 2.5 X $10^{-6}$ M IAA in Figure 13. Note that the callusing increased with increasing levels if inositol. The tube on the left is relatively free of callus. It contains 90mg/L or half the amount of inositol described by Beasley and Ting (1973) for in-ovulo culture of G. hirsutum L. embryos. Although callus was reduced with this medium, no embryos were observed growing after 28 days.

The medium supporting the one embryo which did grow contained Beasley and Ting's (1973) salts, 180mg/L inositol, 500mg/L casein hydrolysate, and 5 X $10^{-6}$ M IAA. Although this embryo was firm and white, there were some abnormalities. The radicle was curved approximately $90^{o}$ off the axis of the embryo. Cotyledons were completely absent. The most striking observation concerned the shoot apex. Small protrusions of cells covered the area where the meristem should have been. It appeared that the meristem was absent, or was covered with a callus-like growth.

G. arboreum L. X Hexaploids

No embryos were observed growing in culture when this diploid was used as the female parent. Callus developed rapidly over the entire ovule surface after the first few days in culture. The callus was bright yellow and somewhat friable. A comparison of ovule development between G. arboreum L. X hexaploids, and hexaploids X G. arboreum L. may be seen in Figure 14. These are 21-day-old cultures growing in Beasley and Ting's (1973) medium as described originally in the

Figure 13. Four-day post-anthesis cultures of hexaploid X G. arboreum L. -- Callus development increases from left to right with increasing level of inositol. Media contain from left to right: 90mg/L, 180mg/L, and 270mg/L.

Figure 14. Comparison of ovule development between G. arboreum L. X hexaploid (arrows) and hexaploids X G. arboreum L. in Beasley and Ting's (1973) medium.

Figure 15. G. herbaceum L. X hexaploid embryo on Norstog (1973) medium.

Figure 16. Necrotic G. herbaceum L. X hexaploid embryo (left) compared to self-pollinated G. arboreum L. seedling on Norstog (1973) medium.

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literature for in-ovulo culture of G. hirsutum L. Callusing was so extensive in this cross that it made ovule removal difficult at the end of the culture period.

G. herbaceum L. X Hexaploids

This diploid proved to be a much more suitable female parent than G. arboreum L. A total of 15 embryos were observed from 346 cultured ovules. Results of this cross are summarized below (Table 6).

After 28 days in culture, embryo measurements averaged 1.83 ± .55 X 1.15 ± .40mm. All embryos were white and appeared healthy at the time they were removed from the ovule. The radicle typically broke through the seed coat before the end of the culture period. Fiber developed on the ovule surface above the level of the nutrient medium at about the same rate as self-pollinated G. herbaceum L.

Table 6. Results from in-ovulo embryo culture of G. herbaceum X hexaploids.

| Days Post Anthesis | Number of Embryos Observed | Number of Ovules Cultured | Percent Success |
|---|---|---|---|
| 4 | 1 | 121 | .83 |
| 5 | 14 | 154 | 9.09 |
| 9 | 0 | 71 | 0 |

This hybrid combination appeared to prefer nutrient media of high nitrate and relatively low ammonium content. No embryos were

observed growing in media containing less than 55mM $KNO_3$. Likewise, embryos did not develop when the $NH_4NO_3$ concentration was greater than 10mM. The media producing embryos all contained Beasley and Ting's (1973) salts (with known nitrogen content), 250mg/L casein hydrolysate, and 5 X $10^{-7}$ M IAA.

Embryos which developed were transferred to Norstog (1973) medium to induce germination. A few embryos developed a small amount of chlorophyll but never became fully green. The best of these hybrids after 10 days on Norstog (1973) medium is shown in Figure 15. Chlorophyll had developed but the cotyledons did not expand. After 20 days on this medium, the embryo began to turn brown at the tip of the radicle. The discoloration spread and eventually covered the entire embryo. The necrotic embryo is shown along side a healthy G. arboreum L. seedling in Figure 16.

Most of the hybrid embryos transferred to Norstog (1973) medium developed abnormally. Root expansion was never observed. Callus proliferated from the hypocotyl portion of the embryo. This callus grew slowly and took on a reddish, translucent appearance. All hybrids died within 30 days after placement on Norstog (1973) medium.

## Overall Results

Over 2000 self-pollinated and hybrid ovules were cultured in the course of this study. Ovules were carefully dissected after a 28-day culture period to reveal whether or not embryo development had occurred. A total of 81 embryos were observed in the cultures to yield a success

rate of 3.94%. The overall results obtained during the course of this study are summarized in Table 7.

Table 7. Overall results from *in-ovulo* embryo culture of self-pollinated hexaploids, diploids, and hybrid hexaploid-diploid combinations.

| Source of Ovules | Number of Ovules Cultured | Number of Embryos Observed | Percent Success |
|---|---|---|---|
| Self-pollinated | | | |
| Hexaploids | 626 | 10 | 1.59 |
| *G. davidsonii* Kell. | 251 | 22 | 8.76 |
| *G. arboreum* L. | 87 | 0 | 0 |
| *G. herbaceum* L. | 40 | 32 | 80.00 |
| Hybrid Combinations | | | |
| Hexaploid X *G. arboreum* L. | 262 | 1 | 0.38 |
| Hexaploid X *G. herbaceum* L. | 255 | 1 | 0.39 |
| *G. arboreum* L. X hexaploid | 187 | 0 | 0 |
| *G. herbaceum* L. X. hexaploid | 346 | 15 | 4.34 |

## DISCUSSION

A problem encountered early in many hybridization programs concerns cross pollination and fertilization. Pundir (1972) observed that the rates of pollen tube growth through the stigmatic surface and style in crosses between G. arboreum L. and G. hirsutum L. were much slower than in the self pollinated parents. The slow rate of pollen tube growth in the cross G. arboreum L. X G. hirsutum L. may be attributed to physical restrictions on the G. hirsutum L. pollen. Tetraploid pollen tubes are nearly twice the diameter of diploid pollen tubes. The stigmatic surface or style might also exert an antagonistic effect on the growing pollen tube.

Differences in style length might delay or prevent fertilization from occuring. Maheshwari and Rangaswamy (1965) stated that when one was attempting to produce hybrids between parents having a large difference in style length, the short style X long style hybrid might be easy to produce. In the reciprocal cross, long style X short style, one may find that the hybrid seeds always abort. These workers attributed the seed abortion in this hypothetical situation to be due to the pollen's inability to grow the entire length of the style. The pollen simply runs out of energy before entering the ovule and accomplishing fertilization.

It was observed that the style length of hexaploids was normally 2.5 times the length of G. herbaceum L. styles at anthesis. G. arboreum

L. styles were approximately half the length of hexaploid styles. This could explain why a low percentage of ovules were fertilized in hexaploid X diploid crosses. In the reciprocal crosses, 15 G. herbaceum L. X hexaploid embryos were observed. Although hexaploid pollen tubes are larger in diameter, they may have been able to fertilize a higher percentage of G. herbaceum L. ovules. Pollen tubes did not have to travel as far to reach the ovary. This may have accounted for the higher percentage of embryos observed developing in this cross. Style length is only one factor associated with the imcopatibility in these crosses. The reciprocal cross involving G. arboreum L. as the female parent was completely unsuccessful. Hexaploid pollen should have been able to travel the required distance to accomplish fertilization. Non-dividing endosperm or failure of the proembryo to differentiate may have caused the early abortion of these ovules.

In crosses involving hexaploids as the female parent, there was no sign of endosperm development. Since embryos were observed in these crosses, the nutrient medium must have successfully replaced the absent endosperm. Workers have been successful raising embryos in nutrient medium free of all maternal tissues. Artificial media can adequately mimic the role of endosperm. But since such a low percentage of embryos were observed in the hexaploid-diploid crosses, either embryos ceased to develop due to some genetic imbalance, or the medium was inadequate in supplying nutrients essential for embryo growth and development.

The variation in embryo size at the end of the 28 day culture period caused problems in the evaluation of nutrient media. Larger

embryos certainly produce more vigorous seedlings. But many times, embryo size was quite variable within the same nutrient medium. Ideally, embryos would grow to a certain size and stage of development at a uniform rate in the same nutrient medium. Frequently, within any given medium, some embryos would be three times the length of other embryos. After 28 days in culture, embryos could be found anywhere from the globular to the late heart stage growing in the same medium. This causes problems when conducting a statistical analysis that compares the effects of various media on embryo growth. Perhaps it is better to find the medium producing the highest percentage of embryos. Some embryos would probably be too small to attempt to germinate. These could possibly be subcultured to produce larger embryos. The larger embryos could be readily germinated on a proper medium.

Callusing of the ovule surface appeared detrimental to embryo development. All embryos observed in this study came from ovules relatively free of callus. One suspects that callus must be abnormal growth. Callus grew at a much higher rate than the ovule tissues or embryo. The callus definitely takes nutrients out of the medium that might be necessary for embryo growth. There is also a conduction problem. It would be difficult to transport nutrients to a developing embryo through nearly an inch of undifferentiated callus. Possibly periodic removal of callus during the culture period might allow for more embryos to develop.

The callus observed on the ovule surface may be representative of an improper nutrient balance. The hormone regime in the medium is

certainly a factor associated with callus development. Vitamins and salts might also initiate callus development if they are present in the medium at the wrong level. Although media that have a high probability of promoting embryo development of G. hirsutum L. have been found, one must evaluate a wide range of nutrient media with any other species or hybrid cross that one attempts to grow in culture.

Though the mdeium developed by Beasley and Ting (1973), supports the embryonic growth of G. hirsutum L., it appears that this medium is not adequate in supporting the growth of all species of Gossypium. However, this medium would be a good starting place in any study involving in-ovulo embryo culture of Gossypium. Modification of the nitrogen content and hormone regime allowed for embryo development of the species used in this study. The hormones GA and Kin appeared to serve no useful function in the culture medium. When these hormones were included in the medium, callusing of the ovular surface was extensive. No embryos were observed when these hormones were in the culture medium.

Nitrogen content of nutrient media was the factor modified most extensively in this study. Self pollinated hexaploid embryos were observed to be capable of growing in media over a wide range of non-reduced : reduced nitrogen ratios. It seems possible that each species or genome in Gossypium might prefer a slightly different ratio for optimum in vitro embryonic growth. The hexaploids used in this study were produced by the colchicine doubling of a hybrid cross

involving two species. This may account for their ability to grow and differentiate in culture over a wide range of non-reduced : reduced nitrogens.

Many workers now feel that nutrient media should be completely defined. Media should not include organic additives such as coconut milk, yeast or malt extract, or casein hydrolysate. This is certainly true for studies involving specific nutrient uptake or secondary product production. Organic additives might have a complex or undefined effect that could confound experimental results. But when research is aimed at producing particular plant types from in vitro culture, I believe that one should test these compounds for their possibly beneficial effects on in vitro growth. Embryos of many species are known to be capable of utilizing a wide range of amino acids. Including a low concentration of casein hydrolysate in a nutrient medium gives a spectrum of amino acids available to the developing embryo.

Supporting the hypothesis that each species might have different nturient requirements for in vitro embryonic growth are the results obtained from cultured G. herbaceum L. and G. arboreum L. ovules. Eighty percent of all G. herbaceum L. ovules produced embryos after 28 days in culture. Callus development was minimal and fiber frequently developed on the ovule surface. G. arboreum L. embryos failed to develop in culture. Manipulation of medium constituents failed to allow for any embryo development. Callus was abundant on these ovules and medium became rapidly discolored. These species are closely related

and will cross readily to produce vigorous hybrids. But the results obtained from the in-ovulo embryo culture of these self pollinated species show that their nutrient requirements for in vitro growth are different.

A reliable germination medium is necessary to produce viable hybrids from incompatible species. Developing media to support in-ovulo embryo growth is only one step in producing a desired hybrid. The germination medium must be capable of allowing for root and shoot expansion. Stewart and Hsu (1977) medium did not support the germination of hybrid or self pollinated embryos. Norstog (1973) medium did allow self pollinated embryos to germinate but was ineffective in germinating hybrids. The success of Norstog (1973) medium in allowing embryo germination might be partly attributed to the medium composition. Norstog (1973) medium contains a number of amino acids. Embryos need not expend energy reducing nitrate when placed on this medium. Stewart and Hsu (1977) medium contains no amino acids. This medium contains 5.0mM $KNO_3$ and 3.0mM $NH_4HO_3$. Since over half the nitrogen is non-reduced, embryos must have a higher nitrate reductase activity in order to survive on this medium. Perhaps this enzyme's activity in the young embryos is not sufficient to account for the total nitrogen necessary for embryo germination.

A question arises as to why hybrid embryos did not survive on Norstog (1973) medium. Compared to G. herbaceum L. and G. arboreum L. embryos, the hybrids were certainly large enough to be germinated. One

explanation as to why the hybrids failed to germinate is that essential enzyme systems might be lacking or inhibited. Or, perhaps enzyme activity is at such a low level that necrosis is eventually inevitable.

# CONCLUSION

There are several reasons for attempting to hybridize the hirsutum-armourianum hexaploid with the A genome diploids. First, an instant teraploid is obtained when these plants are crossed. Theoretically, the hybrid should be fertile. Chromosomes from the A genome are known to pair with A chromosomes of G. hirsutum L. G. armourianum Kearney chromosomes are capable of pairing with the D chromosomes of G. hirsutum L. Secondly, it was hoped that the caducous bract trait present in the hexaploids would show up in the hybrid. If this trait did occur in the hybrid tetraploid, a backcross program with G. hirsutum L. would be initiated. Plants possessing the caducous trait would be selected after each backcross generation. The overall goal is a G. hirsutum L. plant with caducous bracts and acceptable yield and fiber qualities.

In-ovulo embryo culture was used to produce the hybrids due to the incompatibility between the parents. Thousands of crosses were made in the past two years and no hybrids were produced through conventional breeding procedures. The use of embryo culture as a tool in plant breeding shows promise for the future. Embryos can be produced from incompatible crosses using the proper nutrient media. Higher percentages of viable hybrids will be realized only when the proper nutritional balance is discovered for each particular hybrid in question.

# APPENDIX

Table I. Nutrient medium described by Beasley and Ting (1973) for the *in vitro* culture of cotton ovules.

| Nutrient | mg/L (final) |
|---|---|
| $CaCl_2 \cdot 2 H_2O$ | 441 |
| $CoCl_2 \cdot 6 H_2O$ | .024 |
| $CuSO_4 \cdot 5 H_2O$ | .025 |
| $FeSO_4 \cdot 7 H_2O$ | 8 |
| $H_3BO_3$ | 6 |
| $KH_2PO_4$ | 272 |
| KI | .83 |
| $KNO_3$ | 5055 |
| $MgSO_4 \cdot 7 H_2O$ | 493 |
| $MnSO_4 \cdot H_2O$ | 16 |
| $Na_2$ EDTA | 11 |
| $Na_2MoO_4 \cdot 2 H_2O$ | .242 |
| $ZnSO_4 \cdot 7 H_2O$ | 8 |
| Nicotinic Acid | .492 |
| Pyridoxine HCl | .822 |
| Thiamine | 1.34 |
| Myo-inositol | 180 |
| D-glucose | 18016 |
| D-fructose | 3603 |

Table II. Modified Beasley and Ting medium used as control.

| Nutrient | mg/L (final) |
|---|---|
| $CaCl_2 \cdot 2 H_2O$ | 441 |
| $CoCl_2 \cdot 6 H_2O$ | .024 |
| $CuSO_4 \cdot 5 H_2O$ | 8 |
| $FeSO_4 \cdot 7 H_2O$ | 6 |
| $H_3BO_3$ | 6 |
| $KH_2PO_4$ | 272 |
| KI | .83 |
| $KNO_3$ | 5055 |
| $MgSO_4 \cdot 7 H_2O$ | 493 |
| $MnSO_4 \cdot H_2O$ | 16 |
| $Na_2$ EDTA | 11 |
| $Na_2MoO_4 \cdot 2 H_2O$ | .242 |
| $NH_4NO_3$ | 1200 |
| $ZnSO_4 \cdot 7 H_2O$ | 8 |
| Nicotinic Acid | .492 |
| Pyridoxine HCl | .822 |
| Thiamine | 1.34 |
| IAA | .876 |
| GA | .866 |
| Kin | .054 |
| Sucrose | 40,000 |

Table III. Amounts of $KNO_3$ stock used to produce various volumes of medium at known $KNO_3$ levels.

| $KNO_3$ Level | ml of $KNO_3$ stock*/final volume medium | | |
|---|---|---|---|
| | 250 ml | 500 ml | 100 ml |
| 25 mM | 12.7 | 25.3 | 50.6 |
| 35 mM | 17.7 | 35.4 | 70.8 |
| 45 mM | 22.5 | 45.1 | 90.1 |
| 55 mM | 27.8 | 55.6 | 111.2 |
| 65 mM | 32.9 | 65.7 | 131.4 |

* $KNO_3$ stock is 50g/L.

Table IV. Amounts of $NH_4NO_3$ stock used to produce various volumes of medium at known $NH_4NO_3$ levels.

| $NH_4NO_3$ Level | ml of $NH_4NO_3$ stock*/final volume medium | | |
|---|---|---|---|
| | 250 ml | 500 ml | 1000 ml |
| 5 mM | 2.1 | 4.2 | 8.3 |
| 10 mM | 4.1 | 8.3 | 16.6 |
| 20 mM | 8.4 | 16.7 | 33.4 |
| 30 mM | 12.5 | 25.0 | 50.0 |
| 40 mM | 16.7 | 33.3 | 66.6 |

* $NH_4NO_3$ stock is 48 g/L.

Table V. Preparation of 10x Beasley and Tings (BT) major salts.

| Chemical | ml of Prepared Stock | |
|---|---|---|
| | 500 ml | 1000 ml |
| $KH_2PO_4$ | 1.36 (g) | 2.72 (g) |
| $CaCl_2 \cdot 2\ H_2O$ | 2.20 | 4.41 |
| $MgSO_4 \cdot 7\ H_2O$ | 2.47 | 4.93 |
| $KNO_3$ | 25.27 | 50.55 |
| $NH_4NO_3$ | 6.00 | 12.00 |

Note: Dissolved individually in 1/2 volume DDW and bring to volume. Store under refrigeration.

Table VI. Preparation of 1000x BT minor salts.

| Chemical | ml of Prepared Stock | |
|---|---|---|
| | 500 ml | 1000 ml |
| $H_3BO_3$ | .93 (g) | 1.86 (g) |
| $MnSO_4 \cdot 4\ H_2O$ | 3.35 | 6.7 |
| $ZnSO_4 \cdot 7\ H_2O$ | 1.29 | 2.58 |
| $Na_2MoO_4 \cdot 2\ H_2O$ | .114 | .225 |
| $CuSO_4 \cdot 5\ H_2O$ | .004 | .008 |
| $CoCl_2 \cdot 6\ H_2O$ | .004 | .008 |

Note: Dissolve individually in 1/2 volume of DDW. Bring to volume and store under refrigeration.

Table VII. Formulation of Stewart and Hsu's (1977) medium for germinating cotton embryos.

| Macronutrients (mM) | | Micronutrients (mM) | |
|---|---|---|---|
| $KNO_3$ | 5.0 | Fe EDTA | 15 |
| $NH_4NO_3$ | 3.0 | $H_3BO_3$ | 30 |
| $MgSO_4 \cdot 7\ H_2O$ | 2.0 | $MnSO_4 \cdot 4\ H_2O$ | 30 |
| $CaCl_2 \cdot 2\ H_2O$ | 1.2 | $ZnSO_4 \cdot 7\ H_2O$ | 9 |
| $KH_2PO_4$ | .2 | KI | 1.5 |
| | | $NaMoO_4 \cdot 7\ H_2O$ | .9 |
| Vitamins (mM) | | $CuSO_4 \cdot 5\ H_2O$ | .03 |
| Nicotinic Acid | 4 | $CoCl_2 \cdot 6\ H_2O$ | .03 |
| Pyridoxine-HCl | 4 | Agar | .6% |
| Thiamine-HCl | 4 | Sucrose | 2% |

pH to 5.0

Table VIII. A medium modified from Norstog (1973) and the directions for preparation.

| Stock I (10x) | | Amino Acids (mg/L of final medium volume) | |
|---|---|---|---|
| $KH_2PO_4$ | 9.1 g/L | Glutamine | 400 |
| $CaCl_2 \cdot 2 H_2O$ | 7.4 g/L | Alanine | 50 |
| | | Cystine | 20 |
| Stock II (10x) | | Arginine | 10 |
| KCl | 7.5 g/L | Leucine | 10 |
| $MgSO_4 \cdot 7 H_2O$ | 7.4 g/L | Phenylalanine | 10 |
| | | Tyrosine | 10 |
| Vitamins (100x) | | | |
| Inositol | 5.0 g/L | Malic Acid | 1.0 g/L (final medium volume) |
| Thiamine HCl | 25 mg/L | | |
| Ca-Pantothenate | 25 mg/L | Sucrose | 35 g/L |
| Pyridoxine HCl | 25 mg/L | Agar | .6% |

To prepare 1 Liter of medium:

1. In a 2 L flask, mix 100 ml Stock I, 10 ml vitamins, sucrose, agar, and amino acids dissolved in 100 ml of DDW. Fill flask to 870 ml with DDW and pH to 5.0.

2. In a 250 ml flask, place 100 ml of Stock II and pH to 5.0.

3. Place these two solutions plus enough capped culture vessels for 1 L of medium in the autoclave. Autoclave 20 minutes. While waiting, prepare malic acid.

4. Dissolve 1 gram malic acid in 20 ml DDW. pH to 5.0 with $NH_3OH$. Bring to 30 ml with DDW and then filter sterilize.

5. When autoclave is opened, place Stock II and capped tubes under hood. The major solution is placed in a water bath at $38^{o}C$. When cool to touch, mix Stock II and malic acid into the major solution under hood. Culture tubes are poured immediately under hood.

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