THE EFFECT OF THE *SEMIGAMY* (*Se*) MUTANT ON THE EARLY

DEVELOPMENT OF COTTON (*Gossypium barbadense* L.)

A Dissertation

by

GEORGE LESLIE HODNETT

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

May 2006

Major Subject: Plant Breeding

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Approved by:

Chair of Committee,	David M. Stelly
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ABSTRACT

The Effect of the *Semigamy* (*Se*) Mutant on the Early Development of Cotton

(*Gossypium barbadense* L.).

(May 2006)

George Leslie Hodnett, B.S., Brigham Young University

Chair of Advisory Committee: Dr. David M. Stelly

A stain-clearing method, which facilitated the analysis of large numbers of ovules, was developed using methyl salicylate (MS) and azure C, and used with real-time video imaging and image capture. The ability to modulate contrast and illumination intensity using video made it feasible to reduce stain intensity and thus light interference from the specimen. Samples stained and cleared were used as whole mounts which allowed the specimen to be oriented for precise analysis. Of 440 semigamous zygotes examined 439 had one egg and one sperm nucleus resulting from syngamy without karyogamy indicating *semigamy* is completely expressed. All phenotypes observed in semigamous cotton seedlings appear to arise as products of zygote division. Haploid and tetraploid sectors may result from relative spindle positions, orientation, and the tendency for nearby telophase chromosomes to form a common nucleus. Semigamous endosperm nuclei are triploid, but fusion was never observed. Endosperm may be a result of triple fusion or mitosis-based fusion. A slight delay in endosperm development was observed which could result from failure of the nuclei to fuse. Haploid-diploid ratios of ploidy chimeras were centered on the original 2 : 1, haploid : diploid ratio, but ratios

were widely disparate among seedlings. Dispersion resulted from seeds which were chimeric for ploidy in the hypocotyls, but not in the cotyledons. Additionally, departures from the initial ploidy ratio may be a result of normal development. Differences in cell cycling may occur as cells differentiate and specialize.

To examine expression of the parents on the leaves of chimeras, crosses were made between three semigamous genotypes, $r_1r_1SeSev_7v_7$, $R_1R_1SeSeV_7V_7$, and $r_1r_1SeSeV_7V_7$. Chimeric plants were scored for leaf color distribution. For cotyledons the average percentage of yellow-green sectors was less than the non-yellow sector, while true leaves exhibited sectoral ratios that were higher for the maternal sectors or, when confounded, for maternal plus hybrid sectors in every cross. Differences between cotyledons and true leaves may result from the differences in development. Chimeric sectors in true leaves were disproportionately derived from the maternal parent. The apparent advantage of the maternal parent may result from initial maternal developmental control.

DEDICATION

To my loving and patient wife, Maritza, and my wonderful children, Michael, Ruth, Leslie, Kevin, and Paul. Completing this work has been a family affair. My wife and my children have been intimately involved and have participated over the years in many ways. Their contributions and sacrifices have helped make this possible. This is as much theirs as it is mine.

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I would like to thank Dr. David Stelly who has permitted me to complete this task. In particular he has taken the time to review the manuscript, providing needed editing and advice, when demands on his time were extreme.

And lastly, in memory of Dr. H. James Price who is no longer with us. I am grateful for the interest he took in this work and the advice and direction he gave me.

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INTRODUCTION

PREVIOUS WORK

Semigamy

The term *semigamy* was first used by Battaglia (1945) to describe the failure of synkaryon formation in recurrently apomictic *Rudbeckia laciniata* L. with the subsequent sporophyte arising from the female gamete. *Semigamy* has also been reported in a recurrently apomictic species of *Zephyranthes* (Coe, 1952; Solntzeva, 1978) and *Cooperia* (Coe, 1953). In the "recurrent" forms of *semigamy* the sperm nucleus is sequestered to a small part of the embryo, where it ceases to divide or becomes part of the suspensor. Nonrecurrent forms have been reported in *Gossypium* (Turcotte and Feaster, 1963). The cytology of semigamous reproduction in *Gossypium* has not been studied.

Haploids of cotton typically arise from polyembryonic seed (Blank and Allison, 1963; Endrizzi, 1959; Harland, 1936). The frequency of polyembryonic seed ranges from *ca*. 1/60,000 in *G. hirsutum L.* (Kimber, 1958) to *ca*. 1/130 in certain lines of *G. barbadense L.* Owings et al., 1964). The *Semigamy* mutant produces monoembryonic seed that include haploids at frequencies up to 60% (Turcotte and Feaster, 1963).

The *Semigamy* mutant (*Se*) in cotton was initially recovered in a doubled haploid, 57-4, from Pima S-1, an obsolete commercial variety of *G. barbadense* (Turcotte and

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This dissertation follows the style of Crop Science.

Feaster 1963). The semigamous nature of *Se*-induced apomixis was deduced because the seed produced were monoembryonic, yet gave rise to normal hybrids, maternal haploids and paternal haploids, as well as chimeras composed of paternal, maternal and/or tetraploid tissue (Chaudhari, 1978; Turcotte and Feaster, 1967, 1969, 1973). It has been suggested that polyspermy might be involved in chimeras with hybrid sectors (Turcotte and Feaster, 1973), but this has not been cytologically confirmed.

Turcotte and Feaster (1969, 1973) deduced that semigamous reproduction occurred only if the egg nucleus possessed *Se*, and that the level of *semigamy* expression increased if the sperm nucleus also had *Se*. The frequency of haploids and chimeras increased from a low of 10% in *SeSe* x *sese* to up to 60% for *SeSe* x *SeSe*. (Chaudhari, 1978; Turcotte and Feaster, 1963, 1967, 1969, 1973). When Turcotte and Feaster (1974) backcrossed a 57-4 F_1 hybrid with a reproductively normal line, they obtained 35 progeny that reproduced semigamously and 39 that reproduced normally, i.e. approximately a 1:1 ratio. Thus they proposed the ratio and indicated that a dominant allele conferred facultative semigamous reproduction. However these results might be explained in several ways, depending on the time and mode of *Se* action (Gwyn, 1995). For example, if *Se* expression were determined megagametophytically, expression would occur in the hemizygous (haploid) state, regardless of the dominant or recessive nature of the mutant allele. In an attempt to identify the time(s) and mode(s) of *Se* gene action, Gwyn and Stelly (1990) developed a genetic-statistical test involving 3x3 factorial crosses of genetically marked *SeSe*, *Sese*, and *sese* genotypes. Their data indicated that *Se* is incompletely dominant, and that it is expressed sporophytically in both parents. The genotype of the zygote appears not to be a

factor in *Se* expression. However, auxiliary tests intended to deconfound additive gene action of the sporophyte and hemizygous gene action in the gametophyte were not completed due to the difficulty of doubling the chromosomes of a large number of haploid plants.

Expressivity of semigamous reproduction seems to be environmentally influenced. Turcotte and Feaster (1963) reported haploid production rates of 24.3% in 1961 from S_3 seedlings from field grown Dbl. Hap. 57-4. Percent haploids from S_3 seedlings in 1962 were 43.4 % and 61.3% when grown in the field and greenhouse, respectively. In College Station no more than 10% chimeric or haploid progenies were recovered from 57-4 from 1985 to 1990 (Gwyn, 1995). Since then, three other semigamous lines, $R_1R_1SeSeV_7V_7$, $r_1r_1SeSeV_7V_7$, and $r_1r_1SeSev_7v_7$, have been developed from the original 57-4 by Stelly, Mac Stewart, and Dolan, respectively, and these averaged between 30% and 40% chimeric or haploid progeny in College Station.

Fertilization and Embryogenesis

Cotton embryogenesis has been studied extensively and thus has served as a model of double fertilization common to angiosperms (Gore, 1932; Jensen, 1964, 1965, 1968a, 1968b; Jensen and Fisher, 1967, 1968a; Joshi et al., 1967; Pollock and Jensen, 1964; Reeves and Beasley, 1935; Schultz and Jensen, 1977). Megasporogenesis and megagametogenesis result in formation of an 8-nucleate, 7-celled megagametophyte, via the "*Polygonum*" type of development (Maheshwari, 1950). On the path to fertilization the pollen tube (PT) grows through the stigma and style to the ovule. It will enter the ovule through the micropyle, grow through the nucellus, and enter the embryo sac between

the synergids. Immediately after entry into the embryo sac the pollen tube makes a right turn into one of the synergids entering through the filiform apparatus. Gore (1932) and Joshi et al. (1967) reported one synergid is destroyed by entrance of the pollen tube, whereas Jensen and Fisher (1968a) observed one synergid begins to break down at the time of pollination. The degenerating synergid remains in contact with the egg and the central cells during the process of double fertilization (Jensen and Fisher, 1968a). The pollen tube ceases to grow before it leaves the synergid and discharges its contents through a pore which forms on the chalazal side of the pollen tube near its tip (Gore, 1932; Jensen and Fisher, 1968a; Joshi et al., 1967). The two sperm are discharged with pollen tube cytoplasm, which forms a cone shaped mass of spheres extending toward the chalazal end of the disintegrating synergid. These spheres stain positively with periodic acid-Schiff's (PAS) for insoluble carbohydrates (Jensen and Fisher, 1968a), and consist of polysaccharides involved in cell wall synthesis (Jensen, 1973). Jensen (1973) suggested these spheres may help gel the outer portion of the pollen tube cytoplasm, and thus help form a "tube" that directs the sperm cells to the chalazal end of the disintegrating synergid, where they are carried to the egg and central cells. There is evidence that the cytoskeleton plays a direct role in transporting the sperm to the egg (discussed elsewhere).

Just before the pollen tube ruptures, the plasma membrane of the synergid disappears (Jensen and Fisher, 1968a). The egg and central cells have no cell walls where they come in contact with the degenerating synergid (Jensen, 1965), enabling the sperm cells to come in direct contact with their plasma membranes. Sperm cells encounter the egg and central cells, where their membranes fuse, providing passage for the respective

sperm nucleus, exclusive of any sperm cytoplasm (Jensen and Fisher, 1968a). One sperm nucleus moves by an unknown mechanism through the egg cytoplasm to the egg nucleus, as does the other sperm nucleus through the central cell to the two polar nuclei. Jensen and Fisher (1967) suggested the sperm nuclei are passively carried via cytoplasmic streaming, since only the sperm enters the cell and has no evident means for locomotion. Stelly (per. comm., 1993) suggested a more controlled mechanism, such as filament- or tubule-mediated movement, and (or) perhaps a novel system involving the tubular endoplasmic reticulum (ER) formed near the time of nuclear fusion. Evidence of cytoskeletal involvement will be discussed in the next section.

Cytoskeletal Development Pre and Post Fertilization

Huang and Russell (1994) have described the cytoskeleton during the process of fertilization in tobacco (*Nicotiana tabacum L.*). Prior to fertilization, the egg cell cytoskeleton is essentially uniform and nonpolar. Cortical microtubules (MT) are found at the chalazal end of the cell near the wall-less interface with the synergids and the central cell in the area where fusion most often occurs. Little association with organelles is observed. MTs are mainly cortical, randomly oriented and mostly restricted to the chalazal and micropylar cytoplasm near the edge of the cell. Actin filaments are organized as a fine network at the cell cortex where they are associated with the MTs.

In contrast to the egg cell, the cytoskeletal structure of synergids is highly polarized, with most of the structure located near the micropylar end. Such a highly polarized cell may reflect the ability for rapid organelle movement (Huang and Russell, 1994). Microtubules, oriented mainly longitudinally, are usually located near the

micropylar end, the densest arrays being adjacent to the filiform apparatus (FA). A few MTs form a meshwork. MTs also frequently appear to be associated with one another. Microfilaments (MFs) occur in isolated regions and are associated with mitochondria and plastids. Huang and Russell (1994) believe MFs and MTs associated with the surface of organelles may be involved with establishing their intracellular location maintaining the polarized distribution of major organelles in the synergid cytoplasm.

In the central cell, MTs are mostly cortical occurring in bundles and singly, closely associated with the cell wall. MTs in lateral regions are mainly longitudinal but occasionally form a loose network. In the micropylar and chalazal regions, MTs are more random and more densely distributed terminating in cortical arrays next to the egg apparatus. A few MTs associated with organelles are frequently co-localized with actin. Although MFs are found only occasionally in the central cell, significant actin binding was observed (Huang and Russell, 1994).

The central cell and both synergids are highly polarized with prominent microtubule arrays. They are both highly active with rapid directional movement of organelles (Huang and Russell, 1994) which is presumably assisted by the cytoskeleton.

As the synergid begins to break down, two coronas form prior to PT arrival, inferably along the pathway the gametes follow after PT discharge to the fusion site (Huang and Russell, 1994). In *Plumbago zeylanica* L., which has no synergids, only one corona forms (Huang et al., 1993). The coronas are composed of aggregates of electron-dense homogeneous material approximately 0.2 to 1.5 μm in size which strongly stain for rhodamine-phalloidin. However, no filaments were observed. Whether that is due to

the "nature of the coronas" (Huang and Russell, 1994) or if they consist of F-actin fragments was not determined. In tobacco, the coronas are always oriented at an angle to one another. One begins at the midlateral region of the synergid and extends to the chalazal end. The other band forms along the side of the egg cell and extends along its chalazal boundary with the central cell. Ultrastructurally, these coronas correspond to electron dense bodies deposited at the boundary of the degenerated synergid, between the synergid and the central cell, and between the egg cell and the central cell.

The origin of the electron-dense aggregates that make up the "coronas" is not clear. Huang and Russell (1994) believed they originated mainly from cytoplasm from the degenerating synergid. Huang et al. (1993) observed that cytoplasmic bodies appeared to be "pinched off" from the surface of the degenerated synergid before pollen tube arrival. As the synergid degenerates it may be that this material is liberated and either passively or actively transported to the site. Other sources may be from migrating cytoplasm after plasma membrane breakdown (Dute et al., 1989; Hause and Schroder, 1987), or from cytoplasm originating in the PT (Engell, 1989; Jensen and Fisher, 1967; Fisher and Jensen, 1968a; van Went and Cresti, 1988; Yan et al., 1991). These coronas appear to coat the egg and central cell membranes (Huang and Russell, 1994)

During fertilization both sperm cells presumably pass along one of these coronas to the position necessary for respective cell fusion, one fusing with the egg and the other with the central cell. Such transport would probably include motor proteins associated with actin such as myosin. Myosin-like proteins have been observed in pollen tubes (Tang et al., 1989) and on the surface of generative cells (Heslop-Harrison and Heslop-

Harrison, 1989). If such myosin-like proteins are active during the fertilization process, they could actively transport each male gamete to its fusion site (Huang and Russell, 1994). After gamete fusion, a very light actin label can be seen on the nuclear envelope with heavier labeling in the cytoplasm near the sperm nucleus. In *Pelvetia fastigiata* (J. Agardh) De Toni (brown algae), F-actin was associated with gamete fusion; whereas, the migration of the sperm nucleus to the egg nucleus was dependent on microtubules (Swope and Kropf, 1993). Initially, MTs from the cortex presumably "pushed" the sperm nucleus toward the egg nucleus, but later, as the sperm nucleus approached the egg nucleus, MTs from the nuclear envelope of the egg nucleus connected with the sperm nucleus.

MTs commonly originate from MT nucleation sites from the cortical area of the cell or from sites around the nucleus (Flanders et al., 1990). Microtubules have been observed radiating from the nucleus of plant cells (Flanders et al., 1990; Schmit et al., 1983; Stelly, 1983; Wick et al., 1985). Several lines of evidence indicate that the plant nuclear envelope can serve as a microtubule organizing center (MTOC). Purified pig brain tubulin was used to nucleate MTs on the surface of isolated maize nuclei (Stoppin et al., 1994). In addition at least one protein located at the plant nuclear surface shared common antigenic determinants with the nucleating domain of mammalian centrosomes which are MTOCs (Stoppin et al., 1994). Chevrier et al. (1992) produced a monoclonal antibody against mammalian centrosomes that also bound to the plant nuclear surface, suggesting the mammalian centrosome and plant nuclear envelope share some common antigens and thus function(s).

It may be that MTs radiating from the nuclear envelope of the egg and sperm nucleus draw the sperm nucleus to the egg nucleus. Microtubules are reportedly associated with nuclear congression in both yeast (Kurihara et al., 1994) and brown algae (Kropf, 1994). In conjunction with a kinesin-like motor protein, which can pull MTs past each other, the sperm could be drawn toward the egg nucleus. The egg cell nucleus is surrounded by cytoplasm and from my observations, does not appear to move, but it is clear the sperm nucleus does. If these MTs or motor proteins are in some way defective, the nuclei may not be drawn in close enough proximity to begin the fusion process. From my preliminary observations, as the egg and sperm nuclei come into the general proximity of each other, they remain far enough apart to be easily observed with stain-cleared specimens. If apposition does occur, then the fusion mechanism may be defective, and the nuclei move apart.

Karyogamy

Karyogamy is the process of gametic nuclear fusion. In triticale (*Triticale hexaploide* Lart.), karyogamy occurs about 60 min after pollination (Hause and Schroder, 1987); whereas, in cotton it occurs approximately 14 to 16 h after pollination in *G. hirsutum* (Jensen, 1968a), and approximately 23 to 26 h post pollination in *G. barbadense* (this study). In maize (*Zea mays* L.), gametic nuclei were apposed to each other within 10 to 55 min after gametic fusion *in vitro* fertilization (Faure et al., 1993). In cotton, karyogamy seems to begin within minutes of gametic fusion (Jensen, 1968a; my observations, 1996). During karyogamy, ER that seems to originate from the nuclear envelopes of the participating nuclei, is associated with the membranes in the unfused

regions (Hause and Schroder, 1987; Hoffman, 1974; Janson and Willemse, 1995). Hause and Schroder (1987) consider the contact between the ER and the nuclear envelope as the initiation of karyogamy. Multiple contacts form between the ER and the outer membranes of the nuclei (Hoffman, 1974; Hause and Schroder, 1987; Janson and Willemse, 1995; Jensen, 1964). The outer membranes then shorten, forming bridges. Fusion among bridges leads to complete outer membrane fusion. The inner nuclear membranes fuse in a similar manner. As they enlarge, the connective nuclear bridges push all cytoplasmic elements aside (Hoffman, 1974). Chromatin in the sperm nucleus is highly condensed (Faure et al., 1993; Hause and Schroder, 1987; Hoffman, 1974; Janson and Willemse, 1995), but decondensation begins with the initiation of fusion (Faure et al., 1993; Hause and Schroder, 1987). In maize, the sperm nucleus is seen in a nuclear "side bud" 45 to 60 min after karyogamy and is recognized by its denser chromatin. After 60 to 195 min only one nucleus was observed in the maize zygote (Faure et al., 1993).

Post Fertilization

Cotton zygotes remain undivided for approximately two and a half days after fertilization. During this time, a number of changes begin to take place. At fertilization, the egg cell is about the same size as the synergid and is highly vacuolated. Shrinkage occurs after fertilization, reducing the zygote to about half the size of the egg in eight to ten hours (Jensen, 1968a).

Prior to fertilization, the central cell contains two large partially fused polar nuclei, each containing one large nucleolus, or occasionally two or more smaller ones (Joshi et al.,

1967; Schultz and Jensen, 1977). Fusion of the polar nuclei is completed after the sperm nucleus arrives, i.e. as part of the "triple fusion". The cytoplasm is concentrated around the polar nuclei and contains many plastids and mitochondria (Schultz and Jensen, 1977). Rough endoplasmic reticulum (RER) occurs parallel to the nuclear membrane and between the organelles. Ribosomes are found singly and as polysomes, both free and attached to membranes (Schultz and Jensen, 1977).

Soon after the sperm and polar nuclei fuse, the primary endosperm nucleus divides (Jensen and Fisher, 1967). Nuclear endosperm divisions are accompanied by a rapid increase in organelles such as dictyosomes, mitochondria and starch-containing plastids, along with an increase in RER (Schultz and Jensen, 1977). At the same time, there is a sharp decline in starches and lipids in the central cell. Helical polysomes, consisting of 15 to 20 ribosomes, appear shortly after fertilization, but disappear after the initial nuclear divisions (Schultz and Jensen, 1977), indicating a decline in protein synthesis.

The zygote is highly polarized at this stage. A large vacuole occupies most of the micropylar end; whereas, the nucleus and most of the cytoplasm and organelles are located at the chalazal end (Jensen, 1968a). Cleavage commences three to five days after pollination, with the first cleavage plane forming transverse to its central axis (Gore, 1932; Joshi et al., 1967; Pollock and Jensen, 1964; Reeves and Beasley, 1935). The cell at the micropylar end develops into a small suspensor consisting of three to four cells; whereas, the cell at the chalazal end develops into the embryo. The second zygotic cell division can occur in the suspensor cell or the chalazal cell, either transverse to or along its central axis

(Pollock and Jensen, 1964; Reeves and Beasley, 1935). Many different types of cell

displacement and arrangements occur as the embryo develops.

A RAPID STAIN-CLEARING METHOD FOR VIDEO-BASED CYTOLOGICAL ANALYSIS OF COTTON MEGAGAMETOPHYTES[*]

INTRODUCTION

Large numbers of cytological specimens are required for reproductive and cytogenetic analysis of angiosperms. Nondestructive methods such as optical "clearing" can ease the task of ovular analysis (Herr 1971; Crane 1978; Young et al. 1979; Crane and Carman 1987) by reducing or eliminating the time-consuming preparation and analysis of sectioned specimens. Unlike classical clearing in NaOH, which destroys cellular contents, "optical" clearing media allow one to obtain a focused view of nuclei and vacuoles inside a specimen. An optical clearing medium approximates the refractive index of cell walls, nuclei, and cytoplasmic organelles, rendering them relatively nonrefractive. In addition, optical clearings require simple laboratory equipment, preserve three-dimensional relationships within the specimen, and allow reorientation of the megagametophyte during examination. Optical clearing in plants is most commonly used for analysis of sexual and asexual reproduction in whole ovules and ovaries, but other potential applications may be found in pathological and genetic studies. A common clearing agent, Herr's 4½-type clearing fluid, was limited initially by "the temporary nature of the clearing preparations" (Herr 1982) and low refractive index.

Both of these limitations were avoided by using the aromatic esters methyl salicylate

(MS) (Crane 1978; Young et al. 1979), benzyl benzoate (BB), and dibutyl phthalate

(DBP) (Crane and Carman 1987) as clearing agents. In addition, they permit long-term

specimen storage. The refractive index of a BB:DBP medium can be adjusted from

1.490 to 1.568 to optimize for the species, tissue, or organelle type to be examined.

Analysis of cleared, unstained specimens requires contrast-enhancing optics,

such as Nomarski differential interference contrast optics, to visualize organelles

adequately. Limitations are the cost of such optics and the low contrast they may impart

to objects of similar refractive index. Simple brightfield optics can be used if the cleared

specimen has been stained. Pfeiffer and Bingham (1983) cleared alfalfa (*Medicago*

sativa L.) ovules in MS after staining with basic fuchsin in acid ethanol (Horobin and

Kevill-Davies 1971) giving very low contrast. To achieve clarity, resolution, and

contrast approaching that of paraffin sections, Stelly et al. (1984) regressively stained

ovaries of *Solanum* with Sass's modification of Mayer's hemalum, prior to clearing in

MS. Hemalum has subsequently been used in a number of studies, including white

clover, *Trifolium repens L.*, (Pasumarty et al. 1993) and alfalfa (Mariani et al. 1993;

Tavoletti 1994 and Tavoletti et al. 1991). Other researchers have stained and cleared

ovules with this technique with varying results. Both Jongedijk (1987) and Palser et al.

(1992) found destaining of the hematoxylin to be variable. Palser et al., however, found

that the stain improved optical contrast in the preparations. I have found that acid-

mediated destaining of Sass's hemalum is relatively difficult to predict and is often

insufficient for thick specimens. For example, hemalum-MS stain-cleared cotton

nucelli, which are approximately 300 μm in diameter near the egg apparatus at fertilization, have such deeply stained nucellar cells that the details in the underlying megagametophyte are obscured. At this stage, the light providing an image of the gametophyte must also pass through 12 - 35 layers of somatic cells.

CCD cameras, improved electronics, and modern image-processing software now provide additional tools to examine biological specimens. These tools can be expensive. Although the limit of resolution remains a function of microscope and specimen optical properties, specimen contrast and resolution can be adjusted at the microscope, the video camera, and the computer. Electronic image enhancement permits the use of specimens with low contrast for brightfield microscopy. Therefore, we have developed a method by which ovules could be stained lightly before clearing, with facile control over stain intensity.

MATERIALS AND METHODS

Ovaries of *G. barbadense* were selected one to five days after anthesis. Ovules were immediately excised and fixed in FAA_{50} for at least 24 h. Because numerous starch grains, which are objectionably birefringent, form in the integuments early in ovule development, so nucelli were dissected from ovules under a dissecting microscope. Nucelli were bulk-stained for 12 to 24 h in 0.08% azure C (CI 52002) in aqueous buffer or saturated azure C in buffered 50% or 70% ethanol. All azure C solutions were buffered to pH 4, 7, or 9, using citric acid/sodium citrate, sodium phosphate, and sodium borate/potassium chloride buffers (0.05 M), respectively. Destaining occurred during ethanolic dehydration and was optionally accelerated by the

addition of acetic acid and formalin to respective concentrations of 1% and 2%. Stained nucelli were washed in the current buffered solution (water, 50%, or 70% ethanol at the same pH), then dehydrated to 95% ethanol at 5% increments in unbuffered ethanolic solutions. With the addition of formalin and acetic acid, fluids were changed every 10 to 15 minutes owing to accelerated destaining. Without acetic acid and formalin, destaining was checked approximately every 15 minutes to observe how much stain was left and to change fluids when necessary. Nucelli were gradually infiltrated with MS through 15 to 30 min each of 5:1, 2:1, 1:1, 1:2, 1:5, 0:1, and 0:1, 95% ethanol:MS.

Raj slides were prepared by gluing two 22 x 40 mm #1 cover slips together with cyanoacrylate glue (super glue), cutting them lengthwise into thirds with a diamond pencil, and gluing them with cyanoacrylate ca. 7 mm apart on the slide. Nucelli were placed in the channel, which was then filled with MS and capped with a 22 x 40 mm #1 or #0.5 coverslip. Methyl salicylate slowly dissolves cyanoacrylate. Continuous exposure over three or four weeks caused the coverslips to detach from the slide. Coverslips remained attached for three to four months when the slides were cleaned daily after examining the specimen. The slide was examined with a Zeiss Universal II microscope, either directly or through the video camera and monitor that are described below. Because of the large size of the cotton nucellus, two objectives with long working distances were used: a planapochromat 40 X oil immersion, N.A. = 1.0, W.D. = 0.38 mm, and a 63 X Neofluar, N.A.= 1.25, W.D. = 0.5 mm. kindly loaned to me by Dr. Spencer Johnston.

Brightfield images were obtained using an Optronics VI-470 CCD camera (470-line, RGB/grayscale) attached to a 0.7- 7 X zoom lens mounted on the microscope. Magnification of each image was adjusted with the Optovar™ and/or the zoom lens. Camera controls and/or software were used to adjust light intensity, exposure time, and contrast for the desired image. For images to be saved digitally, camera noise was reduced by 4- or 8-frame averaging. Effects of uneven illumination due to foreign particles in the light path were subtracted by adding the inverse of an averaged image of the background. To illustrate results of various treatments without differential photographic effects, the plate was assembled digitally. The images were cropped, rotated, and in some cases contrast-enhanced, using Adobe Photoshop™ or Corel Photo-paint™. Images in the figure plate were adjusted to approximately equal brightness, assembled into a plate using Corel Draw™ and printed on dye-sublimation paper with a Kodak XLS 8600 PS printer courtesy of Meyer Instruments, Houston, TX.

All images were stored and processed in a 486-66 EISA computer with an Imaging Technologies CFG 24-bit RGB imaging board, driven by the Bioscan Optimas™ program (v. 4.0). Live images were viewed on a Sony 1343D monitor.

RESULTS AND DISCUSSION

Analysis of megagametophytes within stain-cleared nucelli or ovules requires a stain that is specific for the structures of interest. Otherwise, excessive staining of the nucellar cells can easily obscure details of the megagametophyte. I found that hemalum-based stain-clearing (Stelly et al. 1984) of cotton ovules insufficiently controlled intensity, making it difficult to obtain high quality preparations. Alternative stains and

staining conditions were therefore investigated. Azure C gave the best results. The intensity of azure C staining was controlled by the pH and polarity of its solvent. Staining was maximal at pH 7, less at pH 4, and negligible at pH 9. Staining also decreased with increasing ethanol concentration of the stain solvent (Fig. 1 A & B).

Nucleoli and synergids stained very darkly. The contents of the synergids were obscured in nucelli stained darkly with azure C. With lighter staining or greater destaining, the interior of the synergids could be seen clearly (Fig.1 C & D). Light transmission through less-stained nucelli was more uniform, simplifying both viewing and image analysis since camera and software settings required less adjustment across the specimen. On the other hand, greater staining with azure C enhanced the membranes of the cells and nuclei in the megagametophyte, making them easily distinguishable (Fig.1 E & F).

A generic limitation of clearings is that the clearing medium is optically isotropic and can match only one refractive index at a time. Plant cells contain cell walls and organelles that differ in refractive index. The zygote nucleus in cotton is usually surrounded by somewhat birefringent amyloplast-borne starch that has a different mean refractive index. These amyloplasts confused the visual detail to the image, but azure C-stained nuclei were generally easy to recognize (Fig1. G and H).

The strategy employed here was to reduce specimen stain intensity to allow ample light transmission through thick specimens and to compensate electronically for the lighter contrast within lightly stained specimens. Optimal video-based viewing was achieved when contrast of the stained specimens was too low for effective cytological

Fig. 1. Images from brightfield microscopy of specimens stained in azure C in the designated solution at the designated pH and cleared in methyl salicylate. Each image was digitally stored and processed with the image analysis system. The plate was constructed digitally and printed with a Kodak XLS 8600 PS printer on dye-sublimation paper. All images were stained in 0.8% aqueous azure C at pH 7 unless otherwise indicated. Each bar = 10 μm. (A & B) Comparison of stain intensity of polar nuclei prior to fertilization: Nuclear membranes and nucleoli are clearly visible in both A and B, but they are most distinct in the more darkly stained specimen (A), which was produced using an aqueous stain at pH 7. The more lightly stained specimen (B; stained in 70% ethanol at pH 4) was nevertheless informative, and still very unambiguous (distinct). Each image was grabbed as a single frame, and unprocessed. (C) A lightly stained degenerating synergid after pollen tube penetration. Although the light path passes through the synergid (Syn), the interior of the pollen tube (PT) can be seen in some detail. The pollen tube wall is clearly visible as it protrudes from the nucellus and makes an abrupt turn into the micropylar end of the synergid. Stained in saturated azure C in 50% ethanol at pH 4. Single-frame image, with slightly adjusted contrast and brightness. (D) A darkly stained degenerating synergid after pollen tube penetration. This is an optical section of a nucellus that was heavily destained for viewing the synergid contents. Remnants of the nucleus (Nuc), filiform apparatus (Fil), and vacuole (Vac) are visible in this section. This image, an 8-frame average, was adjusted for contrast and brightness. (E & F)   Distinction of small, closely associated organelles made more visible by more intense staining. The sperm nucleus (SN) can be seen just below the egg nucleus (EN) in E, and jammed between the two polar nuclei in F. Even though the sperm nucleus is small and does not stain very intensely, it can still be clearly seen as a distinct body. The Optovar™ and zoom magnified the images, while contrast, brightness, and gamma were adjusted for clarity of the nuclei. Background noise was subtracted from these 8-frame averaged images. (G & H) Zygotes with starch: The sperm and egg nuclei are clearly visible in this 8-frame-averaged, heavily destained semigamous zygote (H), but they are not as distinct as in the more darkly stained zygote of G. Staining, even when light, enhanced the relative contrast. A blue filter was used to reduce contrast on the 4-frame- averaged image of H. (I) Post-plasmogamous sperm and egg nuclei prior to karyogamy, magnified using a zoom lens system external to the microscope. Background was subtracted from this image, an 8-frame average. (J & K) Processing of a stored image. Through histogram equalization of the original 4-frame image average (J), this egg nucleus became much more appealing to the eye (K), although no more information was added. About 100 gray levels with values from 90 to 190 are present in J, versus the same number of values spread from 0 to 255 in K.
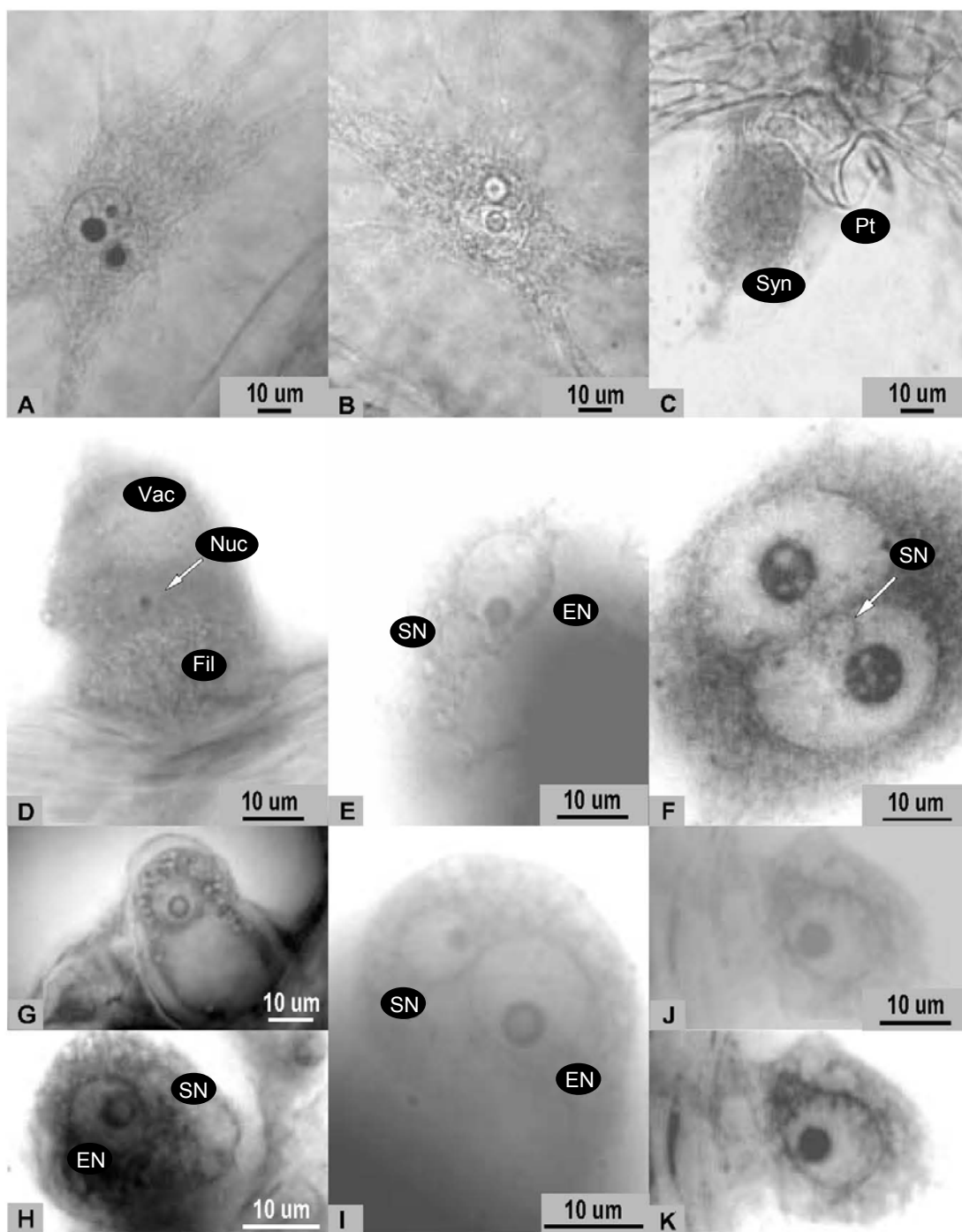
analysis by direct observation under Köhler brightfield illumination. Many samples

were stained sufficiently to distinguish cell walls, nuclei, and membranes without image

Since typical video camera and digital image resolutions are far below those of

color and especially grayscale films, sizing the image to fill the CCD chip was

particularly important. Magnifying lenses between the objective and the camera are

used for this purpose but can exacerbate deleterious effects of particles, etc., that may be

in the light path. Once the region of interest was scaled to fit the CCD, the microscope

and camera were adjusted to obtain the optimal image for digital processing. Camera

noise was reduced by averaging four or eight images of the specimen. Uneven light or

foreign particles in the camera path, whose effects can be compounded by a zoom

system, were reduced by background subtracted. Adjusting contrast and brightness

caused local saturation (pixel value = 255, Fig.1 D, E, H, & I) or extinction (pixel value

= 0, Fig.1 E & I) in some images. Because the pixel values of an unprocessed image

often cover only part of the value spectrum, spreading the values more equally over the

entire spectrum of gray values by "contrast equalization" or simply adjusting contrast,

brightness, and gamma usually clarify the image (Fig.1 J & K). None of the operations

of the image processing, except perhaps background subtraction, added any new

information to the image. Nevertheless visualization and reproduction were facilitated

(Fig.1 J & K).

Stain-clearing with azure C can be performed rapidly with simple equipment.

The entire process requires two or three days: one day or less for fixation, one for

staining, and one for dehydration, destaining, and clearing. Only the third day demands

manual working time. Stain intensity can be controlled progressively with an ethanolic staining solution, or regressively by the degree of destaining. Controlled stain intensity allows a wide applicability among organisms, structures, and goals. In this study I looked at cotton nucelli, which are very large**.** An image analysis system in conjunction with a brightfield microscope allowed me to stain lightly enough to see through the nucellus and at the same time provided acceptable contrast within the megagametophyte, including the synergids. Because of the ease of simultaneously processing large numbers of samples, stain-clearing combined with image analysis lends itself well to a broad range of studies requiring large sample sizes or thick specimens. The technique should be applicable to many botanical problems including reproduction, genetics, plant pathology, and plant development.

ANALYSIS OF SEMIGAMOUS COTTON USING STAIN-CLEARED NUCELLI

INTRODUCTION

Semigamy is an abnormal type of fertilization where syngamy, the union of the sperm and the egg, occurs but synkaryon formation, fusion of their nuclei, does not. The term "semigamy" was first used by Battaglia (1945) to describe reproduction in recurrently apomictic *Rudbeckia laciniata* L., in which there is a failure of synkaryon formation, and the sporophyte arises from a 2n female gamete. The 2n egg nucleus reportedly arises from a restitution nucleus from a previous division during embryo sac development. After fertilization the sperm nucleus divides but the products are confined to the basal cell of the embryo with respect to the micropyle. The basal cell usually contains one egg-derived nucleus along with the two sperm-derived nuclei. Additional divisions of the sperm-derived nuclei may occur, but they are limited to the basal area and do not participate in the development of the sporophyte. Cell walls form rarely between the sperm-derived nuclei or between sperm-derived nuclei and the egg-derived nucleus of the basal cell. Triploid nuclei have also been derived from joining of the chromosomes of the egg and sperm nuclei if the nuclei are sufficiently close to each other at the time of mitosis.

Semigamy has also been reported in a recurrently apomictic species of *Zephyranthes* (Solntzeva, 1978). Egg and sperm nuclei do not divide at the same time, and walls form between the four nuclei yield a four-celled embryo. The second division of the sperm-derived nuclei usually is not accompanied by cell wall formation. The apical

cell is derived solely from the egg cell, and is devoid of sperm-derived nuclei. Thus the *Zephyranthes* sporophyte arises from a genetically unreduced 2n egg nucleus. In semigametic "zygotes" of *Cooperia drumondii* Herb. (Coe, 1952;1953), the egg and sperm nuclei divide at the same time and undergo cytokinesis to form four cells. The two cells containing the derivatives of the sperm nucleus occupy a space lateral to the basal daughter cell which came from the egg. No additional cell divisions of the male derived cells were observed. The male-derived nuclei were very small. The apical cell is derived solely from the egg cell, devoid of sperm-derived nuclei. The *Cooperia* egg nucleus is "tetraploid", in that meiosis does not occur during megasporogenesis (Coe, 1953). In *Cooperia pedunculata* Herb. (Solntzeva, 1978), sperm nuclei may form polyploid cells. The basic characteristics of these recurrent forms of semigamy as reported in *Rudbeckia laciniata* L., *Zephyranthes*, and *C. pedunculata* can be summarized in the following way. The egg nucleus is tetraploid and the sperm nucleus does not take part in the development of the sporophyte. The sperm nucleus (or its derivative) is sequestered to a small part of the embryo and ceases to divide or is developmentally confined to part of the suspensor.

Nonrecurrent forms of semigamy have been reported in *Gossypium* (Turcotte and Feaster, 1963) and *Coix aquatica* Roxb. (Rao and Narayana, 1980), an Asian relative of maize. *Semigamy* has also been induced in *Arabidopsis thaliana* (L.) *Heynh* (Gerlach-Cruse, 1970). The *Coix aquatica* progeny reportedly include chimeras of the 2*n*-2*n* and the 2*n*-*n* variety (Rao and Narayana, 1980). These sectors appear as forked seedling stems

instead of the single stem of a wild type plant. Each fork resembled the male or female parent.

In mutant *Gossypium* that undergo semigamous reproduction (Turcotte and Feaster, 1963), both gametes are haploid, and the sperm nucleus takes part in development of the sporophyte. Progeny families include normal hybrid plants as well as maternal and paternal haploids. In addition, progeny can include chimeras with haploid maternal and paternal, and/or hybrid tetraploid tissue.

Haploids of cotton typically arise from polyembryonic seed (Blank and Allison, 1963; Endrizzi, 1959; Harland, 1936). The frequency of polyembryonic seed ranges from *ca.* 1 /60,000 in *G. hirsutum L.*(Kimber, 1958) to *ca.* 1/130 in certain lines of *G. barbadense L.* (Owings et. al., 1964). In contrast, semigamous reproduction in *Se* mutants leads to monoembryonic seed and yields haploids at frequencies up to 60% (Turcotte and Feaster,1963). The *Semigamy* mutant (*Se*) in cotton was initially recovered in a doubled haploid, 57-4, from Pima S-1, an obsolete commercial variety of *G. barbadense* (Turcotte and Feaster 1963). By using genetically marked parents, semigamously derived chimerism was shown to involve any combination of male haploid, female haploid, and/or hybrid sectors or plants haploid or tetraploid or a normal hybrid (Turcotte and Feaster, 1967). Evidence of the semigamous nature of *Se* was reinforced by observations that the seed produced were uniformly monoembryonic, yet gave varying percentages of normal hybrids, maternal haploids and paternal haploids, as well as chimeras composed of paternal, maternal and/or tetraploid tissue (Chaudhari, 1978; Turcotte and Feaster, 1967,

1969, 1973). It was suggested that polyspermy might be involved in chimeras with hybrid sectors (Turcotte and Feaster, 1973), but cytological investigations were not reported.

Turcotte and Feaster (1969, 1973) deduced from studies of genetically marked parents that semigamous reproduction occurred only if the egg nucleus possessed *Se*, but the frequency of *Semigamy* expression increased if the sperm nucleus also had *Se*. The frequency of haploids and chimeras increased from a low of 10% among progeny of the cross *SeSe* x *sese* to up to 60% from the cross *SeSe* x *SeSe*. (Chaudhari, 1978; Turcotte and Feaster, 1963, 1967, 1969, 1973). When Turcotte and Feaster (1974) backcrossed a 57-4 F_1 hybrid with a reproductively normal line, they obtained 35 progeny that reproduced semigamously and 39 that reproduced normally, i.e. approximately a 1:1 ratio. Thus, they proposed the ratio indicated that a dominant allele conferred facultative semigamous reproduction. However these results might be explained in several ways, depending on the time and mode of *Se* action (Gwyn, 1995). For example, if *Se* expression were determined megagametophytically, expression would occur in the hemizygous (haploid) state, regardless of the dominant or recessive nature of the mutant allele. In an attempt to identify the time(s) and mode(s) of *Se* gene action, Gwyn and Stelly (1990) developed a genetic-statistical test involving 3x3 factorial crosses of genetically marked *SeSe*, *Sese*, and *sese* genotypes. Their data indicated that *Se* is incompletely dominant, and that it is expressed sporophytically in both parents. The genotype of the zygote appears not to be a factor in *Se* expression. However, auxiliary tests intended to deconfound additive gene action of the sporophyte and hemizygous gene action in the gametophyte were not

completed due to the difficulty of doubling the chromosomes of a large number of haploid plants.

Expressivity of semigamous reproduction may be environmentally influenced. Turcotte and Feaster (1963) reported haploid production rates of 24.3% in 1961 from S_3 seedlings from field grown 57-4. Percent haploids from S_3 seedlings in 1962 were 43.4 % and 61.3% when grown in the field and greenhouse, respectively. The line 57-4 provided by Turcotte and its self-progeny produced no more than 10% chimeric or haploid progenies when grown in College Station, Texas from 1985 to1990 (Gwyn, 1995). Since then the reproductive behaviors of three other semigamous lines have been characterized and found to average between 30% and 40% chimeric and haploid progeny in College Station. These three lines, $R_1R_1SeSeV_7V_7$ (red), $r_1r_1SeSeV_7V_7$ (green), and $r_1r_1SeSev_7v_7$ (yellow-green), were derived by crossing R_1V_7 and r_1v_7 genotypes or were selected from the original 57-4 by David Stelly, James McD. Stewart, and Ian Dolan, respectively.

Given the unusual effects of the *Semigamy* mutation and the uncertainties regarding specific cytological mechanisms involved, a cytological investigation of this mutant was conducted, with emphasis on fertilization and early embryogenesis.

MATERIALS AND METHODS

Flowers and developing fruit of *G. barbadense* were harvested one to five days after anthesis. Ovules were excised and fixed in a solution of 10% formalin, 5% glacial acetic acid, 50% ethanol (95%) and 35% H_2O (FAA_{50}) for at least 24 h. Objectionably birefringent starch grains and dark phenolic pigments formed in the integuments early

during ovule development; therefore, nucelli were dissected from ovules under a dissecting microscope. Nucelli were bulk-stained from 12 to 24 h in 0.08% azure C (CI 52002) in 0.05 M phosphate buffer pH 7. Destaining occurred during ethanolic dehydration and was optionally accelerated by the addition of acetic acid (1%) and formalin (2%). Stained nucelli were washed in the buffered solution and then dehydrated to 95% ethanol at 5% increments in unbuffered ethanolic solutions. With the addition of formalin and acetic acid, fluids were changed every 10 to 15 minutes because of accelerated destaining. Without acetic acid and formalin, destaining was checked at 15 minute intervals to observe how much stain was left and to change fluids when necessary. Nucelli were gradually infiltrated with MS through 15 to 30 min each of 5:1, 2:1, 1:1, 1:2, 1:5, 0:1, and 0:1, 95% ethanol:MS.

To avoid compression during microscope observation, nucelli were mounted on Raj slides. Raj slides were prepared by gluing two 22x40 mm #1 cover slips together with cyanoacrylate ("super glue"), cutting them lengthwise into thirds with a diamond pencil, and gluing them with cyanoacrylate *ca.* 7 mm apart on a conventional glass slide. Nucelli were placed in the channel, which was then filled with MS and capped with a 22x40 mm #1 or #0.5 coverslip. Methyl salicylate slowly dissolves cyanoacrylate so continuous exposure over three or four weeks causes the coverslips to detach from the slide. The slide was examined with a Zeiss Universal II microscope, either directly or through a digital camera and CRT monitor. Because of the large size of the cotton nucellus, two objectives with long working distances were used: a planapochromat 40X

oil immersion, N.A. = 1.0, W.D. = 0.38 mm, and a 63X Neofluar, N.A.= 1.25, W.D. = 0.5 mm. kindly lent to me by Dr. Spencer Johnston.

Brightfield images were obtained through an Optronics VI-470 CCD camera (470-line, RGB/grayscale) attached to a 0.7- 7X zoom lens mounted on the microscope. Magnification of each image was optimized with the Optovar™ and/or the zoom lens. Camera controls and/or software were used to adjust light intensity, exposure time, and contrast for the desired image. For images to be saved digitally, camera noise was reduced by frame averaging four or eight frames. Macros (short programs) were written in Optimas to expedite the repetitive task of acquiring and averaging of multiple frames. Effects of uneven illumination due to foreign particles in the light path were subtracted by adding the inverse of an averaged image of the background. The images were cropped, rotated, and in some cases contrast-enhanced, using image-wide operations of Adobe Photoshop™ or Corel Photo-paint™. Images in the figure plate were adjusted to approximately equal brightness. All images were stored and processed in a Pentium II computer with an Imaging Technologies CFG 24-bit RGB imaging board, driven by the Bioscan Optimas™ program (v. 4.0 or 6.0). Live images were viewed on a Sony 1343D monitor.

Flow cytometry was used to determine the ploidy levels of the endosperm nuclei in the central cell . Samples of semigamous and nonsemigamous endosperm were excised prior to cellularization. As a check, tetraploid nuclei from the nucellar tissue was used from one of the nonsemigamous lines. The buffer used to suspend the nuclei for flow cytometry was made by adding 4.26 g $MgCl_2$, 8.84 g sodium citrate, 4.2 g 3-[N-

morpholino] propane sulfonic acid (MOPS), 1 ml Triton X-100^{TM} and 1 mg boiled

ribonuclease A to make one liter of solution (Galbraith et al., 1983). The pH was

adjusted to 7.2. Samples were chopped with a single-edge razor blade in the buffer and

filtered through a 53-μm nylon mesh. To the filtrate, which contained the nuclei, was

added propidium iodide (PI) to a final concentration of 50 ppm. Solutions were kept on

ice. The fluorescence of each sample was quantified using a Coulter Epics Elite (Coulter

Electronic, Hialeah, FL, USA) flow cytometer.

RESULTS

Prior to entry of the pollen tube into the female gametophyte, the egg cell nucleus

is typically located chalazally and cortically (Fig. 2 A&B) and, with few exceptions is

located, away from the synergids (Fig. 2A). In a few samples, the egg cell nucleus was

positioned in the middle or near the base, as shown in Figs. 2 C&D. A small amount of

cytoplasm was distributed around the nucleus and along the cortex, while much of the

egg cell is occupied by a large vacuole. There was no discernible relationship between

the location of the polar nuclei and the location of the sperm. In both semigamous and

nonsemigamous samples, the polar nuclei were positioned variously, anywhere from

adjacent to the egg cell (Fig 2E) to deep within the central cell (Fig. 2F). Polar nuclei

were usually positioned side-by-side (Fig. 2G), but were occasionally observed end-to-end (Fig. 2H).

Growth of the pollen tube into the embryo sac was as described by Jensen and Fisher (1967, 1968a). The pollen tube enters the ovule through the micropyle and travels directly to the embryo sac, entering it between the synergids. Soon after entering the embryo sac, the pollen tube makes an abrupt turn into the base of one of the synergids, and enters it through the filiform apparatus (Fig. 3A).

In most cases the pollen tube could not be identified beyond its entry into the synergid because the cytoplasm stained more intensely as the cell degraded. A limited number of synergids were not as intensely stained. In one sample the pollen tube had grown to the chalazal end of the synergid (Fig. 3 B-D). Beginning at the base of the synergid (Fig 3B), the pollen tube had traversed the cell (Fig 3 C&D) and had emerged at the chalazal end where the sperm were released. In all cases the sperm emerged along

Fig. 2. Egg, embryo sac and polar nuclei of semigamous cotton. Typical semigamous egg cells at 22 (A) and 23 (B) hours post-anthesis (HPA) show the range of nuclear locations. The nuclei were invariably associated with the cortex and were almost always located on the far side of the egg with respect to the synergids. The unfertilized egg was mostly occupied by one very large vacuole and the egg nucleus . A small amount of cytoplasm was associated with the nucleus and the cortex. C) Atypical semigamous egg cell with the nucleus positioned in the middle of the zygote, along with surrounding cytoplasm. The vacuole can be seen both above and below the nucleus. D) Atypical zygote with nuclei located in a basal position. (E and F) Positions of polar nucleivaried within the central cell, from the cell membrane nearest the egg apparatus (E) to deep within the central cell (F). (G and H) Positions of adjacent polar nuclei relative to each other. All polar nuclei were partially fused prior to fertilization and cytoplasm around the polar nuclei was always connected to the cytoplasm near the egg apparatus by a cytoplasmic strand. Polar nuclei generally occurred side-by-side (G), but occasionally end-to-end (H).
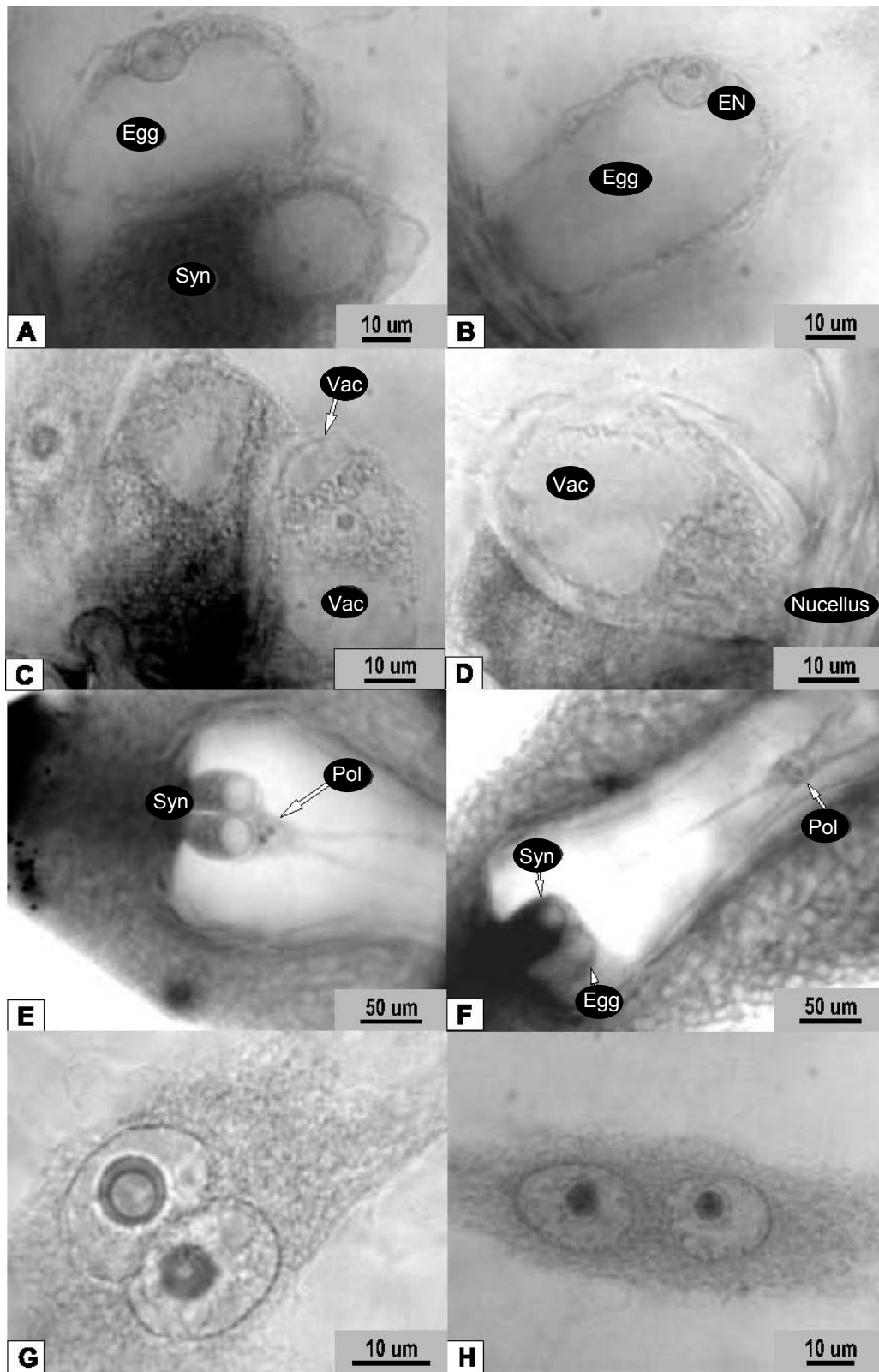
with the vegetative nucleus at the chalazal end of the synergid in the intercellular space between the egg and the central cell (Figs 3 E&F). When the sperm emerged, the nuclei were slightly elongated. Cytoplasm was not apparent, however the sperm cell membrane was visible (Fig. 3E).

Sperm in intercellular space

The egg nucleus is cortical, nearly always opposite the release point of the sperm (Fig 4A) and usually located in the chalazal half of the egg. Beginning at the synergid and extending into the intercellular space between the egg and the central cell is a darkly stained finger of synergid cytoplasm with the sperm near the apex (Fig. 4A). Sometimes the cytoplasm extends from the synergid through the intercellular space to a position chalazal to the egg and between the egg and the central cell (Fig 4B). The cytoplasm can be seen extending around the egg cell to its chalazal extreme. As the synergid shrinks from degradation, this extension remains visible. Sperm were observed anywhere along the apical portion of the egg (Figs 4 C&D) and flattened in the intercellular spaces between the egg and the central cell as seen in Figs 4 E&F. From
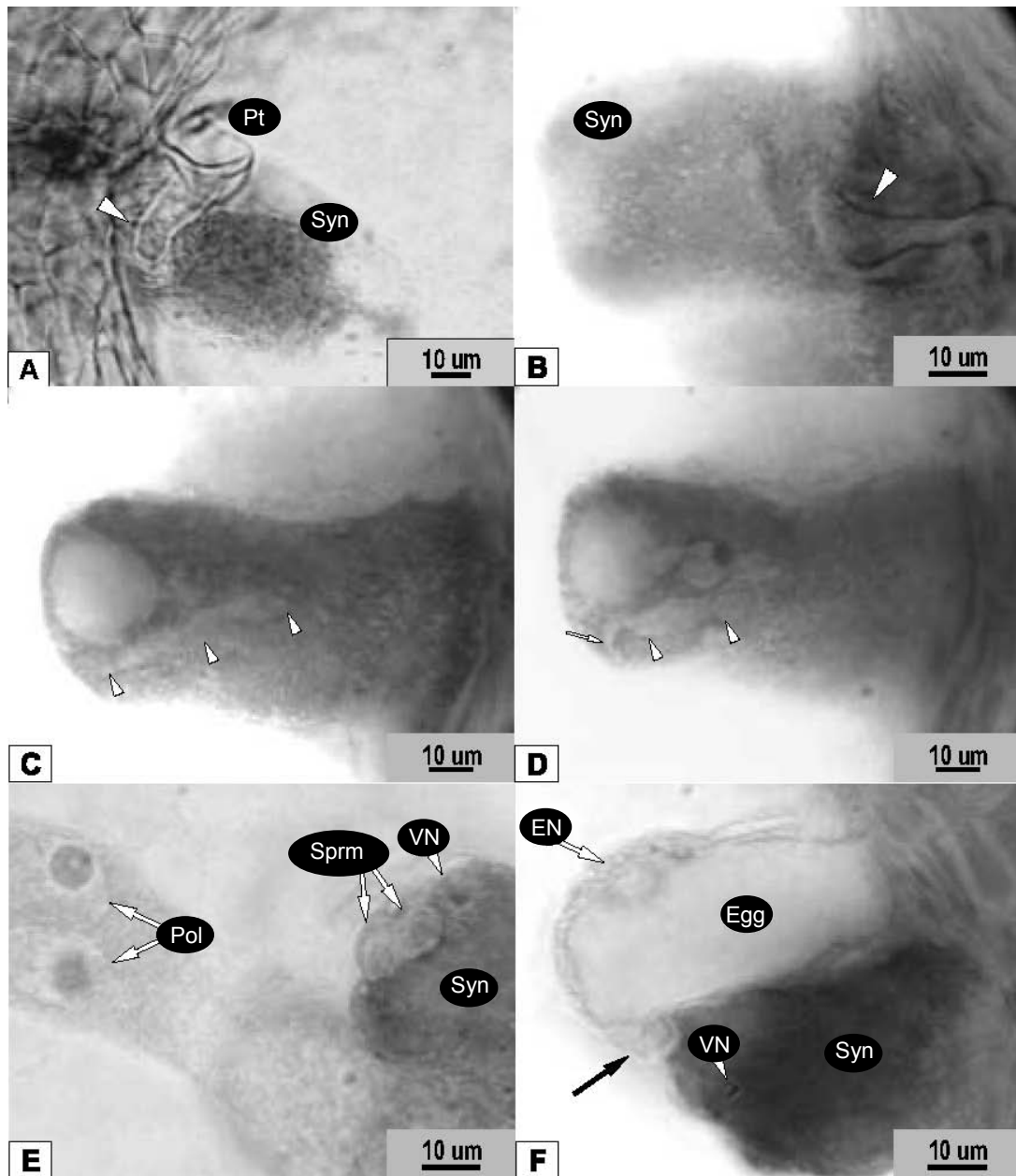
Fig. 3. Pollen tube growth and sperm release. A) A pollen tube that has entered the egg apparatus between the synergids and made an abrupt turn to enter one of the synergids through the filiform apparatus. The sperm can be seen near the tip of the pollen tube (arrowhead). (B-D) Degenerating synergid with pollen tube. The pollen tube (arrowhead in B) has entered the egg apparatus, turned into the synergid, grown to the chalazal end of the cell, where it has turned sharply and ceased growth (arrow in D). The path of the pollen tube is marked by arrowheads in C and D. Sperm cells are expelled at the extreme tip of the synergid next to the egg (E). Dark-staining synergid cytoplasm extends into the intercellular space, up to the more lightly stained cone-shaped area that contains the sperm (black arrow in F).
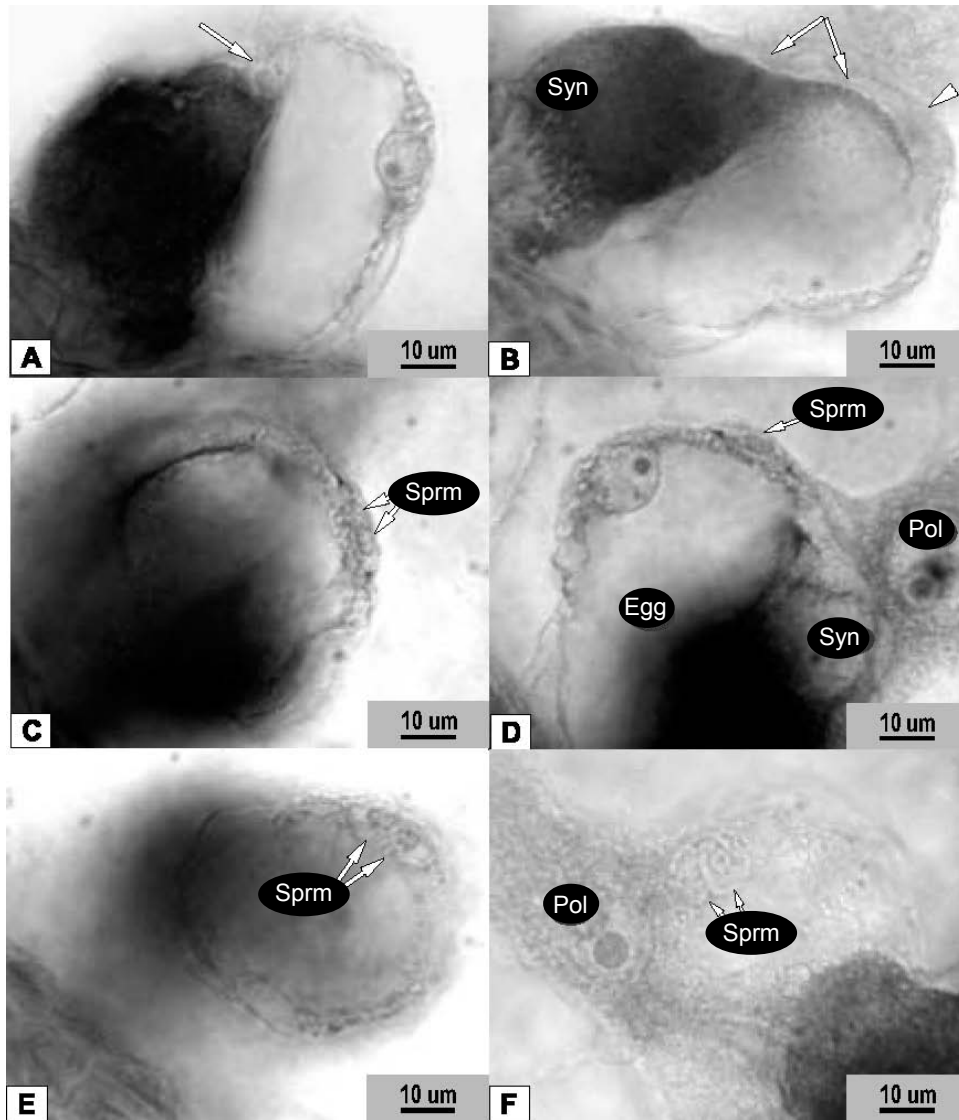
Fig. 4. Movement of sperm cells in the embryo sac after they emerge from the synergid and before syngamy. A) The egg nucleus is located cortically and away from the cone-shaped area where the sperm are released (arrow). B) Sometimes cytoplasm from the synergid stretches beyond the cone-shaped area, actually extending around the egg (arrows). The sperm cells (arrowhead), are above the narrow ribbon of cytoplasm, but out-of-plane in this optical section. (C and D) Two views of the same embryo sac. The sperm shown in the intercellular space, which is dynamically shaped to create a larger intercellular space around the migrating sperm cells. (C) Both sperm nuclei are visible, one elongate and one round. D) Migrating sperms seen in relation to the egg nucleus. They are closer to the egg nucleus than when originally released from the synergid, but are still in the intercellular space. E) Polar view of egg cell apex, which in contrast to D, reveals spheroid rather than spherical shape of sperm cells. The nuclei and cell membranes are visible, but the sperm cytoplasm is not. F) Polar view of egg cell apex. The polar nuclei are adjacent to the spheroid sperm.

this prospective of the embryo sac, the sperm nuclei are prominent with a visible cell

membrane but no visible cytoplasm.

By 25 HPA the non-semigamous line had just completed or was in the process of

completing karyogamy (Figs. 5 A&B). As karyogamy progressed the sperm nucleus

flattened against the egg nucleus (Fig. 5B). Shortly after fusion two nucleoli were seen

in the newly formed zygote nucleus (Fig. 5C) but quickly formed a single nucleolus.

Egg volume decreased over time with a reduction of the vacuole while at the same time

cytoplasm surrounding the zygote nucleus increased. At the first mitotic division of the

zygote, most of the remaining vacuole was relegated to the basal cell with little or no

vacuole observed in the chalazal cell (Fig. 5D)

Syngamy in semigamous cotton occurred some time between 23 and 26 hours

post-anthesis (HPA). At 23 HPA, the first sperm were observed just beyond the

synergid in the intercellular space between the egg apparatus and the central cell. After

26 HPA, no sperm were observed outside of the egg or the central cell. Immediately

following syngamy the sperm nucleus in the zygote was near the egg nucleus but

karyogamy did not occur (Fig 6A).

Egg and sperm nuclei of heterokaryotic *SeSe* zygotes

In semigamous cotton, following syngamy, egg and sperm nuclei were routinely

separate. Relative to the long axis of the megagametophyte, the sperm nucleus was

usually observed to be on the same plane with the egg nucleus, but occasionally above or
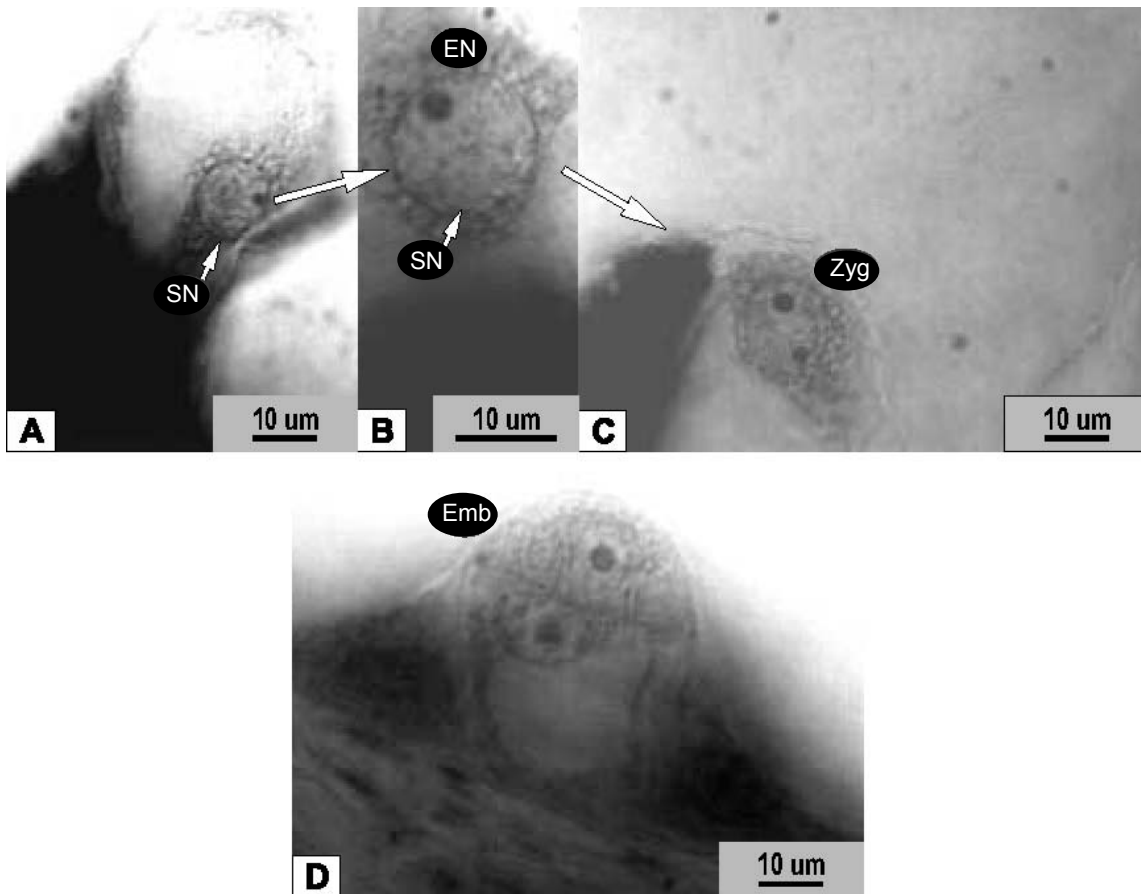
Fig. 5. Images of early development in a non-semigamous embryo sac. A) Karyogamy in progress at 25 HPA. The sperm nucleus has flattened against the egg nucleus. B) A close-up of the same process. C) Post-karyogamy. Two prominent nucleoli are visible in the zygote nucleus, the maternal one being larger. D) 2-celled embryo. The basal cell is vacuolated and larger than the chalazal cell, which is filled with cytoplasmic substances.

below it. As the zygote developed, the two nuclei were seen at any orientation with respect to each other and were closely apposed to highly separated. In no instance however, did the sperm nucleus seem to be appressed to the egg nucleus.

The diameter of the egg nucleus at the time of syngamy was about twice the diameter of the sperm nucleus at about 12 μm with 8 times its volume. The sperm

nucleus increased in size to about the initial volume of the egg nucleus, while the egg nucleus nearly doubled in volume (Fig 6B).

At the time of syngamy, a nucleolus was present in the egg but not the sperm nucleus. However, by about ten hours later, a nucleolus was clearly visible in the sperm nucleus, with a diameter slightly less than 0.5 micrometers. It progressively became prominent, increasing to about 1 micrometer in diameter at zygote division. The egg nucleolus increased from approximately one micrometer in diameter to one and a half micrometers. One nucleolus was observed in each of the zygotic nuclei, each increasing in size until division of the zygote.

During analysis some samples had a single nucleus. These were unfertilized eggs distinguished from zygotes by the amount of cytoplasm that had accumulated. Unfertilized eggs remained highly vacuolated with no additional accumulation of cytoplasm.

The egg cell cytoplasm was sparse with the greatest concentration surrounding the nucleus. After fertilization, the cytoplasm increased in quantity until cell division. As in the egg throughout the development of the zygote, most of the cytoplasm surrounded the nuclei. The egg cell volume decreased slightly as the vacuole shrank but shrinkage of the zygotes seemed to be less extreme than in non-semigamous zygotes.
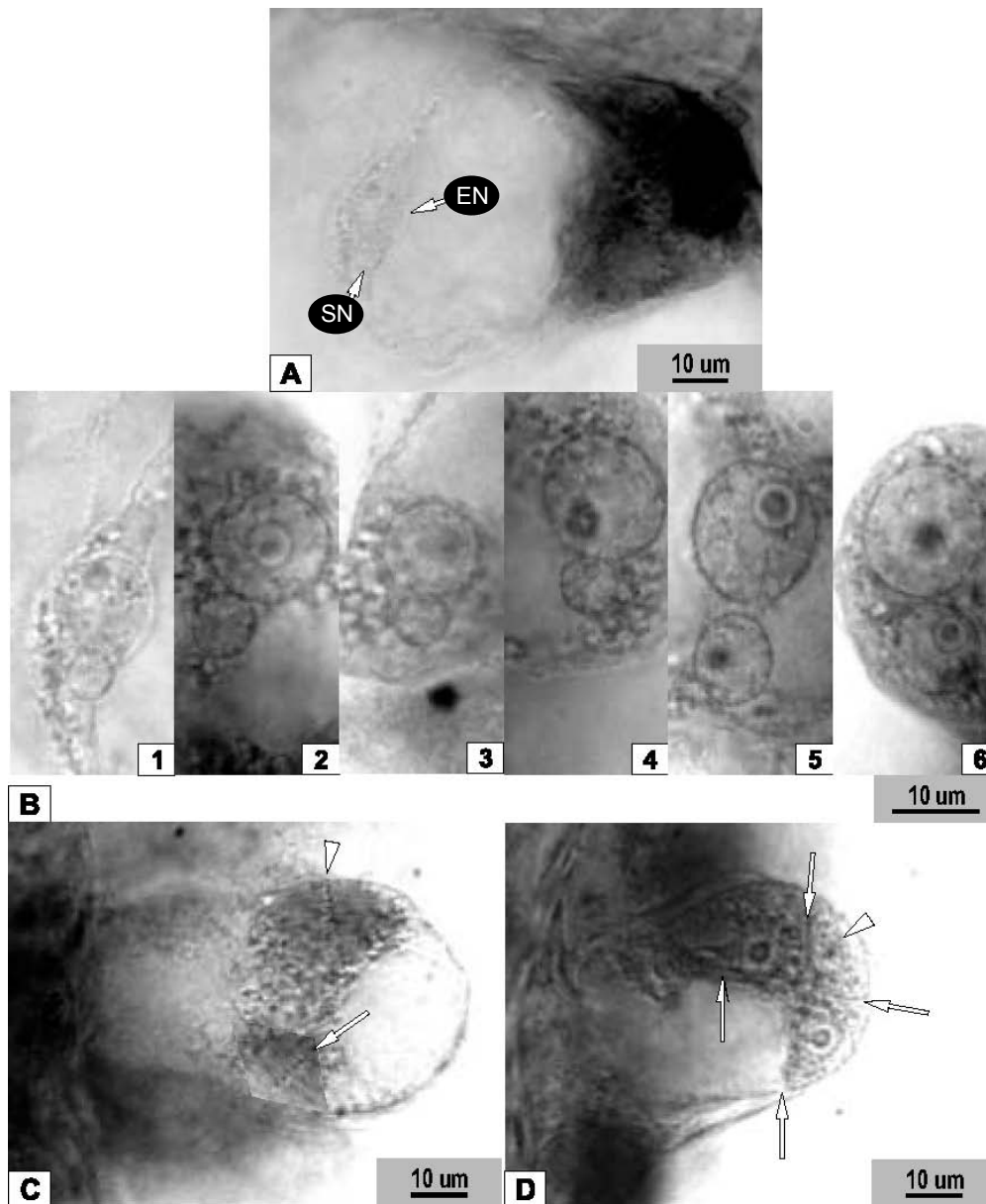
Fig. 6. Development of a semigamous zygote and early embryo. A) In this newly fertilized egg the small sperm nucleus is just below the egg nucleus. Although adjacent, both nuclei are spherical and not appressed or fused. The heterokaryotic zygote is highly vacuolate. B) Series of images (1-6) showing the progression of nuclear development in semigamous heterokaryotic zygotes. Sperm and egg nuclei differ in size but both increase in size and develop a prominent nucleolus after syngamy and prior to mitosis. C) Fused image from two opitical sections of one semigamous zygote undergoing mitosis. Two spindles are visible, one larger and one smaller, respectively orchestrating mitosis of egg (arrowhead) and sperm (arrow) nuclei. D) Embryo immediately following division of heterokaryotic zygote containing four haploid cells. The arrows point to the cell wall and the arrowhead points to the nucleus which is out of focus. One basal cell is highly vacuolated.

Zygote division

The timing of initial embryo development was similar for both semigamous and nonsemigamous zygotes. Mitosis of the zygote was first observed at 95 HPA, but zygotes were still present in some ovules at 125 HPA, a span of nearly 30 hours. In the first division of a non-semigamous zygote the cell divided transversely with respect to the long central axis of the megagametophyte, producing a smaller daughter cell chalazally and a larger more vacuolate daughter cell nearer the micropyle. Semigamous zygotes remained heterokaryotic until the first mitotic division. Nevertheless, the gametic nuclei divided synchronously. Individual spindles were seen if the nuclei were far enough apart (Fig. 6B), but if they were close together, individual spindles could not be distinguished. The egg nucleus spindle was larger than that formed by the sperm nucleus, being both wider and longer. Spindles were parallel to each other when viewed together but usually not in the same plane as one spindle was slightly more chalazal. Upon completion of the first mitosis, cell walls formed with one nucleus in each cell. Three different embryo types resulted from the zygote cell division: 1) four-celled (Fig 6C), all haploid, two derived paternally and two maternally; 2) three-celled, two haploid (one paternal and one maternal), and one tetraploid hybrid cell formed; and 3) two tetraploid hybrid cells formed with a single nucleus in each. It was not possible to determine differences in nuclear volume due to obstructions caused by dense cytoplasm. A total of 477 semigamous zygotes and embryos were examined in ovules collected from 95 to 114 HPA (Table 1). Three hundred and twenty-two of these were zygotes, all of which had an egg and a sperm nucleus, and 155 were embryos. One embryo was

eight celled indicating it had passed through the second cell division. Of the 154 post-

first mitosis embryos collected, 97 had three or more cells, whereas 52 were two-celled

embryos. The frequencies of three- and four-celled embryos compared to total embryos

was similar to the frequencies of chimeras and hybrids from progeny tests using

seedlings from parents homozygous for *Semigamy*.

Table 1. Zygotes and embryos from 95 to 114 hours post anthesis (HPA). Numbers of one- and two-nucleate zygotes and of 2-,3-,4-, and 8-celled embryos observed

| | Zygote | | Embryo | | | |
| | Number of nuclei | | Number of cells | | | |
HPA	1	2	2	3	4	8
95	0	24	1	0	0	0
96	0	65	1	0	5	0
97	0	31	0	0	0	0
98	0	7	1	0	1	0
99	0	13	1	0	4	0
100	0	9	3	0	3	0
101	0	14	8	0	12	0
102	0	9	6	0	2	0
103	0	6	0	0	0	0
104	0	3	5	0	5	0
105	0	30	11	0	16	0
106	0	64	4	2	31	0
107	0	7	2	0	2	0
108	0	15	0	0	0	0
111	0	7	0	0	0	0
114	0	18	14	0	14	1
totals	0	322	57	2	95	1

At zygotic division of non-semigamous zygotes, the vacuole was positioned

toward the micropylar end of the cell, which led to its inclusion in the basal cell product

that gives rise to the suspensor. The apical cell product was smaller and more densely packed with cytoplasmic substances. At comparable stages, the vacuole of semigamously derived heterokaryotic zygotes was not consistently positioned in the micropylar region but was variably distributed in any of the daughter cells. After mitosis, all cells in some embryos were highly vacuolated and thus were very dissimilar in appearance to first-division products of non-semigamous zygotes.

The central cell

Arrival of sperm cells at the egg and central cells was first observed at approximately 23 HPA. At the time of sperm nucleus entry, polar nuclei ranged from being immediately adjacent to the egg apparatus to deep into the central cell. After the sperm nucleus entered the central cell, sperm nuclei were seen only in association with the polar nuclei (Fig. 7), an indication that the sperm nucleus is carried very quickly to the polar nuclei. In semigamous central cells, the sperm nucleus regularly fused with the polar nuclei, but the fusion was slightly delayed . Table 2 summarizes endosperm development in semigamous and nonsemigamous embryo sacs.

In nonsemigamous embryo sacs, 24 HPA was the earliest time at which a primary endosperm nucleus was observed, and at 25 HPA was the earliest a two-nucleate endosperm was observed. Thus, a period of up to two hours separated the arrival of the sperm nucleus, the formation of the primary endosperm, and its mitotic
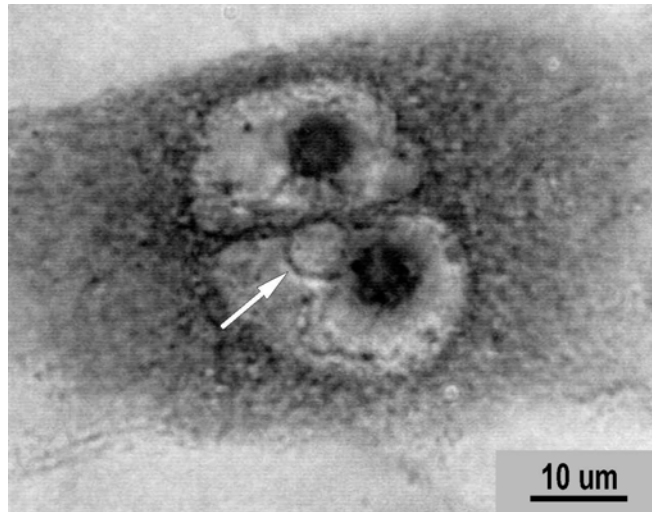
Fig. 7. Partially fused polar nuclei and the sperm nucleus (arrow). Image is from a sample collected 39 hours post anthesis of semigamous cotton.

division. Nine of 16 fertilized samples had at least the primary endosperm by 25 HPA. At 40 HPA, polar nuclei and primary endosperm nuclei were never observed, while as many as 8 endosperm nuclei were present in the central cell. At 53 HPA, no fewer than 28 endosperm nuclei were present. Of the 14 ovules examined at 53 HPA, four had more than 50 endosperm nuclei.

In semigamous ovules, the first division of a primary endosperm nucleus occurred at 32 HPA. Of 27 ovules examined at 39 HPA,17 still had the polar nuclei present, 3 had formed a primary endosperm nucleus and 7 had 2 endosperm nuclei. At 40 HPA, half of the ovules still had their polar nuclei. By 42 HPA, two of 15 ovules still had their polar nuclei. Less than 1% of the ovules from this time forward still had polar nuclei.

Table 2. Endosperm development. Numbers of semigamous (SeSe) and normal (sese) zygotes from 25 to 59 hours post anthesis (HPA) with polar nuclei (Polars) or the indicated number of endosperm nuclei

Genotype	HPA	Polars	Number of endosperm nuclei in the central cell								
			1	2	4	8	9-15	16	17-31	32	33+
SeSe	25	30	0	0	0	0	0	0	0	0	0
	26	15	0	0	0	0	0	0	0	0	0
	27	4	0	0	0	0	0	0	0	0	0
	29	27	2	0	0	0	0	0	0	0	0
	30	8	1	0	0	0	0	0	0	0	0
	31	11	1	0	0	0	0	0	0	0	0
	32	6	6	4	0	0	0	0	0	0	0
	33	1	0	0	0	0	0	0	0	0	0
	34	7	11	11	0	0	0	0	0	0	0
	35	8	5	12	0	0	0	0	0	0	0
	36	12	8	10	2	0	0	0	0	0	0
	37	12	0	1	0	0	0	0	0	0	0
	38	10	0	1	0	0	0	0	0	0	0
	39	17	3	7	0	0	0	0	0	0	0
	40	13	3	10	0	0	0	0	0	0	0
	41	2	1	11	3	2	4	0	1	0	0
	42	2	0	9	3	0	0	0	0	0	0
	50	1	1	4	7	3	8	7	0	1	0
	51	0	0	1	1	1	0	1	0	0	4
	52	1	1	3	8	3	3	1	2	2	0
	59	0	0	1	0	14	11	8	4	2	5
	total	187	43	85	24	23	26	17	7	5	9
sese	24	15	2	0	0	0	0	0	0	0	0
	25	7	7	2	0	0	0	0	0	0	0
	30	41	18	12	0	0	0	0	0	0	0
	40	0	0	1	5	7	0	0	0	0	0
	53	0	0	0	0	0	0	0	7	1	6
	total	63	27	15	5	7	0	0	7	1	6

Although endosperm formed, it was necessary to determine the ploidy level of the nuclei because triple fusion in the central cell was not directly observed. Because karyogamy failed in the zygote, it could be that the same process in the central cell also failed. Semigamous endosperm nuclear ploidy levels were measured using flow cytometry and compared to ploidy levels of endosperm nuclei from normal plants using tetraploid nuclei from nucellar tissue. Nucellar tetraploid cells had a mean of 106.6 channels while the nonsemigamous endosperm had a mean of 157.8 channels. Three endosperm samples from the semigamous plants had a mean of 167.6 channels. Tetraploid nuclei were not observed indicating triploid endosperm development. Endosperm nuclei from both normal and semigamous samples were triploid. Although endosperm development in semigamous samples was slightly delayed when compared to the nonsemigamous ovules, numerous endosperm nuclei were present when the zygote divided.

The synergids

Prior to synergid degeneration, their cytoplasm stained uniformly but with a grainy medium intensity. All synergids had a large vacuole at the chalazal end of the cell and a large spherical nucleus just under the vacuole. Some synergids exhibited signs of degeneration prior to the arrival of the pollen tubes in the nucellus, but others showed no signs of change with a pollen tube approaching the egg apparatus.

At pollen tube entry, the synergid stained very deeply but also variably. In addition, the synergid nucleus and vacuole began to degrade. The speed at which a synergid underwent degeneration, presumably a form of apoptosis, varied from sample

to sample. In Fig. 3B, synergid deterioration appears to be slight even though the pollen tube has penetrated the cell and the sperm are at the chalazal end. In most circumstances, the pollen tube was not visible beyond its the entry point into the synergid, as the tube wall was not visible using the cytological methods employed. There were a few exceptions in that the pollen tube was seen and traced across optical sections, thereby revealing the path to its ultimate destination. For example, the entrance of the pollen tube through the filiform apparatus is visible in Fig 3B. After rotating the specimen, the pollen tube is visible in the lower middle portion of the synergid and through successive focal planes emerges at the chalazal end where it has turned sharply (Figs 3 C&D). The region into which the sperm cells were released is just behind the pollen tube shown in this image (Figs 3 C&D).

Upon pollen tube entry, an organized synergid nucleus was no longer visible, but other objects were sometimes visible, including spherical and birefringent objects. The vegetative nucleus was also observed near the chalazal end, at the point of sperm release (Figs 3 E&F). As the disintegrating synergid decreased in size, it seemed to maintain contact with the egg. For example, a finger of cytoplasm from the synergid was observed at 34 HPA (Fig 8).   At 50 HPA, some of the persistent synergids exhibited symptoms of "breaking down", including deterioration of spherical nucleus shape, and shrinkage of the chalazal vacuole. By the first zygotic division, very little remained of either synergid.

Fig. 8. Cone-shaped finger-like cytoplasmic extension of the disintegrating synergid toward apical region of egg cell bordering with the central cell at 34 HPA.

DISCUSSION

Semigamy expression is complete in the zygote

Semigamy in cotton has been evaluated principally according to sporophytic phenotype. The classification of phenotype and genotype at the *Se* locus has typically been based on progeny testing, i.e., by characterizing a family of progeny from self-, open-, or cross-pollination scored as seedling, flowering plants, and/or mature plants for the occurrence of haploids, tetraploids, and chimeras. The occurrence of haploid and chimeral progeny indicate the presence of the *Semigamy* mutant allele, though haploid-

tetraploid twins can occur at appreciable frequencies in some genotypes of *G. barbadense* (Owings et al.,1964).

Turcotte and Feaster (1974) and Chaudhari (1978) concluded that a single dominant gene, *Semigamy* (*Se*), confers semigamous reproduction in cotton, and that it exhibits varying levels of penetrance. The occurrence of tetraploid progeny was attributed to karyogamy, whereas haploid and haploid chimeric progeny were attributed to expression of the *Semigamy* allele. The percentages of haploids produced from crosses of 57-4 between 1959 and 1962 ranged from 25% to 61.3% (Turcotte and Feaster, 1963, 1974). In these same reports *Semigamy* expression as defined by haploids and chimeras is slightly higher because chimeras with haploid and tetraploid sectors were also produced. Table 3 summarizes reports of *Semigamy* expression. Total expression from crosses of parents homozygous for *Se* ranged from about 40% to 75% when analyzing seedlings and plants. If the male parent was not semigamous, the proportion of haploids produced was greatly reduced, ranging from 1.7% to 8.9%. When the female parent was not semigamous, haploids were not recovered from the cross.

The level of *Semigamy* expressivity has been considered the determinant of the frequency of haploid and chimeric plants (Turcotte and Feaster, 1967). According to this interpretation, expression of *Se* leads to karyogamy failure and thus the production of haploids (2n = 26) and chimeras, but lack of expression leads to normal fertilization and production of normal progeny (2n=52). In this study, a different means of analysis was used, i.e., one based on cytological examination rather than progeny testing. Four

hundred and forty ovules of genotype *SeSe* were examined that contained zygotes, and karyogamy failure was universal among them. Every zygote, except for one, had one egg and one sperm nucleus. – i.e., a heterokaryon, resulting from syngamy without karyogamy. In *SeSe* ovules where a single nucleus was observed, the egg cell was devoid of the rich cytoplasm characteristic of a zygote, and it was highly vacuolated, as in egg cells prior to syngamy. Both features strongly indicate fertilization had not occurred in these ovules.

A rapid accumulation of cytoplasmic substances was observed when gametes fused. In just one zygote of *SeSe*, a single nucleus was observed. This could have been a result of karyogamy, or could be due to failure to discern the sperm nucleus amidst densely packed cytoplasm which included starch. Starch grains caused birefringence making it difficult to accurately see the organelles. This sample was collected 39 HPA, where the sperm nucleus would be expected to be small and not easily detected when surrounded with such dense cytoplasm.

Early investigations of *Se* revealed the production of ploidy chimeras (Turcotte and Feaster, 1967). In an attempt to explain their occurrence, Turcotte and Feaster (1973) proposed the possibility of polyspermy. They theorized the sperm nucleus may have divided in the pollen tube or, more likely, in the egg before karyogamy. One sperm nucleus fused with the egg nucleus forming tetraploid hybrid cells while the other sperm nucleus developed autonomously. In the present study no polyspermy was observed. No more than one pollen tube was seen at the synergid and no more than one sperm nucleus was observed in the zygote. In other species that undergo semigamous

reproduction, the observation of coenocytic cells could potentially increase the possibility of nuclear fusion, which could lead to sectors with higher ploidy levels (Solntzeva, 1974, 1978). Indeed, Zhang et al. (1996) reported failure of cytokinesis in semigamous cotton. If cytokinesis failed at any appreciable frequency in semigamous cotton, somatically doubled haploid sectors should arise and be regularly observed. Somatically doubled haploid sectors have not been reported to date, except after treatment with chromosome-doubling agents, such as colchicine. Turcotte and Feaster (1967) initially proposed that the tetraploid sector in ploidy chimeras was the result of failure of cytokinesis, but later they observed that these sectors were not only tetraploid but also hybrid (Turcotte and Feaster, 1973). Cytokinesis failure was not observed in this study.

In this study, all phenotypes that have been observed in semigamous cotton are thought to have arisen as products of zygote division. Haploid and tetraploid sectors are not determined by karyogamy or its failure but rather when the zygote divides. Of 163 embryos formed, 61 were two-celled and 100 were four-celled. Two embryos had three cells. This reflects the variability of relative spindle positions, orientation, and the generic tendency for nearby telophase chromosomes to form a common nucleus. The tetraploid two-celled embryos formed from the union of the sperm and egg chromosomes when the zygote divided. It is only at this time when the sperm and egg chromosomes unite. Formation of a common nuclear envelope around the two haploid sets of chromosomes, one from the egg cell and one from the sperm cell, results in a tetraploid hybrid product at the common spindle pole. Four-celled embryos form when a

separate nuclear envelope develops around each haploid set of chromosomes, resulting in two maternal and two paternal uninucleate haploid cells. Three-celled embryos result from a tetraploid cell being formed at one pole and two haploid cells forming at the other pole as described above. This appears to be the result of the spatial relationship of the spindle poles, which vary from being close together to relatively far apart, and the angle between spindles at mitosis. When the poles are close, the neighboring chromosomes form a common tetraploid nucleus, and when the poles are farther apart, the haploid groups of chromosomes independently form nuclei. The angle between the spindles also may determine ploidy level. There would be a greater chance of a tetraploid nucleus forming at the pole where the angle between metaphase plates decreased; whereas, at the pole where the angle is increased there is a better chance of two haploid nuclei forming. Unification of chromosomes when spindle poles are in close proximity was reported in *Rudbeckia laciniata* (Solntzeva, 1974).

Sixty-one percent of the embryos in this study were haploids, which compares closely with percentages that have been observed among progeny tests (Table 3). When individual ovaries were examined, the ratio of 3- and 4-celled embryos vs. 2-celled embryos ranged from 37.5 % to 100%. Such variations in expression also have been reported in semigamous cotton plant progenies. Zhang et al. (1998) reported *Semigamy* expression of 57-4 to be stable as a population but varied greatly among individual plants. Progenies of 50 selfed 57-4 parental plants were grown and scored for haploids. Haploid production varied from 14.3% to more than 60%. The plants that produced less than 30% haploids were thought to be heterozygous for *Semigamy*. Since all chimeric

ploidy combinations are products of zygote division, the simplest explanation for these products is the spacial relationship of the egg and sperm nucleus and of the spindle poles which form during mitosis. This relationship may be affected by the shape and size of the zygote, and the cytoplasmic and cytoskeletal architecture. Centrosome-like activity may fix the orientation of the spindles. These and other genetic and environmental factors may affect the final embryo type that results.

The central cell

Nuclear fusion was not directly observed in the {[(*Se*)(*Se*)][(*Se*)]} heterokaryotic central cells of *Se* gametophytes, and if it occurs, it is delayed. In *Semigamy* genotypes, the sperm nucleus was seen in the central cell at 23 HPA, but a primary endosperm nucleus was not observed until 29 HPA. Moreover, primary endosperm nuclei were still seen in some nucelli as late as 34 HPA. By contrast, the primary endosperm had divided at least once by this time in all non-semigamous samples. Thus, whereas the endosperm developed rapidly in non-semigamous ovules, development in semigamous ovules was delayed. As late as 52 HPA, only two endosperm nuclei were observed and 4 endosperm as late as 60 HPA, but endosperm nuclei were nevertheless abundant by the time the zygote divided.

Without direct observation of triple fusion of the sperm with two polar nuclei, it is not possible to unequivocally state that nuclear fusion gives rise to the primary endosperm nucleus in semigamous cotton. Moreover, without direct observation of the first mitotic division, the possibility remains that mitosis-based fusion events at the spindle poles could lead to functional endosperm nuclei with a 2:1 ratio of

Table 3. Summary of literature reporting semigamy expression in *Gossypium barbadense* L.

| Cross | | Phenotype | | | Method of | Expression | |
Female	Male	Normal	*Se*	Total	analysis	(%)	Reference
Dolan	Dolan	1	439	440	Cytological	~100	Present work
57-4	57-4	516	361	877	Progeny test	41.6	Turcotte and Feaster, 1963
57-4	$SeSe v_7 v_7$	30	90	120	Progeny test	75	Turcotte and Feaster, 1974
$SeSe v_7 v_7$	57-4	22	59	81	Progeny test	72.8	Turcotte and Feaster, 1974
VSG-7	VSG-7				Progeny test	~40	Chaudhari, 1978
VSG-7	57-4	60	109	169	Progeny test	64.5	Chaudhari, 1978
57-4	Pima vir	356	35	391	Progeny test	8.9	Turcotte and Feaster, 1967
57-4	Pima glandless	235	9	244	Progeny test	3.4	Turcotte and Feaster, 1967
57-4	Pima markers	1,589	70	1,659	Progeny test	4.2	Turcotte and Feaster, 1969
57-4	Pima markers	3,158	197	3,355	Progeny test	5.9	Turcotte and Feaster, 1973
$SeSe v_7 v_7$	Pima stocks	5,435	209	6,544	Progeny test	3.7	Turcotte and Feaster,1974
VSG-7	*G. barbadense* x *G. hirsutm*	2,035	59	2,094	Progeny test	2.8	Chaudhari, 1978
57-4	*G. hirsutum*	236	4	240	Progeny test	1.7	Turcotte and Feaster, 1967
VSG-7	*G. hirsutum*	15,949	647	16,596	Progeny test	3.9	Chaudhari, 1967

maternal:paternal genomes. The limited sampling of endosperms C-values provided no evidence contrary to either hypothesis. Thus, available data favor neither hypothesis, i.e., that triple fusion does or does not occur in semigamous ovules. While the two possibilities lead to distinctly different sorts of hypotheses of governance, the end result from a practical standpoint is that a reproductively functional endosperm often results.

Additional information on the regularity with which functional *SeSeSe* endosperm forms and, if possible, the first division in *SeSeSe* endosperms would provide some additional insight into *Se* expression and its governance. For example, the regular occurrence of triple fusion prior to the first mitosis would indicate the effect of *Se* on karyogamy is relegated to the egg cell and zygote, and is not gametophyte-wide or, if so, expression is modulated by differences between the two sperm and(or) by the respective milieus – the egg cell and(or) prekaryogamic zygote versus the central cell and(or) the prekaryogamic endosperm. Conversely, regular absence of the triple fusion would indicate that the effect of *Se* is homogenous across the egg cell/prekaryogamic zygote and central cell/prekaryogamic endosperm. Nonhomogeneity of expression in the prekaryogamic zygote and endosperm would suggest transcription or translation occur after gametophytic cell differentiation and mitosis, or if not, that functionality of products depends on the two cellular environments. Additional insight into the mechanism might also be afforded by examination and comparison to the polar nuclei, behavior of which seems normal, including their partial fusion prior to what would be the time of triple-fusion. This suggests, that the molecules underlying initial phases of polar nucleus fusion are distinct from those that underlie early stages of karyogamy of

sperm and egg nuclei, and perhaps also triple-fusion. Given the existence of gene families and regulatory specificity, this need not imply that the mechanisms are different, though that too seems plausible.

Does DNA replication occur in the sperm and egg?

Direct measurements of the DNA content of the sperm and egg nucleus have not been reported in any cotton species. However, in cotton, fusion and primary division of the endosperm occur so rapidly that it may not be possible for DNA synthesis to occur following karyogamy. Jensen and Fisher (1967) reported that the division of *G. hirsutum* endosperm nuclei began soon after karyogamy, and a primary nucleus was rarely observed. The interval from the formation of the primary endosperm until nuclear division also was very short in this study, i.e., in *G. barbadense*. At 23 HPA, sperm were not observed in the egg or central cell, though the pollen tube was present. By 25 HPA, two endosperm nuclei were typically present in the central cell. Approximately two hours spanned entry of the sperm into the central cell to development of two endosperm nuclei. Since replication of the sperm DNA must occur before the primary endosperm nucleus divides, one of several possibilities must occur: 1) DNA synthesis occurs while the sperm is in the pollen grain and/or the pollen tube; 2) DNA synthesis occurs in the sperm nucleus after it enters the central cell; or 3) DNA synthesis occurs after primary endosperm formation.

Are sperm in some flowering plants capable of replicating their DNA? DNA contents for the sperm and egg nuclei has been reported to be 1C in barley (Bennett and Smith,1976b and Mogensen and Holm, 1995), maize (Mogensen et al., 1995), and

Tradescantia (Woodard, 1956) either by direct measurement or by inference. DNA synthesis in these plants reportedly did not occur until after fertilization. Studies with other higher plants indicate that the sperm nucleus in some Angiosperm species may be capable of DNA synthesis. Ermakov et al. (1980) reported *Crepis capillaries* L. and *Elytrigia elongata* (Host) Nevsky sperm were in G_2 at the time of fertilization while *Chlorophytum elatum* R. Br. and *Ligularia dentate* (A. Gray) Hara were in the S-phase at the time sperm DNA was measured.

Friedman (1999) measured DAPI-stained sperm in *A. thaliana* from the division of the generative cell to just prior to karyogamy. Using the generative cell just prior to mitosis as a baseline for relative fluorescence, fluorescence from the sperm nuclei was measured up to pollen germination on the stigma and after entry into the ovary. Fluorescence was not measured from sperm of pollen tubes in the style and pistil. Newly formed sperm began almost immediately to show higher levels of fluorescence than the 1C DNA expected in G_1. By the time of pollen shed, sperm DNA averaged 1.4C. Fluorescence showed that DNA quantities continued to increase to 1.98C when the sperm arrived at the embryo sac. Thus, at syngamy *A. thaliana* sperm are in G_2. Van't Hof et al. (1996) reported that the S-phase in *A. thaliana* somatic cells is about three hours long. The estimated time for the pollen tube to travel from the stigma to the ovary is eight to ten hours (Kandasamy et al., 1994), thus providing sufficient time for S-phase to finish while the sperm travel from the style to the embryo sac.

In their condensed state, do sperm nuclei in higher plants have the capability of replication? Sperm have been considered by most to be incapable of DNA synthesis, in

part because in most eukaryotes the sperm nucleus is small and chromatin is highly condensed. Lower plants, such as algae, bryophytes, and ferns, have highly condensed chromatin (Reynolds and Wolfe, 1984). In contrast gymnosperms in general may have little or no sperm chromatin condensation as reported in *Ephedra trifurca* Torr. Ex S. Wats., *Ephedra nevadensis* S. Wats., and *Gnetum gnemon* L. (Friedman, 1991, 1990 and Carmichael and Friedman, 1995), *Agathis australis* (Lambert) Steud. (Owens, et al., 1995), *Picea glauca* (Moench) Voss (Dawkins and Owens, 1993), *Ginko biloba* (Gifford and Lin, 1975) and *Zamia* (Norstog, 1975). In terms of the degree of sperm chromatin condensation, angiosperms appear to be intermediate between these two groups (Bloch, 1976).

Pogany et al. (1981) calculated the nuclear volume necessary for nucleosomal solenoidal packaging of the same amount of DNA found in mouse sperm (3.3 ρg) to be 15.5 μm^3. Dimensions of sperm nuclei of *Petunia hybrida* and *G. hirsutum* have been measured from which approximate nuclear volumes can be calculated. The nuclear volume of the male germ units in *P. hybrida* were measured at 18.2 μm^3 and 20.0 μm^3 for the trailing and leading sperm cell respectively (Wagner and Mogensen, 1988). The 1C value for *P. hybrida* was 1.68 ρg (Bennett and Smith, 1976a) which would need a minimum nuclear volume of 7.9 μm^3 if the chromatin is to maintain nucleosomal solenoidal packaging. *G. hirsutum* sperm nuclei are reported to have the dimensions of 6×3 μm (Jensen and Fisher 1968b) and a 1C amount of 3.23 ρg (Bennett and Smith, 1982). This means the nucleus has a volume some where near 50 μm^3. If that were the case, then *G. hirsutum* sperm nuclei have a volume 60% greater than what is needed to

house a 2C amount of DNA as a nucleosomal solenoidal package. In addition, a nucleolus is present in cotton sperm nuclei, which although small, indicates possible gene activity (Jensen and Fisher, 1968b).

Translatable pools of mRNA have been identified in sperm cells of *Lilium longiflorum* (Knox et al.,1993: Ueda et al., 2000). Transcripts have been identified that only appear in the generative and sperm cells (Ueda et al., 2000). The proteins expressed by these transcripts did not appear until mid-maturation of the generative cell and accumulated in the condensing nucleus of the generative cell. They were most abundantly found in the sperm nucleus. In addition, mRNAs of histone cDNA clones accumulated exclusively in the generative cells at late stages of pollen maturation (Xu et al., 1999a), indicating male gamete-specific gene expression in *Lilium*. Activation of a gene specifically in the generative and sperm cells was also detected (Xu et al. 1999b). These are indications that the sperm cell may be transcriptionally active. In addition sperm cell mitochondria were found in the condensed or active form in sperm of *H. vulgare* (Cass, 1973).

An intact nucleus is required *in vivo*. Nuclear pores are necessary for selective import and export of proteins. Therefore an intact nucleus is necessary to permit transport of proteins for initiation of DNA replication (Newport 1987; Blow and Sleeman, 1990; Cox, 1992). Additionally, proteins essential for initiation need to be transported out of the nucleus, or they must be inactivated in order to prevent unwanted initiation during the S-phase (Rowles and Blow, 1997).

The few reports of nuclear pores in the envelope of the sperm nucleus of angiosperms indicate nuclear pore numbers vary with species but that sperm nuclei in general have a reduced number of nuclear pores (Russell and Cass,1981; Southworth et al., 1997; Straatman et al., 2000; LaFountiane and LaFountaine, 1973; Mogensen and Wagner ,1987; Shi et al., 1991). In contrast, Jensen and Fisher (1968b) reported numerous pores in the sperm nucleus of G. *hirsutum* in samples taken from the pollen tube, whereas very few occurred once the sperm nuclei were in the egg and central cells (Jensen and Fisher, 1967).

To determine if DNA synthesis does occur in cotton sperm, either of two studies might be followed. PCNA antibodies could be used to target the protein needed for DNA replication. This could be used on sperm cells in the pollen tube or sperm cells in the embryo sac. The other approach is to germinate cotton pollen on medium that contains tritiated thymidine. DNA replication would be detected by the presence of the marker being incorporated into the DNA. Previous research has used indirect approaches to determine DNA in the sperm nucleus.

If synthesis did not occur until after sperm entry into the egg or central cell but before karyogamy, a sufficient delay between entry and karyogamy must occur. Cotton sperm nuclei begin fusion almost as soon as they enter the egg. No time is available for DNA synthesis. Indeed, if nuclear pores are an indicator of the ability to synthesize DNA, then DNA synthesis in sperm nuclei, the egg, and central cell would be less likely than sperm nuclei in the pollen tube (Jensen and Fisher, 1967).

There is sufficient time in the zygote nucleus for DNA replication since mitosis does not occur for several days. However, for replication to occur after karyogamy in the central cell of cotton, synthesis would have to occur much more rapidly than in somatic cells. Time for DNA synthesis is known to be reduced under certain conditions. Plant cell cycles are reduced by activating quiescent replicon origins when a vegetative meristem switches to a reproductive meristem and begins to form flowers (Jaqmard and Houssa, 1988; Ormrod and Francis, 1986). In animals, DNA replication under embryonic conditions is very rapid. For example, in surf clam, early embryonic cell cycles take 20 to 30 min. while in Drosophila the initial cell cycles are even shorter (Collas and Poccia, 1998). As in many animals, G_1 and G_2 are eliminated because the egg contains a stockpile of proteins necessary for cell division (Poccia, 1986). Such protein stores have not been documented in plants. The central cell shows a much more rapid cell cycle pattern than the zygote, partly due to the fact that cytokinesis is not occurring. I have estimated the cell cycle in the cotton central cell to be approximately seven hours based on mitosis observed in the central cell. If DNA replication occurs after karyogamy, there must be a one time increase in initiation sites reducing the cell cycle to less than two hours.

FLOW CYTOMETRIC ANALYSIS OF SEMIGAMOUSLY
DERIVED CHIMERIC COTTON SEED

INTRODUCTION

In 1967 Turcotte and Feaster first reported the production of ploidy chimeras in cotton by a new mutant, *Semigamy* (Turcotte and Feaster, 1967) In that study they used 57-4 as the ovule parent in crosses with pollen parents homozygous for recessive mutations conferring either reduced-virescence leaf or glandless plant phenotypes. In progeny tests, some of the chimeric seedlings had 2n sectors of maternal phenotype, i.e., green and glanded. They concluded that the 2n sectors formed when cytokinesis failed at zygote division with some of the products being maternal or paternal restitution nuclei. In later studies, all 2n sectors were demonstrated to be hybrid (Turcotte and Feaster, 1973). The line 57-4, which was green and glanded ($r_1r_1Gl_2Gl_2Gl_3Gl_3$), was crossed as female with plants homozygous for the glandless genes gl_2 and gl_3 and heterozygous for red plant color (R_1r_1). Among progeny, paternally derived tissue would be red or green and glandless, maternally derived tissue would be green and glanded, while hybrid tissue could be either green or red and glanded. Sectors that were both green and glanded might be either maternal or hybrid, so plants were grown until flowering to reveal flower size and fertility, and thus their ploidy and origin. Haploid flowers were small and sterile while hybrid flowers were larger and fertile. Out of 195 chimeras from this cross, at least 67 were ploidy chimeras (~34%). At the time it was proposed that tri-chimeric plants resulted from two male and two female gametes (Turcotte and Feaster, 1973). Here, I report all phenotypes that have been observed in

semigamy are produced at the time of embryo formation, as was proposed initially by Turcotte and Feaster (1967). However, the products are different. The angle of the mitotic spindles in relation to each other and the distance between them may determine the products. If the poles are close at one end and distant at the other, three nuclei form; one is hybrid, one is paternal and haploid and one is maternal and haploid. In another study ploidy chimeras made up 10% of the progeny (Turcotte and Feaster, 1974). In section three of this dissertation 1.2% of the newly formed embryos were ploidy chimeras. Two ploidy chimeras were found in 165 embryos examined. Initially ploidy chimeras have two haploid cells, one maternal and one paternal, and one hybrid cell. What happens to this ratio as the embryo develops? This study was undertaken to answer that question.

MATERIALS AND METHODS

Mature seeds of three lines homozygous for *Semigamy* were used to study the effects of *Semigamy*, per se. I also compared results of the three lines, 57-4, R_1Se, and Sev_7, which are related to unknown degrees by way of pedigrees, since the latter two were derived from crosses of marker lines with 57-4 or one of its derivatives by David Stelly (unpublished) and Turcotte et al (1969).

Nuclei for cytometric analyses were isolated and prepared from self-seed obtained by open pollinations of 57-4, R_1Se, and Sev_7 plants grown in closed greenhouses. The seed were placed in a beaker containing ice-cold Galbraith buffer (Galbraith et al, 1983) and left in a refrigerator overnight. Just prior to chopping, the seed coat was removed and each seed was individually chopped with a new single-edge

razor blade in two ml of ice-cold Galbraith buffer. Additional seeds were each partitioned into three parts, the root cap, the hypocotyl, and the cotyledons. Each part was prepared as indicated above. One liter of Galbraith buffer consisted of 4.26 ml of 4.9m $MgCl_2$ solution (Sigma), 8.84g sodium citrate, 4.2 g 3-[*N*-morpholino] propane sulfonic acid (sodium salt, $C_7H_{14}NO_4SNa$), 1 ml Triton X-100 and 1 mg boiled ribonuclease A (Galbraith et al., 1983; Price and Johnston, 1996). All chopped samples were kept on ice. The chopped tissues were filtered through a 30-µm nylon filter. To the filtrate, which contained the nuclei, was added propidium iodide (PI) to a final concentration of 50 ppm.

Each seed or seed part was analyzed individually with a Partec CyFlo® laser flow cytometer. FloMax® software by Partec was used to produce and analyze the data. Tetraploid seed samples were analyzed to set the peak near channel 200. Seeds were classed as haploid (tetraploid), tetraploid or chimeric (haploid-tetraploid) based on the presence of peaks near channel 100, 200, or both, respectively.

Cytometric data on the nuclei were used to estimate the ploidy constitution of each chimeric seed, i.e., in terms of the relative abundance of haploid versus tetraploid cells. A χ^2 test for heterogeneity of phenotypes and genotypes and of cell ratios in ploidy chimeras was done. Additionally Chi-square values for ploidy chimeras were calculated as compared to an expected haploid:tetraploid ratio of 2:1, the ratio of cells in a newly formed embryo, if chimeric.

Chimeric seed were initially analyzed using the raw data. However, data were then transformed to facilitate graphical analysis of changes in haploid versus tetraploid

cell populations, which had the expected ratio of 2:1. For a sample size of N, the

expected numbers of haploid and tetraploid cells would be would be N*2/3 and N*1/3,

respectively. Before transformation, a shift in the frequency of cells across the ploidy

levels, e.g. +n and –n in total sample of N, would result in relatively smaller percentage

change of haploid than tetraploid cells, because the haploid cells would be twice as

abundant. As a proportion of the expected, a 5% shift (0.05*N) between 2x and 4x

categories would lead to a smaller (half) proportion change the haploid numbers

(0.05*N/(2N/3) = 0.15/2N) than in the tetraploids (0.05*N/(N/3) = 0.15/N). The aim of

transformation was to indicate *relative* frequency changes of haploid or tetraploid cells,

according to the 2:1 ratio expected. Thus, variable "*y*" was derived by conditional

transformation of the ratio between observed numbers of haploid:tetraploid nuclei (*h*:1),

otherwise denoted as *h.* When **$h > 2$**, then **$y = h/2$**, whereas when **$h < 2$** then **$y = -2/h$**. If

an instance were to arise where **$h = 2$**, which was highly unlikely given the large sample

sizes involved (thousands), then an arbitrary assignment was to be made for **$y = h/2$**.

The effects of this transformation were examined by comparison of actual and

transformed data. Following transformation, haploid frequencies above the expected

haploid frequency resulted in positive **y**-values; whereas, increases of tetraploid

frequencies above the expected frequency (1/3) resulted in a negative *y*-value. At **$y = 1$**

or -1 the observed ratio would exactly match the ratio expected in a newly formed

chimeric embryo, i.e., 2. At **$y = 2$**, the ratio is 4, twice the number of haploids expected.

At **$y = -2$** the ratio is 1 indicating there are twice as many tetraploids with respect to

original haploid-tetraploid ratio. Thus, after transformation, the positive values (**y**)

would indicate an abundance of haploid cells, negative values would indicate an

abundance of tetraploid cells, and departures from symmetry about 0 would indicate

non-random occurrence, persistence or detection.

RESULTS

Analysis of ploidy chimeras

A total of 663 mature cotton seeds from three genotypes (*R1Se,* 57-4, and *Sev₇*)

were analyzed. Forty-eight percent of the seeds were tetraploid, 30% were haploid and

22% were ploidy chimeras (Table 4). Nearly equal numbers of ploidy chimeras were

produced by each genotype.

Table 4. Number of haploids, tetraploids, and chimeras determined by flow cytometry in semigamous cotton seeds

| Genotype | Phenotype | | | |
	Haploid	Tetraploid	Chimera	total
R_1Se	42	140	52	234
57-4	94	85	45	224
Sev_7	61	97	46	204
total	197	322	143	662
percent	29.76%	48.64%	21.60%	

Table 5 is a list of ploidy chimeras measured from chopping whole mature seed.

The number of tetraploid and haploid cells counted and the χ^2 based on the expected

haploid to tetraploid ratio (2) are listed. For all chimeric seed, the populations of nuclei

differed significantly from the expected 2 : 1 ratio of haploid : tetraploid cells. A

Table 5. Number of haploid and tetraploid nuclei, nuclear ratios, and Chi squares of ploidy chimeras in semigamous cotton seeds

Genotype											
R_1Se				57-4				Sev_7			
Number of nuclei				Number of nuclei				Number of nuclei			
Haploid	Tetrapld	Hap/Dip	x^2	Haploid	Tetrapld	Hap/Dip	x^2	Haploid	Tetrapld	Hap/Dip	x^2
11743	102544	0.1145	163546	7528	34474	0.2184	44908	22992	98103	0.2344	123882
12445	106924	0.1164	169907	39794	147142	0.2704	173228	41298	64335	0.6419	36134
18411	107327	0.1715	153141	33319	97215	0.3427	99425	162617	241441	0.6735	126924
17006	93629	0.1816	130997	18436	45837	0.4022	41727	66019	88318	0.7475	39641
23218	105917	0.2192	137747	26190	60883	0.4302	52455	13589	17049	0.7971	6864
22963	100737	0.2280	128804	15219	35065	0.4340	29982	172242	169747	1.0147	40898
25477	93335	0.2730	109346	36594	63400	0.5772	40688	126655	115444	1.0971	22438
39092	111970	0.3491	113095	40234	60264	0.6676	32076	82148	66525	1.2348	8714
33993	95890	0.3545	95843	74580	108955	0.6845	55966	89056	64435	1.3821	5164
82773	216195	0.3829	204423	43081	49832	0.8645	17229	70876	45497	1.5578	1739
15409	26799	0.5750	17276	40634	45844	0.8864	15070	100982	63294	1.5954	1996
55042	73961	0.7442	33436	34614	36174	0.9569	10057	171833	86923	1.9768	8
36880	49393	0.7467	22211	55776	58083	0.9603	16015	83709	35560	2.3540	664
66358	67915	0.9771	17972	57580	57255	1.0057	14112	191479	76964	2.4879	2626
60454	58383	1.0355	13342	31971	31551	1.0133	7628	132963	43234	3.0754	6135
76692	50211	1.5274	2219	27910	26528	1.0521	5808	125489	29630	4.2352	14138
90538	38812	2.3327	645	74619	63028	1.1839	9611	106617	18572	5.7407	19277

Table 5. Cont'd

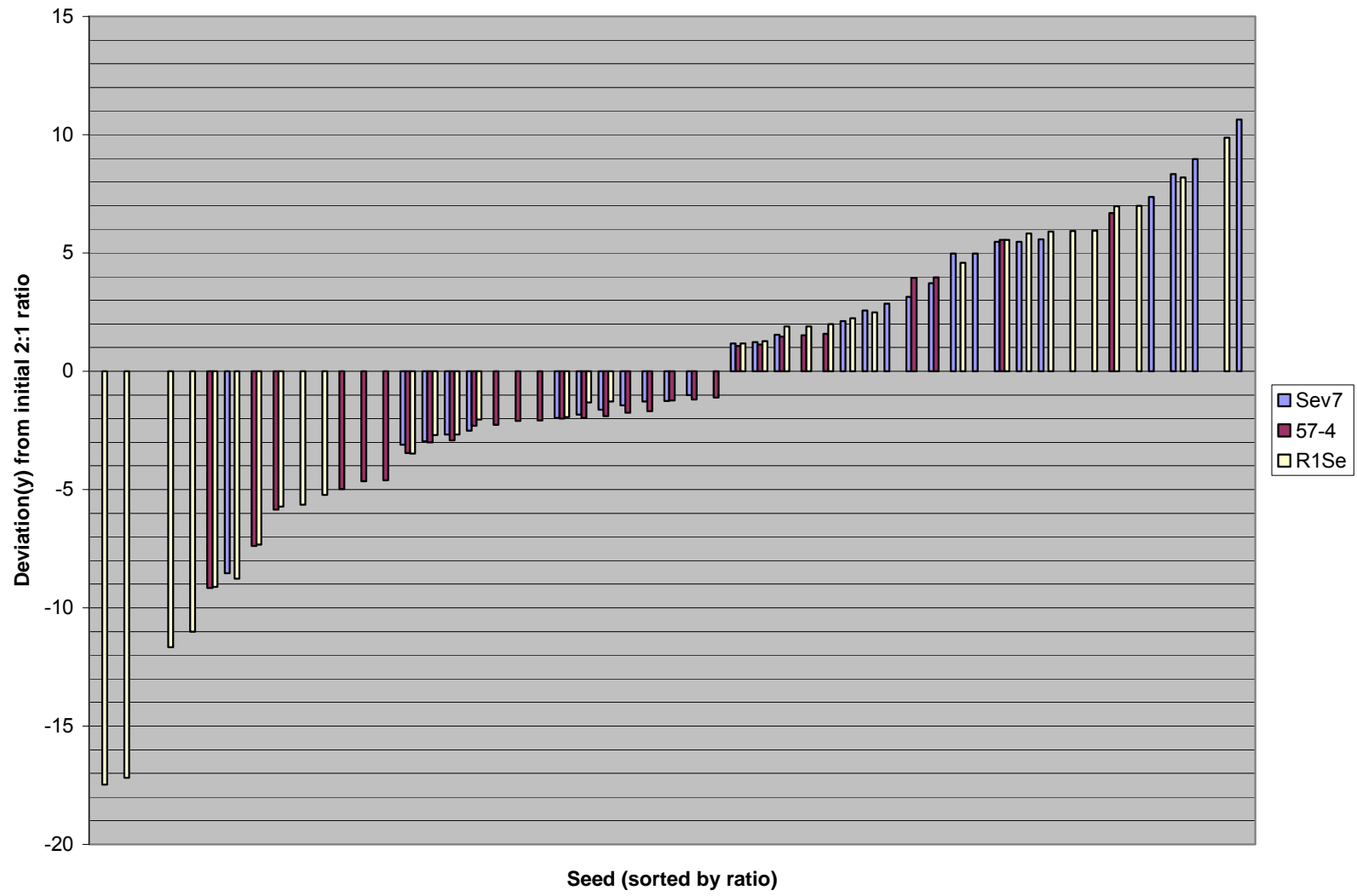|	Genotype										
	R_1Se				57-4				Sev_7		
Number of nuclei				Number of nuclei				Number of nuclei			
Haploid	Tetrapld	Hap/Dip	χ^2	Haploid	Tetrapld	Hap/Dip	χ^2	Haploid	Tetrapld	Hap/Dip	χ^2
152813	60079	2.5435	2504	38476	23827	1.6148	676	191302	30427	6.2872	38373
75588	19989	3.7815	6634	64371	38691	1.6637	821	235892	31597	7.4656	55749
96246	25451	3.7816	8448	24581	13672	1.7979	100	253687	25451	9.9677	73658
111407	27986	3.9808	11023	17087	7920	2.1574	31	108732	10899	9.9763	31587
107207	23935	4.4791	13424	93538	41097	2.2760	478	436671	39893	10.9461	133630
269685	54084	4.9864	40288	116269	40000	2.9067	4209	22683	2069	10.9633	6947
130124	14192	9.1688	35862	76040	24961	3.0464	3377	147124	13189	11.1551	45472
261968	23591	11.1046	80777	48428	15207	3.1846	2550	78307	5312	14.7415	27392
27583	2368	11.6482	8714	79568	10063	7.9070	19711	19286	1156	16.6834	7047
61281	5195	11.7962	19480	210849	26564	7.9374	52390	24595	1370	17.9526	9198
31042	2620	11.8481	9889	101339	9131	11.0983	31238	39609	1862	21.2723	15526
102941	8658	11.8897	32848	87165	6512	13.3853	29340				
118647	8504	13.9519	40623								
115784	8274	13.9937	39690								
36652	2235	16.3991	13317								
27968	1416	19.7514	10751								

summary of the haploid-tetraploid ratios in Table 5 are given in Table 6, including the

mean, median, range and standard deviation for each genotype. Genotype 57-4 had a

median of 1.03, and produced more seeds with excess tetraploid cells, whereas Sev_7

which had a median of 3.08 produced more seeds with excess haploid cells. Chimeras

from R_1Se were evenly split, with 17 seeds in each category with a median of 1.95. The

distributions were more dispersed for Sev_7 and R_1Se than 57-4 as indicated by the larger

mean, range and standard deviation.

Table 6. Mean, median, range and Standard deviation   (SD) of hap/dip ratios of ploidy
chimeras in semigamous cotton seeds

Genotype	Mean	Median	Range	SD	#seeds hap/dip>2	#seeds hap/dip<2
R1Se	4.91	1.95	19.64	5.78	17	17
57-4	2.40	1.03	13.17	3.28	9	21
Sev7	5.98	3.08	21.04	6.00	17	12

Transformed haploid-tetraploid ratios are presented in Fig. 9. The extent of

departure from the expected ratio (2:1) was largest in R_1Se measuring -17.5 to 9.9 with

the seeds distributed evenly across this range. The ratios among Sev_7 fell across a

narrower range of -8.5 to 10.6. Deviations from the expected ratio were the smallest in

chimeras formed in 57-4 ranging from -9.2 to 6.7 with 63% (19 of 30) having values less

than three times the initial embryo ratio.

Fig. 9. Transformed haploid-tetraploid ratios, where the transformation renders changes in ratios proportional. Under the transformation, the expected non-transformed ratio (2:1) is rendered into 1 or −1, the values of which are thus equivalent (perfect fit to a 2:1 ratio). Higher numbers on the x-axis indicate increasing haploid frequencies, e.g., for y = 2, the ratio of haploid to tetraploid cells has doubled, i.e., to 4:1. When y = -2, the ratio of haploid to tetraploid cells has been halved, i.e., to 1:1 (fewer haploid and(or) more tetraploid cells than expected). Each seed from the three genotypes has been plotted with the genotype identified by the legend.

Transformed data were tested for homogeneity among genotypes (Table 7). The values of the deviations were grouped into three magnitudes-based classes: 0-3, 3-6, and 6+. The Chi-square is 5.31 with P=0.257; therefore, the samples are accepted as homogeneous and the data were pooled.

Table 7. Test for homogeneity of genotypes for ploidy chimeras using pooled transformed data. Observed and expected values, X^2, and P are listed

	Pooled Categories of transformed data							
	0-3		3-6		>6		X^2	P
genotype	Obs	Exp	Obs	Exp	Obs	Exp		
R1Se	13	17.548	10	9.140	11	7.312		
57-4	19	15.484	7	8.065	4	6.452		
Sev7	16	14.968	8	7.796	5	6.237		
totals	48		25		20		5.313	0.257

Using pooled data, the ratios were grouped according to whole numbers and plotted in Fig. 10. As in Fig. 9, elevated numbers of tetraploid nuclei resulted in negative values, whereas increased numbers of haploid nuclei resulted in positive values. A fairly normal distribution resulted with the mean near the initial ratio. Cell ratios were slightly skewed in favor of tetraploid cells, as the mode is -1. However there are nearly equal numbers of samples that fall between one and five as there are between negative one and negative five which included 88% of the samples.
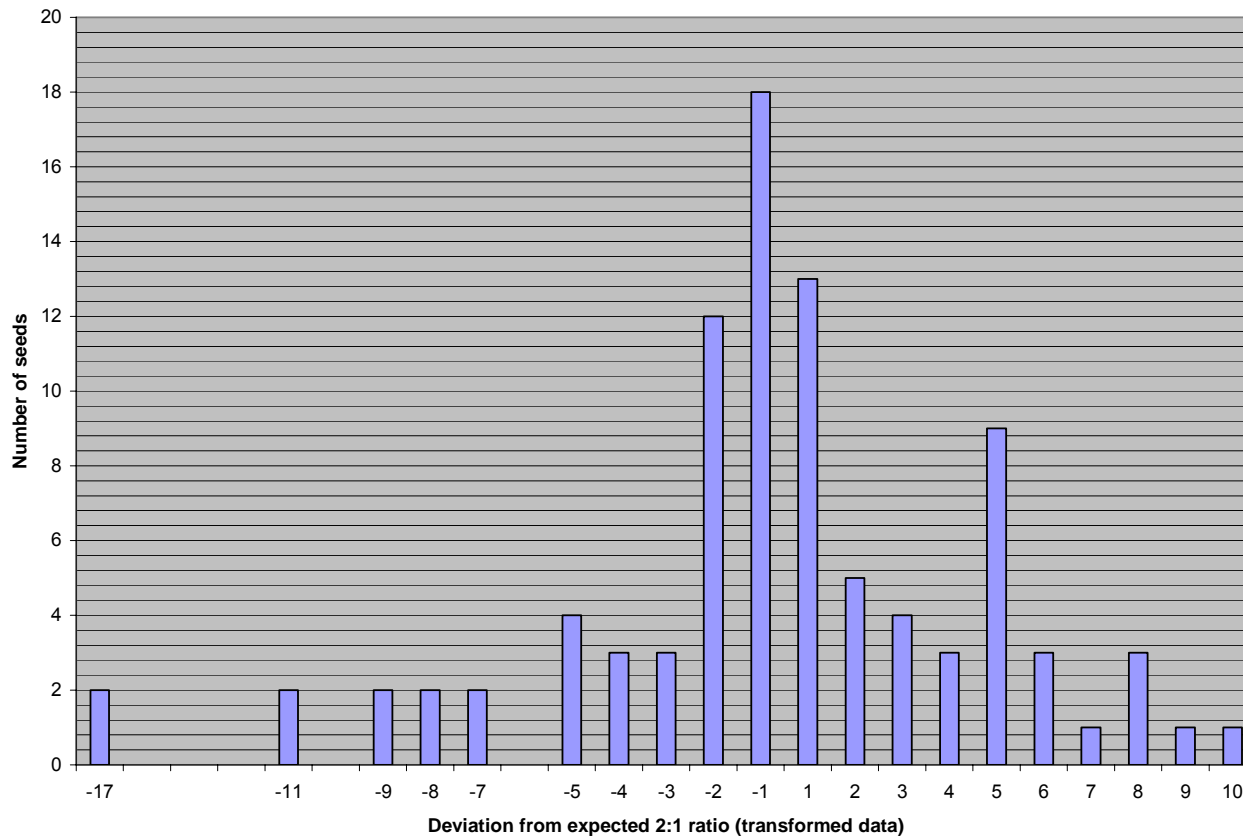
Fig. 10. Transformed data of haploid-tetraploid ratios of all genotypes pooled and rounded up to the whole number. Increasing numbers along the x-axis indicate an increase in the haploid-tetraploid ratio. The value "2" along the x-axis denotes a ploidy ratio of 4:1 or a haploid ratio increase to twice that of original 3-celled embryo (2:1). At a x-axis value of –4, the seed ratio is 0.5, denoting an increase of tetraploids to four times the original tetraploid ratio. 1 and -1 are equal to a 2:1 haploid:tetraploid ratio

Seed partition analysis

To determine the distribution of tetraploid and haploid cells within chimeric seed, seeds were partitioned into three parts; the root, the hypocotyl and the cotyledons. The root was distinguished from the hypocotyl because it lacked glands. A total of 159 seeds were partitioned and analyzed with the flow cytometer. Fifty of the 159 seeds were chimeric for ploidy (31%), of which 18 were chimeric in the cotyledons (11%), 47 in the root cap (30%), and 43 in the hypocotyls (27%) (Table 8). If the cotyledons were chimeric, so was the hypocotyl. In seven of the 159 chimeric seeds, the hypocotyl was not chimeric, and the cotyledons were the same ploidy as the hypocotyl. Of the 18 seeds in which the cotyledons were chimeric so was the hypocotyl. The remaining 25 seeds

Table 8. Semigamic seed partitions; total seed examined, total chimeras, root caps, hypocotyls, and cotyledons chimeric for ploidy

| Genotype | Total seed | Ploidy chimeras | | | |
		Total	Root cap	Hypocotyl	Cotyledons
R_1Se	43	18	16	14	4
57-4	59	15	14	15	7
Sev_7	57	17	17	14	7
total	159	50	47	43	18
percent of total seed		31.45%	29.56%	27.04%	11.32%

were chimeric for ploidy level in the hypocotyl but not in the cotyledons. In 22 of the 25 seeds that were chimeric for ploidy, the ploidy level of the cotyledons was the predominant ploidy level in the hypocotyl.

DISCUSSION

Cytometric profiles from the seed were very "clean", and devoid of "noise" from cell cycling. The cytometric data indicated that cells of *G. barbadense* seeds are uniformly arrested at the G_1 phase of the cell cycle. This synchronization phenomenon has been previously reported by Bino et al. (1993), who reported that the cells in mature seeds of some species are uniformly arrested at the G_1 phase of the cell cycle.

Haploid-tetraploid ratios were widely dispersed but centered around the expected ratio of 2:1 haploid : tetraploid. Most (58%) were within the transformed range of 0.5 to 4. Much of the dispersion may; be a result of the cotyledons. Seeds that were chimeric for ploidy but did not have chimeric cotyledons would be expected to have higher deviations from the initial ratio. It is possible that much of the observed variation in ratios resulted from normal variation in development. *Gossypium* does not have clearly defined cleavage patterns through the globular stage of embryogenesis (Pollock and Jensen, 1964). The first cleavage is transverse, but is the only one that is consistent. Later cleavages cannot be predicted. Cell lines cannot be followed in the developing cotton embryo. A mature globular embryo has about 8,000 cells before the cotyledon initials begin to form (Pollock and Jensen, 1964). This contrasts reports on embryo development in *A. thalina* (Barton and Poethig, 1993; Scheres et al.,1994) and *Capsella bura-pastoris* L. Medic (Pollock and Jensen, 1964), where a more definite cleavage

pattern was discernible. In both *A. thaliana* and *C. bura-pastoris*, cell lineages which were established early could be followed.

In *Arabidopsis* the late globular stage is marked by a flattening of the embryo as the cotyledons begin to form and cells in the center begin to elongate during axis establishment. If cotton development is comparable, the axis and the cotyledons must begin developing in a globular embryo with several thousand cells. The cells that form the axis which includes the hypocotyl and the root would originate from cells located more centrally in the embryo while the cotyledons will develop from cells at the periphery. For these seed parts, differences among their origins within the embryo could account for the differences in ploidy chimerism observed. The hypocotyl and root would develop from cells that are less removed from the initial three-celled embryo.   In contrast, the cotyledons are formed by a more limited number of cells in two distil regions that are more distant both spatially and generationally from the initial three cells of the embryo. In addition, cell differentiation may influence the final ploidy ratio as cells differentiate and become specialized. It is clear that not all cells cycle at the same rate. For example the cells that initiate the cotyledons cycle much faster than the rest of the embryonic cells as they develop (Pollock and Jensen, 1964).

Ploidy ratios in the hypocotyls were a good indication of the phenotype of the cotyledon. All cotyledons had the phenotype of the hypocotyls, or if chimeric for ploidy, the cotyledon was either chimeric or had the ploidy that was predominant in the hypocotyl.

The frequencies of ploidy chimeras in seeds in this study are similar to frequencies observed in seedlings by Turcotte and Feaster (1973, 1974). It would have been instructive to examine the shoot meristem but the meristem in the seed is too small for flow cytometry analysis.

A STUDY OF PARENTAL EFFECTS USING RECIPROCAL CROSSES OF SEMIGAMOUS (*Se*) LINES

INTRODUCTION

The *Semigamy* mutant of cotton (*Gossypium barbadense* L.) was first reported in 1963, and was notable for its very high production of monoembryonic haploids (Turcotte and Feaster, 1963). Several years later, they reported another intriguing characteristic of the Semigamy mutant -- its ability to generate haploid-haploid (maternal-paternal) and haploid-tetraploid chimeras (Turcotte and Feaster, 1967). To reduce confusion, it should be noted that they alluded to the normal cotton ploidy level as "tetraploid", whereas it is more commonly recognized today as tetraploid. The latter were characterized as containing a haploid sector of maternal or paternal origin and a non-heterozygous, somatically doubled sector. The doubling was attributed as most likely arising from failure of cytokinesis at the zygotic mitosis, followed by formation of a restitution nucleus. Later, they reported that the tetraploid sectors of chimeric plants were of hybrid origin, as well as the occurrence of "trichimeric" progeny (Turcotte and Feaster, 1973). The finding that some progeny were "trichimeric" was not only highly unusual, but intriguing in that each plant included a tetraploid hybrid sector, a maternal haploid sector and paternal haploid sector. They hypothesized the involvement of polyspermy and two female gametes or egg nuclei (Turcotte and Feaster, 1973).

Se-generated chimeras have been used in a variety of ways. They have been used to mark the production of cytoplasmic substitution lines (Chaudhari, 1978, Umbeck and Stewart, 1985) and to identify haploids of F_1 interspecific hybrids produced via a female

with the *Semigamy* trait (Barrow and Chaudhari, 1976). Christianson used the chimera to construct a fate map of the shoot apex by clonal analysis (Christianson, 1986). Chimeras were also used to examine cotton leaf morphogenesis (Dolan and Poethig, 1998). In their analysis of Le_2^{dav} hybrid lethality systems, Stelly and Rooney (1989) used semigamy to delimit the allelic and interlocus interactions with Le_1 and Le_2 to intracellular effects, rather than intercellular or systemic ones. The occurrence and frequencies of chimeras have also been used as an assay for parental traits (Stelly, personal communication).

In this study the expression of the parents on the leaves of chimeras have been examined. In 1973 Turcotte and Feaster reported the overall results of 170 chimeras from a cross between the mutant line 57-4, which was semigamous, green and glanded (*SeSe r_1r_1 Gl_2 Gl_2 Gl_3Gl_3*) used as female with wild-type, i.e., non-semigamous (*sese*), pollen parents that were homozygous for the glandless genes and heterozygous for red plant color (*sese R_1r_1 gl_2 gl_2 gl_3gl_3*). Of the 170 chimeras 67% or 114 were green and glanded at flowering, i.e., like the maternal parent. In a three-year period Chaudhari (1978) evaluated 461 chimeras from a cross using the semigamous line carrying the recessive trait for reduced virescence (v_7) as the female with three nonsemigamous *G. hirsutum* stocks, Deltapine 16 x Acala 9450, Coker 201 x Acala 9450, and B7424 x B7860, and one interspecific hybrid SISB12B x Acala 9519. About 46% of these plants displayed abnormal virescence at the time of flowering. The male parents in both cases were non-semigamous. I have used three semigamous lines in reciprocal crosses and

measured their effects on chimeric distributions, using leaf color to evaluate the extent of distribution and categorize the sector according to genotype.

MATERIALS AND METHODS

Plants of three lines homozygous for *Semigamy* were grown in a greenhouse during the summer and into the fall of 2005. They are alluded to here as '*Sev₇*', '*R₁Se*' and '57-4'. Their genotypes (phenotypes) were respectively as follows, $SeSe\ r_1r_1\ v_7v_7$ (yellow-green), $SeSe\ R_1R_1\ V_7V_7$ (red), $SeSe\ r_1r_1\ V_7V_7$ (green). The line '57-4' is the original semigamous line developed by Turcotte and Feaster (1963) while the two marker lines, which carry leaf color markers v_7 (reduced virescence leaf) and R_1 (red anthocyanin), were developed by Turcotte and Feaster (1969) and David Stelly (unpublished). The lines were crossed in a full diallel excluding selfs, but including reciprocal crosses. The resulting seed were planted in flats and examined for chimerism on the basis of color distribution.

Chimeric plants were scored for leaf color distribution in the following manner. Each leaf was assigned a total score of one, where this was the sum of values for two possible colors. A leaf of solid color was assigned a score of one for that color, and zero for the other color. When sectored, the proportions of the leaf bearing the respective colors were estimated, e.g. 0.6, and each color was allotted a corresponding score (0.6), the total for both scores being one (0.6 + 0.4).

The pigmentation served as a guide to deducing the parental origins of sectors in chimeric and trichimeric seedlings. Unfortunately, the red leaf color in the crosses *R₁Se* x 57-4 and 57-4 x *R₁Se* was poorly expressed in the environment in which the seedlings

were grown (winter greenhouse) and was not sufficiently distinguishable from the green leaf color of 57-4 for reliable classification. Thus, only two of the three pairs of reciprocal crosses were ultimately used for analysis. For seedlings of these four crosses, the origins of the sectors were assigned to maternal versus paternal and hybrid origins according to the following expectations:

1. Maternally derived yellow-green haploid sectors were distinguished from non-yellow sectors derived from the paternal haploid nucleus or the hybrid nucleus;

    a. $r_1r_1\ v_7v_7$ (yellow-green) x $R_1R_1\ V_7V_7$ (red)

    b. $r_1r_1\ v_7v_7$ (yellow-green) x $r_1r_1\ V_7V_7$ (green)

2. For the reciprocals, paternally derived yellow-green haploid sectors were distinguished from non-yellow sectors derived from the maternal haploid nucleus or the hybrid nucleus;

    c. $R_1R_1\ V_7V_7$ (red) x $r_1r_1\ v_7v_7$ (yellow-green)

    d. $r_1r_1\ V_7V_7$ (green) x $r_1r_1\ v_7v_7$ (yellow-green)

In addition, the differences between "a" versus "b" and "c" versus "d" were expected to reveal differences between 57-4 and *R1Se* in terms of their influences on chimerism rates, *per se* and/or in terms of their interaction with *Sev7*. Moreover, the comparison allowed for possible interactions with the direction of cross (a versus c and b versus d), i.e., maternal versus paternal parentage.

Leaf scores for each cross were summed and averaged on a per cross basis. The mean for each color and the variance, standard deviations and standard error were calculated. A pair-wise t-test was performed comparing the crosses.

RESULTS

Pigmentation differences enabled facile recognition of the v_7 virescence haploid

genotype relative to the green or red haploid (V_7 or R_1V_7) and hybrid (V_7v_7 or $R_1r_1V_7v_7$)

sectors, which allowed reliable data to be collected from four of the six crosses used,

specifically all of those involving the v_7 virescence mutant. Attempts to distinguish

between the two possible types of red sectors, (i.e., haploid red versus hybrid red

sectors), or between the two possible types of green sectors, (i.e., between haploid green

versus hybrid green sectors) were not successful. Thus those non-yellow sectors were

"pooled" in terms of the analysis, for a given cross. The pooling effect would thus

confound the respective maternal or paternal origin, but differ between reciprocal

crosses.

Seed germination rates were similar for the four crosses, and averaged 73%

(Table 9). Rates of seedling chimerism were very high among all four individual

crosses, ranging from 53% to 63%, with an overall average of 58%.

Table 9. Germination rate and number of plants in each phenotype of
green/red, virescent (Vir), chimera (Chim), percent germination, and percent
chimeras of four crosses

Cross	Planted	Germ	% Germ	Grn/Red	Vir	Chim	% Chim
Sev7 x R1Se	640	484	76%	191	6	287	59%
Sev7 x 57-4	563	399	71%	135	10	250	63%
R1Se x Sev7	234	159	68%	69	2	88	55%
57-4 x Sev7	354	270	76%	125	3	142	53%
total	1791	1312	73%	520	21	767	58%

The proportions of chimeric organs that arose from maternal or paternal origin were estimated on the basis of pigmentation distributions in individual leaves that were visually assessed. Individual cotyledons of chimeras ranged from 0 to 1 for maternal leaves of chimeras.

Total plant leaf color percent was higher for maternal or, when confounded, for maternal plus hybrid sectors in every cross with a low of 53% in Sev_7 x 57-4 to a high of 59% in Sev_7 x R_1Se (Table 10). In these two crosses, only maternal haploid sectors would have contributed to the haploid yellow-green color on which the maternal classification was based, i.e., any confounding effect from non-yellow hybrid sectors to the non-yellow paternal sectors would have contributed to the paternal class, not the maternal one. So, the percentages of paternal haploid-derived sectors for these two crosses were 47% or lower and 41% or lower, respectively.

In all four crosses, the cotyledons had a lower percentage of yellow-green haploid sectors than all true leaves (Table 11), and there was significant heterogeneity for the portion of maternal or maternal plus hybrid tissue in the cotyledons. Heterogeneity of cotyledonary chimeric proportions was due largely to one cross, 57-4 x Sev_7. Graphical representation of the overall data (Fig. 11) indicates that this cross behaved somewhat differently than the other three crosses in general, i.e., not just for the cotyledons. The cross of 57-4 x Sev_7 exhibited no strong increase in maternal chimeric portions across leaves, whereas the other three crosses exhibited similar patterns for the maternal or maternal + hybrid portions (Fig. 11). With the exception of cross 57-4 x

Table 10. Summary of crosses of semigamous lines. Total leaves scored, scores for maternal leaf color, the mean and standard error. Pair-wise comparisons with its t-value and P

	Sev7 x R1Se		Sev7 x 57-4		R1Se x Sev7		57-4 x Sev7	
	Virescent	Red	Virescent	Green	Red	Virescent	Green	Virescent
total leaves	1371		1333		236		748	
value	808	563	703.35	630.65	135.15	100.85	416.25	331.75
Mean	0.5894	0.4106	0.5272	0.4728	0.5727	0.4273	0.5565	0.4435
SE	0.0106		0.0113		0.0232		0.0149	

	Sev_7 x R_1Se vs Sev_7 x 57-4	Sev_7 x R_1Se vs R_1Se x Sev_7	Sev_7 x R_1Se vs 57-4 x R_1Se
t	4.0087	0.6086	1.8101
SE	0.1542	0.0429	0.0823
P	0.0001	0.5435	0.0718

	Sev_7 x 57-4 vs R1Se x Sev_7	Sev_7 x 57-4 vs 57-4 x Sev_7	R_1Se x Sev_7 vs 57-4 x Sev_7
t	1.5948	1.5596	0.5465
SE	0.1126	0.0713	0.0408
P	0.1123	0.1204	0.5853

Table 11. Total leaves and mean scores for cotyledons and leaves of virescent and red/green leaf color

| | | Sev7 x R1Se | | Sev7 x 57-4 | | R1Se x Sev7 | | 57-4 x Sev7 | |
		Vir	Red	Vir	Green	Red	Vir	Green	Vir
	total	263	281	231	262	92	76	153	115
Cotyledons	Mean	0.483	0.517	0.468	0.531	0.549	0.451	0.569	0.431
	total	545	282	472	368	43	25	264	216
Leaves	Mean	0.659	0.341	0.563	0.439	0.632	0.368	0.549	0.451

Sev7, the percent of female parent leaf color was lower for leaf-2 than for the other true leaves (leaf-1, -3, -4, and -5).

In the two crosses where *Sev7* was the female parent, yellow-green pigmentation indicated unequivocally that the tissue was of maternal haploid origin. In both of these crosses, all true leaves had a portion of maternal haploid origin above 0.5 (Fig. 11), and with a single exception (leaf-2 of *Sev7* x 57-4), all deviations from 0.5 were statistically significant (Table 12). The magnitude of differences between the overall frequencies of yellow-green sectors observed in reciprocal crosses of *Sev7* and 57-4 was smaller (0.084) than for the reciprocal crosses between *Sev7* and *R1Se* (0.162).

DISCUSSION

The *v7* virescence mutant was more useful than the *R1* anthocyanin-colored plant trait for recognition of sectors in chimeras. In this instance, the seedlings were germinated under winter greenhouse conditions with only marginal supplementary
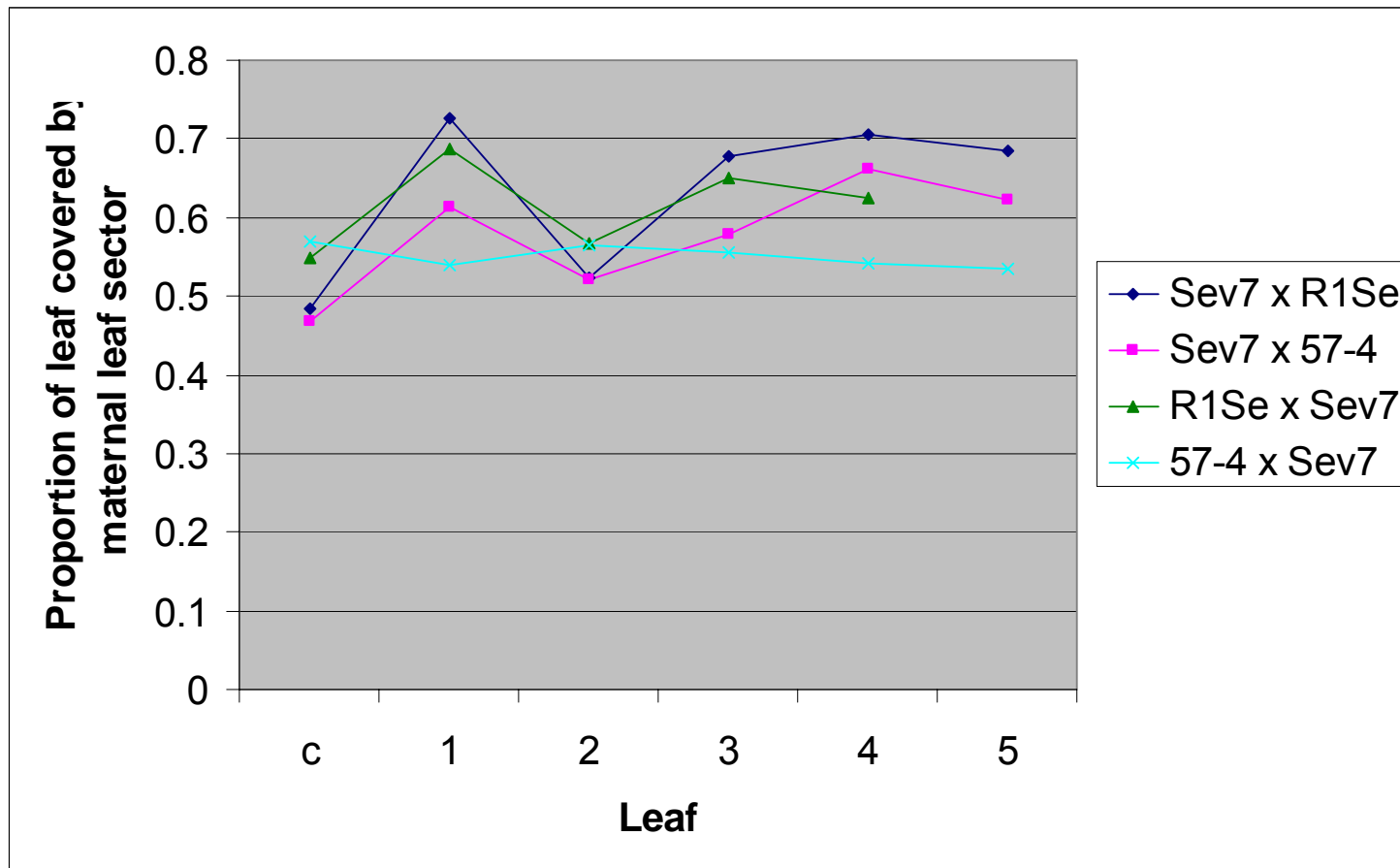
Fig. 11. Proportion of leaf exhibiting maternal leaf phenotype, for chimeric seedlings from reciprocal crosses of $r_1r_1SeSev_7v_7$ (*Sev7*), with $R_1R_1SeSeV_7V_7$ (*R1Se*) and with $r_1r_1SeSeV_7V_7$ (57-4). Cotyledons (C) and leaves 1-5 are indicated.

Table 12. Lowest value of a 95% confidence interval for the cotyledon and leaves of 4 crosses of semigamous cotton lines. Colored blocks indicate they are not different from 0.5

Cross	Cotyledon	Leaf-1	Leaf-2	Leaf-3	Leaf-4	Leaf-5
Sev7 x R1Se	0.505	0.707	0.502	0.655	0.683	0.640
Sev7 x 57-4	0.492	0.592	0.499	0.555	0.637	0.579
R1Se x Sev7	0.524	0.627	0.504	0.549	0.465	
57-4 x Sev7	0.548	0.510	0.534	0.524	0.507	0.482

lighting. One can surmise that the light intensity was insufficient to strongly induce the anthocyanin pathway. Thus, it seems likely that the lack of low efficacy of R_1 for this experiment was probably related to the environmental conditions in which the seedlings were grown prior to and during the screening process. These were greenhouse conditions commencing on December 17, 2005 running through February 18, 2006. Expression of the virescence mutant $v7$ typically varies from yellow-green (strong) to green (weak to none), depending on light, temperature, plant age, and perhaps other factors, but in general it is better expressed in cooler and lower-light conditions. Expression of the R_1-conferred red anthocyanin pigmentation is more or less the opposite – expression is higher in high-irradiance conditions. Thus, it is not too surprising that there was some difficulty in screening for R_1 or R_1r_1 versus r_1 in a V_7 or V_7V_7 background. When screening in a v_7 background, the sectoring for the R-locus and V-locus genotypes were completely confounded, so phenotypic differences among

sectors were accentuated. The pigmentation markers used did not allow facile distinctions to be made between red (green) haploid from red (green) hybrid tissue when crossing R_1Se or 57-4 with Sev_7, respectively. A semigamous line is now being developed that will be of genotype SeR_1v_7. The limitation might have been avoided if one parent carried both red and virescence mutations ($SeSe R_1R_1 v_7v_7$) and the other were 57-4 ($SeSe r_1r_1 V_7V_7$), since the three types of sectors (two haploid types and one hybrid type) would each have markedly different phenotypes red plus reduced virescence ($R_1 v_7$), red green ($R_1r_1 V_7v_7$), and green ($r_1 V_7$), at least if illumination were sufficient to induce R_1-conferred red color, but not so much illumination as to reduce the v_7-conferred yellowness.

Hybrids from the cross 57-4 x Sev_7 exhibited a pattern of sectoral chimerism of leaves that differed from products of the other three crosses. There was no obvious reason why this should be so.

Leaf-2 and the cotyledons exhibited sectoral ratios that differed from the other leaves. On average, a smaller proportion of cotyledon area was occupied by yellow-green sectors than non-yellow sectors. The ontogenic origin of cotton cotyledons is from the two distal portions of the mature globular stage embryo (Pollock and Jensen, 1964). Cotyledons are therefore developmentally separated by time and space from the formation of the remainder of the shoot – which could well account for the observed difference in chimeral patterning. The difference between the proportion of yellow-green between reciprocal crosses were 6.6% for Sev_7 x R_1Se, and 8% for Sev_7 x 57-4.

Leaf-2 was composed of a significantly lower proportion of maternal chimeric sectors than the other true leaves. Christianson (1986) proposed a fate map of the cotton shoot apex based on his analysis of *semigamy*-induced chimerism. His analysis of the patterns of sectors observed in 44 chimeric seedlings led him to propose that the shoot apex is organized into four cadres of hexagonally packed cells. Cells from each cadre represent a distinct cell lineage. The innermost cadre of three cells develops into the apical meristem. One cell from the next cadre of 9 cells develops leaf-2, while two cells from the 17 celled third cadre generates leaf-1. He admitted inferences about leaf-2 origins was quite speculative, since it was based on eight plants that did not have chimeric leaves past leaf-2. In our study, chimerism was clearly evident in leaf-2, indicating that at least two cells initiate the second leaf, thus modifying or eliminating this aspect of Christiansen's model. The idea proposed by Christianson that leaf-1 and -2 develop from cells not of the apical meristem is also in question. It is more likely all true leaves are generated from the apical meristem. Our data, however, concur strongly with those of Christiansen in that leaf-2 is different from that of the other true leaves. In our case, the sector ratios are quite similar to those of the cotyledons and could be taken as concordant with the aspect of Christiansen's model that places the initials for leaf-2 developmentally "near" those of the cotyledon. The developmental proximity may not be spatial, per se, but a matter of cell lineage and generations, i.e., temporal and spatial.

Chimeric sectors observed in this study were disproportionately derived from the maternal parent. This effect was quite pronounced – the rate exceeded 60% for all leaves with the exception of leaf-2. What would endow direct descendents of the

maternal parent an apparent advantage (or confer a disadvantage on paternal haploid descendents)? One possibility is that the rates of cell division are slightly different. If early embryo maternal cells cycle slightly faster than the paternal cells, they would have a competitive advantage. Based on tests of 20 loci, Vielle-Calzada et al. (2000) found that the paternally inherited alleles were not activated for up to three to four days after fertilization in *Arabidopsis*. Early embryo development was therefore under maternal control. Similar phenomena probably occur in cotton reproduction. Under these circumstances, the maternally derived haploid genomes may have a cycling advantage, if some paternal loci are less efficient than their maternal counterparts.

SUMMARY

To study the semigamy mutant in cotton, it was desirable to develop a procedure for preparing large numbers of high quality cytological samples with a high degree of efficacy, in terms of information derived per time investment. A stain-clearing method was developed using methyl salicylate (MS) as the clearing agent and azure C as the stain. Azure C was simple to use and as a regressive stain could be adjusted for intensity. Cell walls were rendered nearly invisible with MS making it possible to clearly see the stained organelles within. Once samples were in MS, azure C was fixed and would not bleed. Samples stained and cleared in this way were used as whole mounts which allowed the specimen to be oriented for precise analysis. For cotton, it is clearly superior to cytological approaches based on sectioning and on other clearing and stain-clearing methods.

Because cotton nucelli are comparatively large, the light providing an image of the gametophyte must pass through 12 to 35 cell layers. If stained too deeply, details of the underlying gametophyte would be obscured. Therefore, a stain-clearing method was devised to be used in conjunction with real-time video imaging and image capture and/or recording. The ability to modulate contrast and illumination intensity using video made it feasible reduce stain intensity and thus light interference from the specimen. Ad hoc illumination and video adjustments allowed for facile optimization of image clarity. With this method it was possible to effectively screen and view the cotton embryo sac and its component cells.

Because of the ease of simultaneously processing large numbers of samples, stain-clearing combined with image analysis lends itself well to a broad range of studies which require large sample sizes or thick specimens. In this study large numbers of cotton nucelli were prepared to study the effects of *semigamy* (*Se*) on fertilization and early embryogenesis.

The purpose of this study was to determine a cytological basis for semigamy in cotton. Based on progeny tests Turcotte and Feaster (1974) and Chaudhari (1978) reported semigamy exhibits varying levels of penetrance. In this study 439 of 440 semigamous zygotes examined had one egg and one sperm nucleus resulting from syngamy without karyogamy indicating *semigamy* is nearly if not completely expressed.

The occurrence of tetraploid progeny was attributed to karyogamy, whereas haploid and haploid chimeric progeny were attributed to expression of the *semigamy* gene. Tri-chimeric progeny consisting of a tetraploid hybrid sector and two haploid sectors, one of maternal and one of paternal origin, was attributed to two eggs or two egg nuclei and two sperm (Turcotte and Feaster, 1973). Zhang et al. (1996) reported failure of cytokinesis in semigamous cotton which should result in doubled haploid sectors. To date no doubled haploid sectors have been reported. In this study, all phenotypes that have been observed in semigamous cotton appear to arise as products of zygote division. Haploid and tetraploid sectors may result from relative spindle positions, orientation, and the tendency for nearby telophase chromosomes to form a common nucleus. When poles are close, the neighboring chromosomes form a common tetraploid nucleus and if farther apart, haploid nuclei form. If spindle poles are not parallel to each other, it is

possible to form a common tetraploid nucleus at one pole and haploid nuclei at the other. This scenario raises the possibility that some species might evolve systems for initial zygotic development that routinely sidestep egg-sperm karyogamic, and rely on mitotic fusion and spatial positioning to selectively transmit the fusion product to the embryo and relegate the maternal and paternal haploid products to the suspensor. Such systems have been reported among apomicts, e.g., *Cooperia*, but seemingly not among sexual types.

It is clear that karyogamy fails in semigamous zygotes but it is not clear if fusion of the polar nuclei and the sperm nucleus occurs. Flow cytometric analysis established the endosperm nuclei are triploid but fusion was never observed. Endosperm may be a result of triple fusion or mitosis-based fusion. A slight delay in endosperm development was observed. Such a delay could result from failure of the nuclei to fuse. If there is a checkpoint governing the first division, it may have limitations, as do some other cell division checkpoints.

Triple fusion and primary division of the endosperm occur so rapidly that it may not be possible for DNA synthesis to occur prior to dividing. Approximately two hours spanned entry of the sperm into the central cell to development of two endosperm nuclei. DNA synthesis in sperm of other angiosperm species reportedly occurs (Ermakova, 1980; Friedman, 1999) and may occur in cotton.

Flow cytometry was used to analyze changes that occur in ploidy chimeras. Initially an embryo chimeric for ploidy will be composed of one tetraploid hybrid cell, one maternal haploid cell, and one paternal haploid cell. While haploid-tetraploid ratios

were centered on the original 2 : 1, haploid : tetraploid ratio, the ratios were widely

disparate among seedlings. Part of this dispersion resulted from seeds which were

chimeric for ploidy in the hypocotyls, but not in the cotyledons -- i.e., 64% of the seeds

in this sample had cotyledons that were not chimeric for ploidy. Additionally departures

from the initial ploidy ratio with the hypocotyls may be a result of normal development.

The globular stage cotton embryo has several thousand cells (Pollock and Jensen, 1964)

and gives rise to the shoot axis. This complexity may increase the opportunity for

differing ploidy ratios between seeds. In addition, cell development may influence the

final ploidy ratios. It is clear that not all cells cycle at the same rate. The cells that

initiate the cotyledons divide much faster then the rest of the embryonic cells for a

period of time. Such differences in cell cycling may also occur as cells differentiate and

specialize during seed development.

In order to examine expression of the parents on the leaves of chimeras, crosses

were made between three semigamous genotypes, yellow-green ($r_1r_1SeSev_7v_7$) with red

anthocyanin ($R_1R_1SeSeV_7V_7$) and green ($r_1r_1SeSeV_7V_7$). Chimeric plants were scored for

leaf color distribution. Cotyledons exhibited sectoral ratios that differed from the leaves.

For cotyledons the average percentage of yellow-green sectors was less than the non-

yellow sector, while true leaves exhibited sectoral ratios that were higher for the

maternal sectors or , when confounded, for maternal plus hybrid sectors in every cross.

These differences between cotyledons and true leaves may result from the differences in

development. Cotyledons are developmentally separated in time and space from the

formation of the remainder of the shoot. Chimeric sectors observed in true leaves were

disproportionately derived from the maternal parent exceeding 60% for all leaves, with

the exception of leaf-2, in progeny from three of the four crosses. Among leaves, leaf-2

was composed of a lower percentage of maternal chimeric sectors but still exceeded

50%. The apparent advantage of the maternal parent may result from initial maternal

developmental control (Vielle-Calzada, 2000). If in the early embryo the maternally

derived haploid cells cycle slightly faster than the paternal cells they would have a

competitive advantage.

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