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Exhibit 3a, #

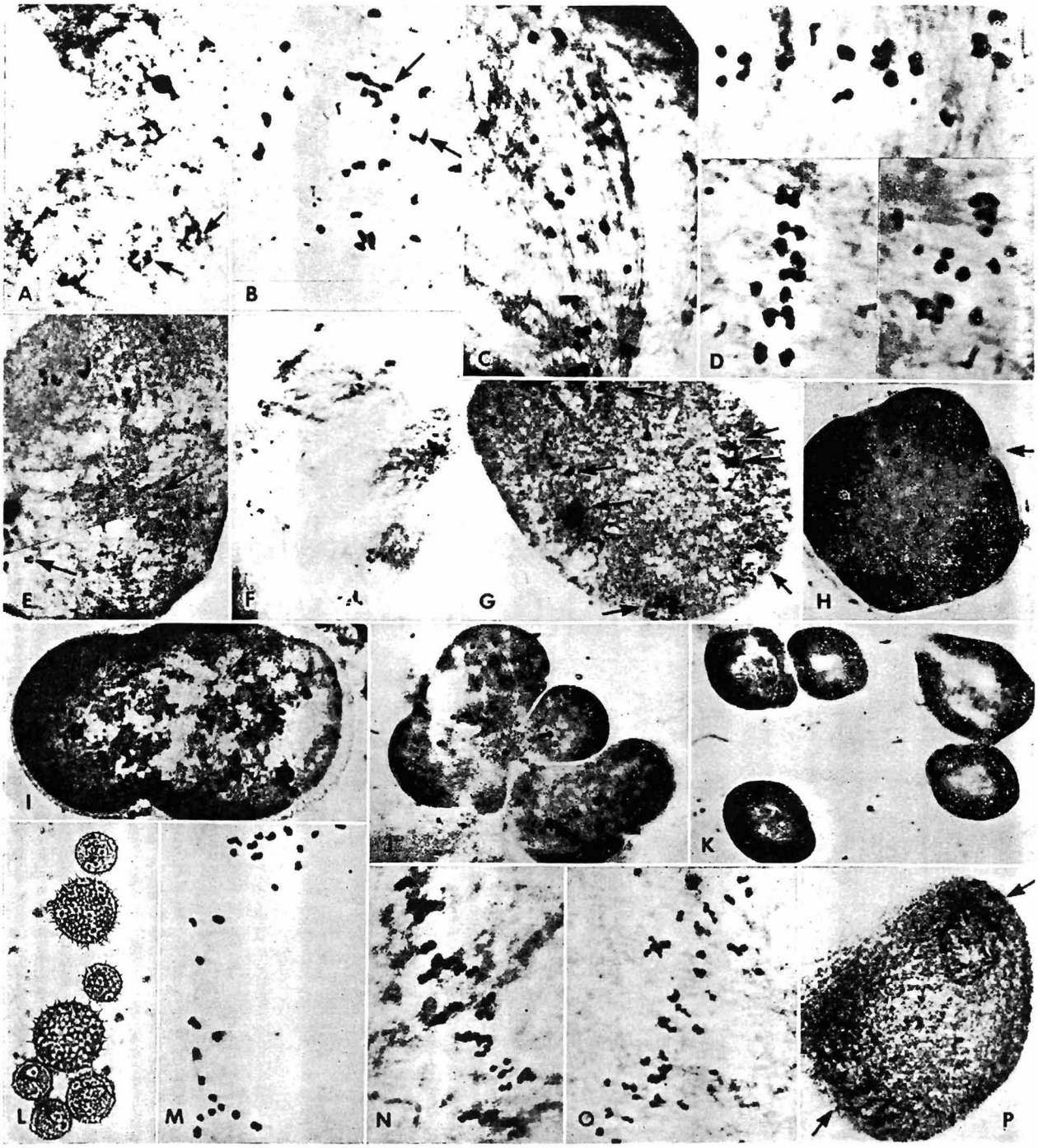

FIGURE 1—Meiosis in haploid cotton plants. *A*—diakinesis showing A and D associations separating. *B*—early meta-anaphase showing two A and D associations. *C*—dispersion of 26 univalents at meta-anaphase. *D*—three metaphase plates from same cell in different planes. *E*—metaphase showing chromatid separation. *F*—eight groups of chromatids at telophase. *G*—five micro-nuclei and two univalents. *H*—exine wall around unequal tetrad. *I*—exine wall around unequal dyad. *J*—furrowing around five nuclei. *K*—five microspores from a single cell. *L*—unequal sized pollen with differential development. *M*—linear arrangement of univalents due to laggards. *N*—26 univalents in one metaphase plate. *O*—anaphase of 26 univalents in one plate. *P*—possible dyad with full complement of chromosomes.

Meiosis and Pollen Development in Haploid Cotton Plants

JERRY R. BARROW

HAPLOID plants, which occur at a low frequency in cotton species *Gossypium hirsutum* L. and *G. barbadense* L. ($n = 2x = 26$), are derived as one or both members of twin embryo seed and have a somatic chromosome number of 26. Turcotte and Feaster[15] reported that a line of Pima cotton produced haploids at a high frequency (approximately 50 percent) from single embryo seeds. Later they demonstrated a phenomenon, termed semigamy, that was responsible for haploid production in single embryo seeds. In semigamy the sperm nucleus fails to unite with the egg nucleus after entering the egg cell. Both nuclei divide independently to form haploid chimeral tissue of maternal and paternal origin in the embryo[16].

American cultivated cottons are allotetraploids composed of two subgenomes designated as A and D[1]. Rhyne[12] demonstrated duplication of linkage groups in the two genomes, indicating a degree of homology between them. Endrizzi[6] postulated that the diploid-like nature of tetraploid cotton was due to a differential rate of chromosome condensation in the two genomes, which prevents the pairing of homologous chromosomes (i.e., chromosomes of similar function in different genomes) and enhances the pairing of homologues. Endrizzi[5] observed an association between chromosomes of the A and D genomes in a polyhaploid of cotton. Brown[3] showed an average of seven to nine paired chromosomes per cell during the pachytene stage in a haploid of *G. hirsutum*; as meiosis progressed, many partners fell apart without chiasmata. The occurrence of bivalents has been reported at less than one or two per cell in AD haploids of cotton by Beasley[1], Endrizzi[5] and Webber[17].

The high level of sterility in AD haploid plants can be attributed to irregularities of the meiotic process. The classical concept of the production of viable pollen grains by normal meiosis requires the pairing of homologous chromosomes in the meiotic prophase, their separation in the first division, and chromatid separation in the second division to provide a full haploid complement of chromosomes in each gamete[13]. Harland[7] obtained a boll containing one seed from a haploid plant derived from a Sea Island cotton, *G. barbadense*, from almost every cross when he used pollen from normal Sea Island cotton; apparently the haploid produced egg cells with 26 chromosomes. Other haploids he observed were less fertile. Webber[17] also collected three open-pollinated seed and one selfed seed from a haploid cotton plant. I observed one open-pollinated seed on one of 12 haploid plants in the greenhouse where natural crossing was less than 1 percent.

Meiosis in haploids has been studied in a number of plant species. Daker[4] observed a low frequency of bivalents and a preponderance of neocentric univalents in a haploid plant of *Pelargonium* ($n = x = 9$). The final products of meiosis included a high frequency of dyads. He concluded that these irregularities were the result of mutations affecting the genetic control of meiosis.

Nishiyama and Tabata[9] found abnormal configurations in the first and second divisions in meiosis of haploid oats ($n = 3x = 21$). Seed fertility was 1.2 percent in open-pollinated plants and 11.7 percent when haploids were hand pollinated with a cultivated oat variety.

Olsen and Hagberg[10] reported a frequency of five to eight bivalents per cell in metaphase I of a haploid rape plant. A few cells contained only bivalents. At anaphase I, bridges and fragments resulted from bivalent separation. Some univalents were moved at anaphase I without division, while others divided at anaphase I or even as late as telophase I. Some univalents were left out of the spindle arrangement and were not included in the second division. One cell, in which a complete haploid set of univalents moved to one end of the cell, divided normally at the second division giving rise to two genetically complete gametes.

Brown[2] saw no evidence of univalents dividing during the first meiotic division in haploid sorghum ($n = 2x = 10$); only the division of bivalents was observed. Univalents were randomly separated at anaphase I and aligned themselves at metaphase II for chromatid separation at anaphase II.

Levan[8] observed that univalents of haploid rye did not respond to the spindle. Only bivalents and trivalents arranged themselves between the poles.

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Considerable uniformity was noted up to metaphase I; irregularities became pronounced in later stages. Univalents moved randomly toward the poles without forming a plate, with some division. Some laggards formed a micronucleus without further division. At second division, chromosomes were seen with either double or single chromatids. Chromosomes with visible chromatids divided, while single chromatids remained at the plate. Tetrads with variable-sized nuclei were the general meiotic product. Some monads were observed and some fertile gametes were thought to result from restitution nuclei.

Person[11] analyzed meiotic chromosome behavior in haploid wheat ($n = 3x = 21$). He found some bivalent formation and some cells containing only univalents. Metaphase I was difficult to define because univalents did not move to a plate. A similar situation was noted by Tometorp[14], which he termed "meta-anaphase." Person did not observe spindle fibers in association with bivalents; univalents moved randomly to polar regions, and formed metaphase II plates either parallel or perpendicular to the equatorial plane between the poles.

The major objective of this investigation was to determine the events of the meiotic process and pollen production in haploid cotton plants and to explain the source of viable gametes observed to occur at a low frequency.

Materials and Methods

Selfed seed of a virescent semigametic line originally obtained from E. L. Turcotte of Phoenix, Arizona, was planted, and 50 out of 62 plants were classified as haploid. The haploid plants were determined by their smaller, narrower leaves, smaller flowers with reduced numbers of nondehiscent anthers, and by cytological examination.

Buds were collected and processed using well-known techniques of cotton cytology. Buds were collected early in the morning (8 a.m.); anthers were removed, killed and fixed in 30 parts acetic acid and 70 parts 95 percent ethyl alchohol (ETOH). The solution was changed immediately after collection, and the buds were stored in the refrigerator for two to three days. The anthers were then smeared in iron-propionic carmine stain. The anther tissue was removed, leaving primarily the pollen mother cells (PMC) or meiotic products on the slide.

Just prior to adding the cover slip a small droplet of 1 percent hematoxylin was mixed with the stain containing the PMC's. The slides were then pressed firmly and heated gently. Approximately 1500 cells were observed from late pachytene to pollen grain formation. More than 1000 cells had condensed meiotic chromosomes at a stage prior to division. Selected slides were made permanent by destaining with 45 percent acetic acid, replaced with absolute ETOH, and infiltrated with Euparal.

Results and Discussion

The cytological examination of PMC's of haploid cotton plants in diakinesis showed some attached A-D chromosomes and some A-D associations that were completely separated (Figure 1*A*). This observation agrees with that of Brown[3] and Endrizzi[5]. The degree of physical attachment decreased as the degree of condensation increased. Figure 1*B* shows one attached A-D association and one A-D association without visible attachments at early meta-anaphase.

Visible chiasmata were observed at a frequency of .005 per cell just prior to spindle formation. No bivalents were observed after spindle formation; consequently 26 univalents were the predominant configuration before division in the haploid cotton used in this investigation.

In metaphase I of haploid cotton referred to by Beasley[1], Brown[3], and Endrizzi[5] bivalents were observed in much higher frequency than in the present study. Beasley[1] presented a drawing showing bridge formation between bivalents at metaphase I, but the material examined in this experiment showed no true metaphase I figures, and is similar to the findings in haploid barley[14] and haploid wheat[11].

Spindle fibers developed among the fully condensed chromosomes (Figure 1*C*). A common observation after spindle formation was dispersion of univalents parallel with the spindle. In most cases the univalents appeared to move freely without any change in shape, but occasionally a single univalent would appear by its shape to be attracted to both poles. This resulted in a division of the univalent with the frequent formation of a univalent bridge. The rate of univalent dispersal was variable, resulting in groups of univalents from 1–26 scattered through the spindle. The term "meta-anaphase"[14] appropriately describes this stage of meiosis in haploid cotton where bivalents are rare.

Individual groups of univalents after meta-anaphase arranged themselves in several metaphase plates similar to the three metaphase plates from a single PMC shown in Figure 1*D*. These plates formed on random planes within the cell. Chromatid distinction could be made in some univalents at this stage (Figure 1*E*).

Figure 1*F* is at telophase after division in which eight incomplete groups of chromatids remained distributed in the cell. In most cases, 26 univalents divided simultaneously and 52 chromatids at telophase were seen in several cells. Figure 1*G* shows the nuclear formation of five groups of chromatids and two univalents that failed to divide. This occurred occasionally in groups of one or two chromosomes.

The sporoderm formed around the entire multinucleate PMC (Figure 1*H* and *I*), or it formed by furrowing around the unequal-sized nuclei as shown in Figure 1*J*. The difference in size of the microspores (Figure 1*K*) is clearly due to unequal distribution of chromosomes in the division of random metaphase groups. The larger microspores tend to develop a normally spined exine, while smaller ones form a thick exine wall with a reduced number of spines (Figure 1*L*).

After dispersal of chromosomes during meta-

anaphase, sometimes the univalents are left in a linear arrangement due to lagging univalents (Figure 1*M*). Regardless of group size, all chromosomes in close proximity aligned themselves in the same metaphase plate. If all 26 chromosomes were in the linear arrangement and were in close proximity, they would all be arranged on the same plate. Five PMC's with all 26 univalents in the same metaphase plate after dispersion of univalents by the spindle were observed in about 250 metaphase configurations (Figure 1*N*). Endrizzi[5] also observed a metaphase with full complement of chromosomes. The division of 26 univalents in a single plate (Figure 1*O* and *P*) would yield two genetically complete gametes. This event in micro- and megasporogenesis would explain the low frequency of haploid fertility in cotton, and is in agreement with the low fertility levels observed by Harland[7] and Webber[17]. Because of the probability that all 26 univalents will go to the same pole is extremely low $[(1/2)^{26}]$, this explanation is more likely for the production of fertile gametes in haploid cotton in the light of the observed frequency of occurrence.

In cotton, haploid anthers do not dehisce and male fertility is less likely to be observed than female fertility. In megasporogenesis the arrangement of 25 univalents at a single metaphase plate would provide 25 chromosomes in a gamete. This gamete fertilized with normal 26-chromosome gametes would be a source of monosomic plants. Haploids could be a source of other chromosomally unbalanced gametes and plants.

Summary

A cytological analysis was made of meiosis in haploid cotton plants obtained from a semigametic line. Because of rare bivalent formation there was only a dispersal of univalents in a meta-anaphase stage with occasional division of univalents. The univalents were dispersed into groups with 1–26 chromosomes at meta-anaphase. Groups with less than 26 chromosomes resulted in smaller than normal pollen grains. Formation of genetically complete gametes resulted when all 26 univalents were in close proximity after dispersal and formed a single metaphase plate. The close proximity of chromosomes after dispersal was explained by lagging chromosomes, rather than by the fact that all univalents moved to the same pole.

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