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Analyses of a Dominant Male-Sterile Character in Upland Cotton. I. Cytological Studies[1]

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ABSTRACT

Cytological observations of microsporogenesis breakdown were used to differentiate a new dominant male-sterile character in Upland cotton, *Gossypium hirsutum* L., from the two known dominant male steriles, Ms_4 and Ms_7. Unlike the Ms_4 genotype where breakdown is either premeiotic or occurs during early stages of meiosis, and unlike the Ms_7 genotype where the latest reported breakdown in microsporogenesis occurs at the onset of pollen wall formation, the pollen in the new male sterile aborted only after the entire pollen wall, both intine and exine, had been fully developed. Thus, breakdown was consistently postmeiotic although a few sporogenous cells may first degenerate. Compared to normal fertile pollen grains, the sterile grains had a significantly thicker and more intensely staining intine. They also possessed plugs at germ-pore regions that failed to stain with toluidine blue. Histochemistry revealed these plugs were composed of hemicellulose or some complex polysaccharide. Histochemical studies also disclosed a unique layer of insoluble carbohydrate located between the intine and exine walls in the sterile microspores. Cytoplasm disintegrated progressively during pollen development resulting in sterile pollen grains that were conspicuously shriveled and vacuolate at maturity.

Additional index words: Gossypium hirsutum L., Germ pore, Histochemistry, Microsporogenesis, Pollen.

MALE sterility in plants may be caused by a breakdown at various stages of pollen production, including stages of undifferentiated anther tissue through microsporogenesis to production of the mature pollen grain. In male-sterile cotton plants (*Gossypium* spp.), pollen abortion occurs at several stages. This differentiation may serve as a basis for distinguishing the various genetic and cytoplasmic-genetic genotypes.

At the premeiotic stage, Allison and Fisher (1) observed no recognizable development of sporogenous tissue in the Ms_4 dominant male sterile. In other work with the Ms_4 genotype, Murthi and Weaver (16) found some pollen mother cells (PMC's) initiating meiosis, but these cells collapsed by midprophase and the tapetal cells were smaller in some locules when compared to fertiles. Pollen production in the cytoplasmic-genetic male sterile (*G. hirsutum* L. nucleoplasm with *G. harknessii* Brandabee cytoplasm) also broke down at the premeiotic stage (16). In this male-sterile strain, sporogenous tissue collapsed and formed necrotic tissue while the tapetum seemed to disorganize and the microsporocytes coalesced. In the Ms_7 dominant male sterile, some locules had a black mass of dead tissue during the premeiotic stage (16).

During the meiotic stage of pollen development, degeneration of the dyad and the tetrad was observed in the Ms_7 male sterile in some instances (16). In Rhyne's male sterile, the tapetal cells died during and after the formation of dyads (16). Dead tapetal tissue disintegrated as meiosis of the PMC's advanced, and microspores degenerated soon after separating from the tetrad mother wall. Asynapsis during meiosis was the cause of male and female sterility in cotton in several instances (3, 5, 19, 22, 26). Sterility has been attributed to small structural differences in the chromosomes in some diploid interspecific crosses by Stephens (25). Kumar (14) found a high number of abnormal tetrads and univalent and multivalent cells after diakinesis in a *G. herbaceum* L. male-sterile plant. Loe and Sarvella (15) also found univalent chromosomes in PMC's of a male- and female-sterile plant.

Breakdown of viable pollen production in cotton also occurs during the post-meiotic stage. Richmond and Kohel (20) found a vacuole-like appearance in pollen grains of the ms_2 genotype and spiny exine missing from the aberrant pollen grains. In the Ms_7 genotype, microspores degenerated during development of the pollen wall, giving shriveled and shrunken nonviable pollen grains (16, 27). Vacuoles were formed during development and maturation of pollen grains in the ms_5ms_6 genotype (16). Vacuoles appeared to crush the chromatin material.

The exact stage of breakdown of pollen production in the ms_1 and ms_3 genotypes was never determined. Justus and Leinweber (13) stated that normal synapsis occurred in the ms_1 genotype.

The objective of this experiment was to differentiate, cytologically, a new dominant male-sterile character from the two known dominant male steriles, Ms_4 and Ms_7. We have determined the inheritance and mode of action of the new male-sterile gene and intend to publish these data later.

MATERIALS AND METHODS

The new dominant male sterile was discovered in an okra leaf (L^o), frego bract (fg), nectariless (ne_1ne_2) cotton strain at the University of Georgia Plant Sciences Farm near Athens, Ga. Flower buds at various stages of development were collected from male-sterile and male-fertile F_1 plants grown in the field in 1976 and in the greenhouse in 1976-77. The light microscope and the scanning electron microscope (SEM) were used in these studies. For light microscopic examination, material was embedded in paraffin and in plastic.

For paraffin embedding, the buds were fixed in a 10% solution of formaldehyde buffered to pH 7.2 (4). The material was dehydrated with ethanol and tertiary-butyl alcohol series and embedded in paraffin. Sections 10 μm thick were cut and stained with 1% safranin in water and 0.5% fast green in 95% ethanol. For plastic embedding, the buds were fixed in a 4% solution of gluteraldehyde buffered to pH 7.0 with a cacodylate (sodium) buffer. The material was then washed in buffer and dehydrated with ethanol before embedding into Spurr's resin. Sections 0.5 μm thick were cut with glass knives and stained with 1% toluidine blue in 1% sodium borate.

Material was prepared for SEM by fixing in 4% gluteraldehyde buffered to pH 7.0 with a cacodylate (sodium) buffer. The material was washed in buffer, fixed in osmium tetroxide

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buffered to pH 7.0 with a cacodylate (sodium) buffer, washed again in buffer solution, and dehydrated with ethanol. The anthers were then critical point dried and broken open before being gold coated. To study exine development, pollen grains were acetolyzed (9).

Histochemical studies included staining for proteins, carbohydrates, callose, and lipids. Fisher's (10) procedure for protein staining was followed by applying a 1% solution of Aniline Blue Black in 7% acetic acid for 10 min at 60 C to the sections.

The PAS stain was used for total insoluble carbohydrates (12). Control slides were prepared to remove starch, mucin, and RNA, all of which may stain with Schiff's reagent. To remove starch, a 1% solution of diastase of malt in a phosphate buffer (pH 6.0) was applied to slides at 37 C for 1 hour. To remove mucin, slides were treated with a solution of 0.1 mg of lysozyme in 10 ml of Soreson M/15 phosphate buffer (pH 6.24) for 1 hour at room temperature. To remove RNA, slides were treated with a solution of 0.01 mg RNAase in 10 ml 0.2 *M* acetate buffer (pH 5.0) at room temperature for 15 min.

The technique of Currier and Strugger (8) was used to stain for callose; a 0.005% solution of water-soluble aniline blue in 0.15 *M* dibasic potassium phosphate (pH 8.2) was applied to plastic sections for 10 min. Preparations were observed for callose using a fluorescence microscope. A control, which had not been stained, was observed in the fluorescence microscope to determine primary fluorescence of the tissue (12).

Lipids were stained by placing plastic sections in 50% ethyl alcohol for 5 min, staining with Sudan black B in 70% ethyl alcohol for 3 min, and differentiating in 50% ethyl alcohol twice for 1 min each (12).

RESULTS AND DISCUSSION

Light Microscopy

Examination of the new dominant male sterile revealed a few anthers contained occasional normal pollen grains (Fig. 1). Several sporogenous cells were observed degenerating (Fig. 2), but this was not the general case. In the majority of anthers examined, normal PMC's and tetrads were observed, indicating no breakdown or abnormalities throughout meiosis. The tapetum did not appear to be the main cause for microsporogenesis breakdown and was not different from that in fertile anthers. The Ms_4 genotype had microsporogenesis breakdown during the premeiotic stage, and the Ms_7 genotype degenerated in some locules during the premeiotic and meiotic stages (16).

The tapetum appeared pulled away from the endothecium in the paraffin sections of young anthers, but it was normal in the plastic sections. The fixation and/or dehydration process of the paraffin embedding process may have caused distortion of the tissue. Coagulation of the cytoplasm, distortion of the tissue, and poor resolution are disadvantages of the paraffin process. Using a plastic embedding medium largely removes these disadvantages. Reexamination of microsporogenesis breakdown in other male steriles may be in order, particularly where there is apparent tapetum breakdown.

A count was not taken, but more pollen grains were seen in fertile locules than in sterile locules. Developing pollen grains in anthers from fertile and sterile plants were misshaped (Fig. 3 and 4). Baranov and Maltzeu (2) stated that cotton pollen grains were angular when young and their walls were not formed.

In fertile and sterile locules, the developing pollen grains were not completely filled with cytoplasm. Baranov and Maltzeu (2) presented a general description of pollen development and showed a diagram of an immature pollen grain full of cytoplasm with exine development not completed. This was not found in the fertile segregates or the commercial cultivar, 'Coker 201', both of which we used as controls. Well-developed exine and intine layers were observed in all pollen grains before cytoplasm filled the interior. Baranov and Maltzeu (2) used four species, *G. hirsutum, G. barbadense* L., *G. arboreum* L., and *G. herbaceum* in their studies. In this study, *G. hirsutum* was the species examined.

Pollen grains in the new dominant male sterile developed exine and intine walls in contrast to the Ms_7 genotype, where pollen grains did not form a fully developed exine layer (16). The intine of sterile pollen grains stained darker than that of the controls, indicating differences in composition or maturity. The intine of pollen grains was measured, and a Student's t-test was performed to determine statistical significance. Measurements were made the day of flowering to insure a comparable stage of development. It had been noticed that flower buds of approximately the same size from fertile and sterile plants did not yield microspores in comparable stages of development. Anthers from fertile plants always appeared in a later stage of microsporogenesis. A highly significant difference in intine thickness was observed between fertile and sterile pollen grains (Fig. 5 and 6). The average thickness of sterile pollen grains was 2.1 μm compared to 1.3 μm in fertile pollen grains.

Pollen development requires large amounts of nutrients for growth and differentiation (7, 24). Sparrow and Hammond (24) suggested that there may be competition between the nucleus and cytoplasm for the same materials. If this is true, there also may be competition between the protoplast and intine for nutrients or materials. Production of a well-structured intine in sterile pollen may be performed at the expense of the protoplast.

Plugs were observed at the germ pores in the developing fertile pollen grains (Fig. 4A). This phenomenon was not described by Baranov and Maltzeu (2) and was not as obvious in sterile pollen grains when stained with toluidine blue. Fukasawa (11) stated that a male-sterile condition in *Aegilotricum* resulted mainly from a failure of nutrient production in pollen grains during germ-pore formation. Germ-pore formation in this particular species occurred together with thickening of the cell wall.

Mature viable pollen grains were spherical and full of cytoplasm (Fig. 5) compared to mature sterile pollen grains, which were misshapen and pratically void of cytoplasm (Fig. 6). These pollen grains were nonviable according to tetrazolium chloride viability tests (21).

Numerous fertile pollen grains at maturity contained two nuclei, and this condition was considered by Baranov and Maltzeu (2) to be necessary for pollination. With the exception of a few, the sterile pollen grains were at no time binucleate. Chu et al. (6) observed degeneration of microspores after the uninucleate stage in rice (*Oryza* sp.) and the developing microspores lost their ability to carry on any nuclear division. This inability for nuclear division was apparent in microspores of the dominant male sterile. It may be due to degeneration of the protoplast after or during intine formation.

Histochemical Studies

Observations of differences in intine size and plugs at the germ pores stimulated interest in their respective components. Plugs at the germ pores during pollen development have not been reported previously in cotton. These plugs stained purple with toluidine blue, which is an indication of RNA (17). The intine stained blue with the intensity determined by the type of pollen grain. Sterile pollen grains had intines staining a darker blue than that of the fertile grains. It is thought that the differences in intensity were due to different wall composition.

The plugs and intine did not stain with stains used for proteins (Fig. 7 and 8). Southworth (23) stated that the intine in three species of Compositae gave positive reactions for protein and insoluble polysaccharides, including pectic acid, callose, and hemicellulose. Her results differed from our findings. A striking difference between cytoplasm was revealed with protein stains (Fig. 7C and 8C). Cytoplasm of sterile pollen grains showed disorganization and degeneration during development. At the time of normal anther dehiscence, no part of the sterile pollen grains stained for protein, indicating complete degeneration of cytoplasm.

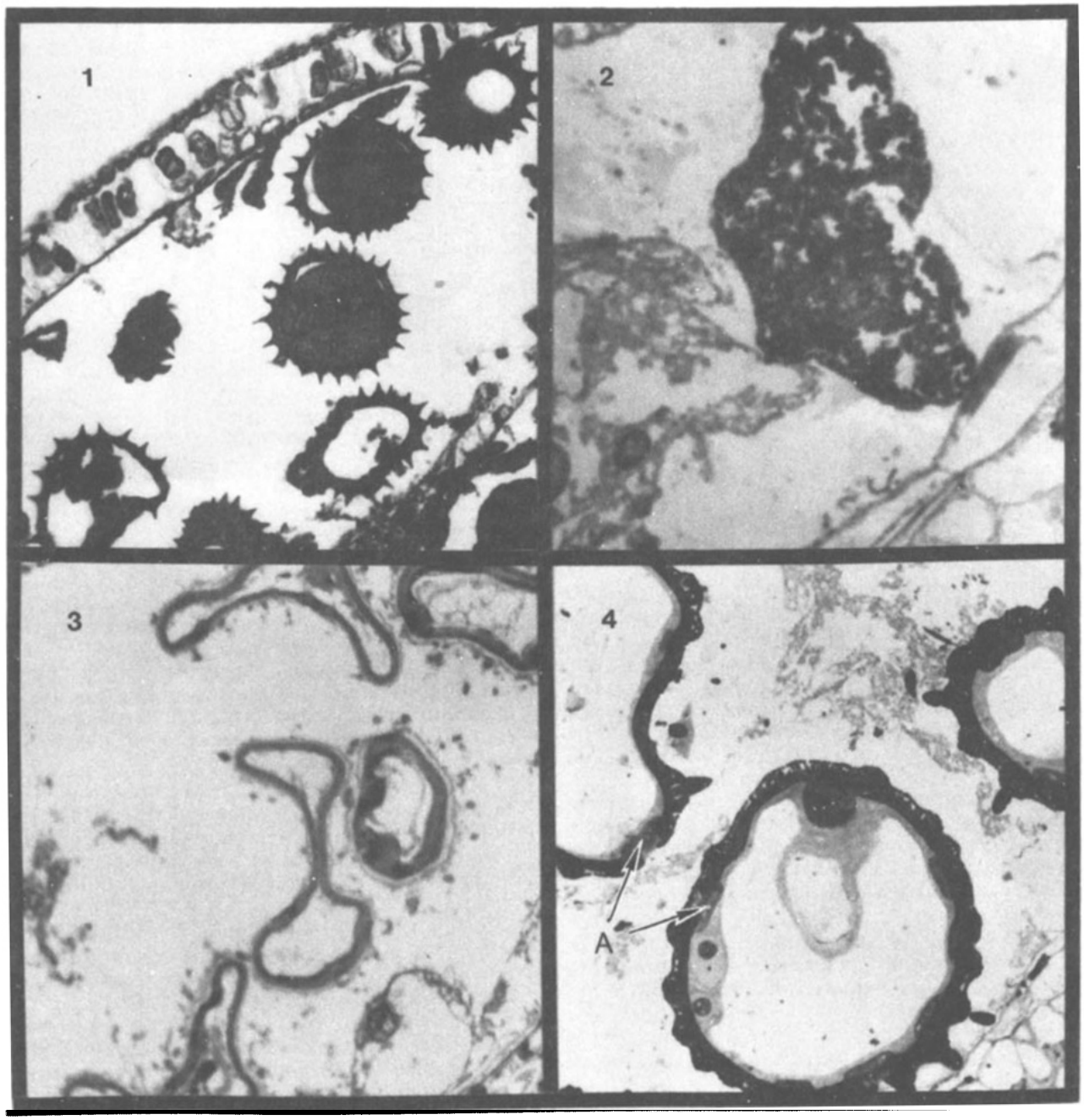

Fig. 1. Transverse section of a sterile anther showing degenerating and fertile pollen grains (×600). Fig. 2. A darkly stained sporogenous cell undergoing degeneration in a sterile anther (×1,200). Fig. 3. Transverse section of an anther from a sterile plant showing developing pollen grains (×600). Fig. 4. Transverse section of an anther from a fertile plant showing developing pollen grains (×960). Plugs (A) are shown in germ-pore regions.

Callose staining revealed very little about the composition of the intine or germ-pore plugs. Primary fluorescence of the tissue is shown in Fig. 9. The stained intine of sterile and fertile grains fluoresced brighter than the unstained intine, indicating a small amount of callose. The area joining the germ-pore plug with the exine and intine fluoresced a brilliant yellow (Fig. 10B). This indicates the presence of callose in this particular region.

The PAS stain for total insoluble carbohydrates stained germ-pore plugs in sterile pollen grains (which were unstained with toluidine blue) (Fig. 11A). Likewise in fertile pollen grains, a positive reaction was obtained for total insoluble carbohydrates (Fig. 12A) in the germ-pore regions. Removal of starch, mucin, and RNA did not alter the staining reaction. Cellulose is generally not stained with PAS (18); therefore, one can conclude that the plugs at the germ-pore regions were probably composed of hemicellulose or some complex polysaccharide. The intines of both sterile and fertile developing pollen grains did not stain uniformly for carbohydrates. Only the outer part of the intine gave a positive reaction. The sterile pollen grains showed a thicker, darker layer of carbohydrates on the outer part of the intine than did fertile pollen grains (Figs. 11D and 12D). In more mature pollen

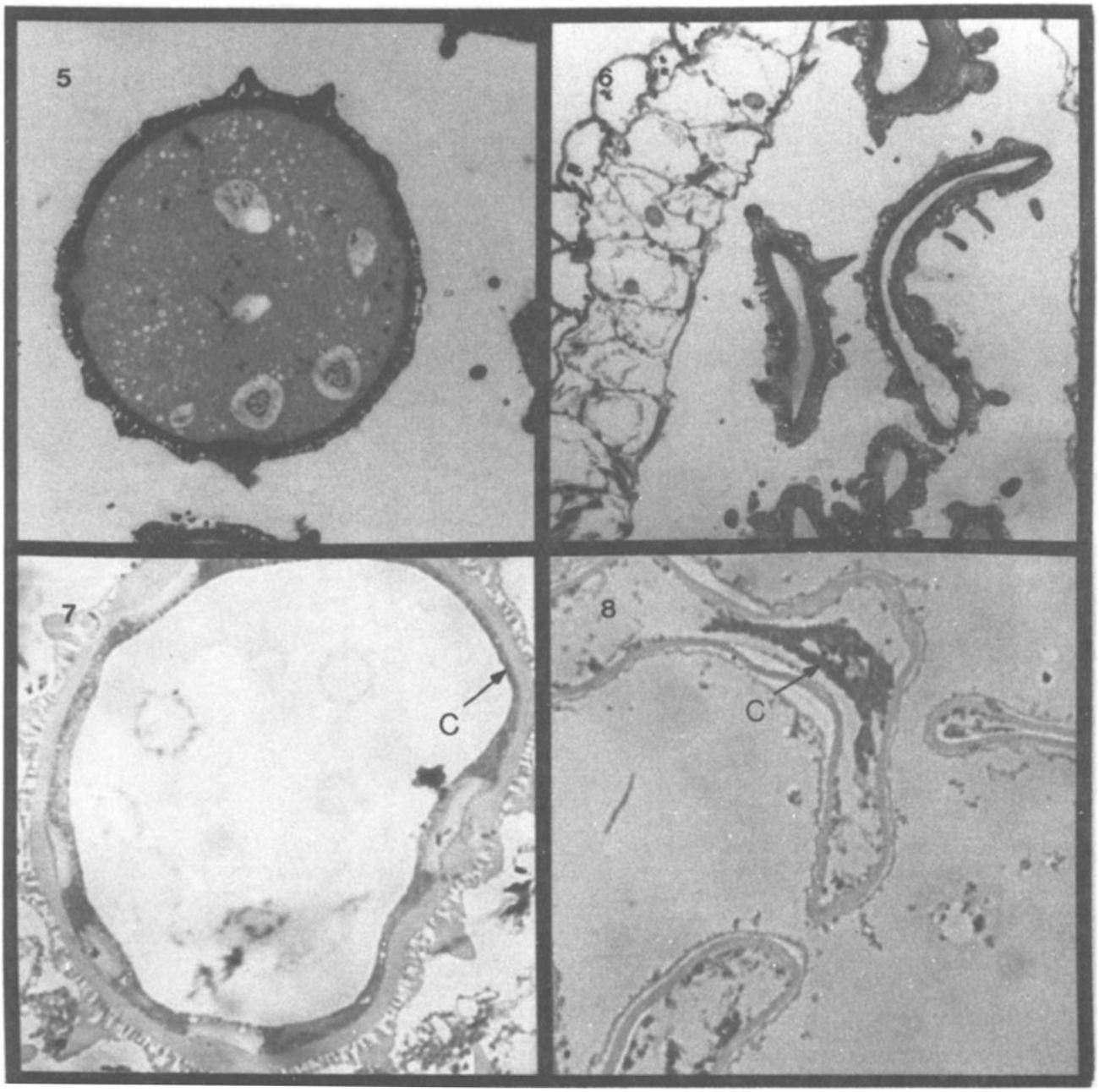

Fig. 5. A fertile pollen grain at maturity (×600). Fig. 6. Sterile pollen grains at maturity showing well developed exine and intine and void of cytoplasm (×600). Fig. 7. A developing fertile microspore stained with Aniline Blue Black for protein (×1,184). The cytoplasm (C) stains uniformly blue. Fig. 8. Developing sterile microspores stained with Aniline Blue Black for protein (×1,200). The cytoplasm (C) is shown degenerating.

grains, the differences were magnified. The layer of carbohydrates in sterile pollen grains increased in width and color intensity, while fertile pollen grains stained lightly for carbohydrates at the exine and intine walls. The histochemical studies revealed this to be the only difference in composition between the sterile and fertile microspore intines.

Negative results were obtained with lipid staining in regard to intine and germ-pore plug composition.

Scanning Electron Microscopy (SEM)

Highly shriveled, shrunken, and misshapen pollen grains from sterile plants were viewed in the scanning electron microscope (Fig. 13). Tapetal tissue covered the majority of these pollen grains. Fertile pollen grains were spherical in shape and cleaner in appearance (Fig. 14). An occasional pollen grain from fertile anthers was similar in shape to sterile pollen.

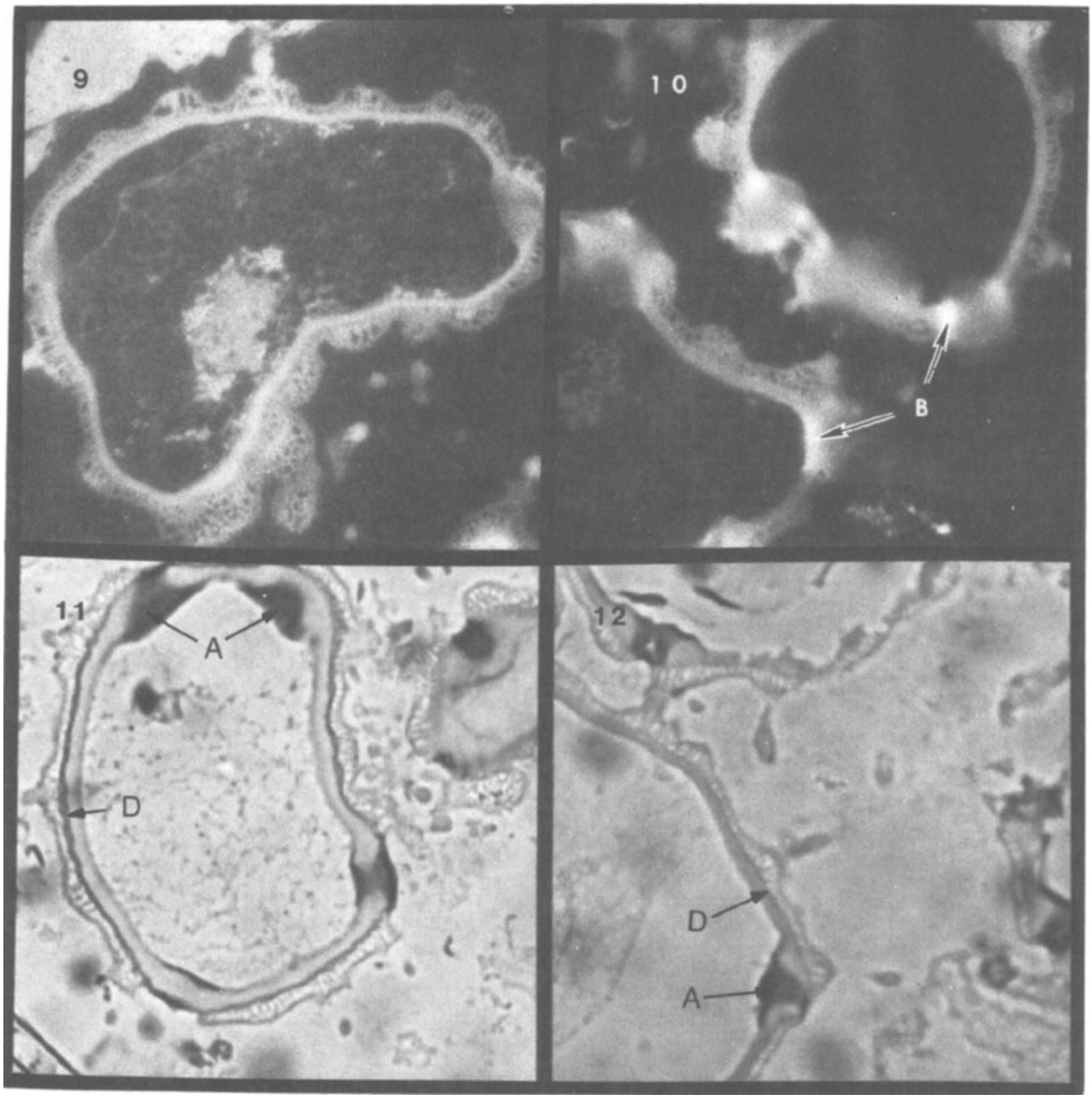

Fig. 9. Primary fluorescence of a microspore (×960). Fig. 10. Fluorescence of microspores stained with Aniline blue showing areas (B) of callose in germ-pore regions (×960). Fig. 11. Sterile microspores stained with PAS for total insoluble carbohydrates showing plugs (A) in germ-pore regions and a layer (D) between the intine and exine (×960). Fig. 12. Fertile microspores stained with PAS for total insoluble carbohydrates showing plugs (A) in germ-pore regions and only a very thin, light area (D) between the intine and exine (×960).

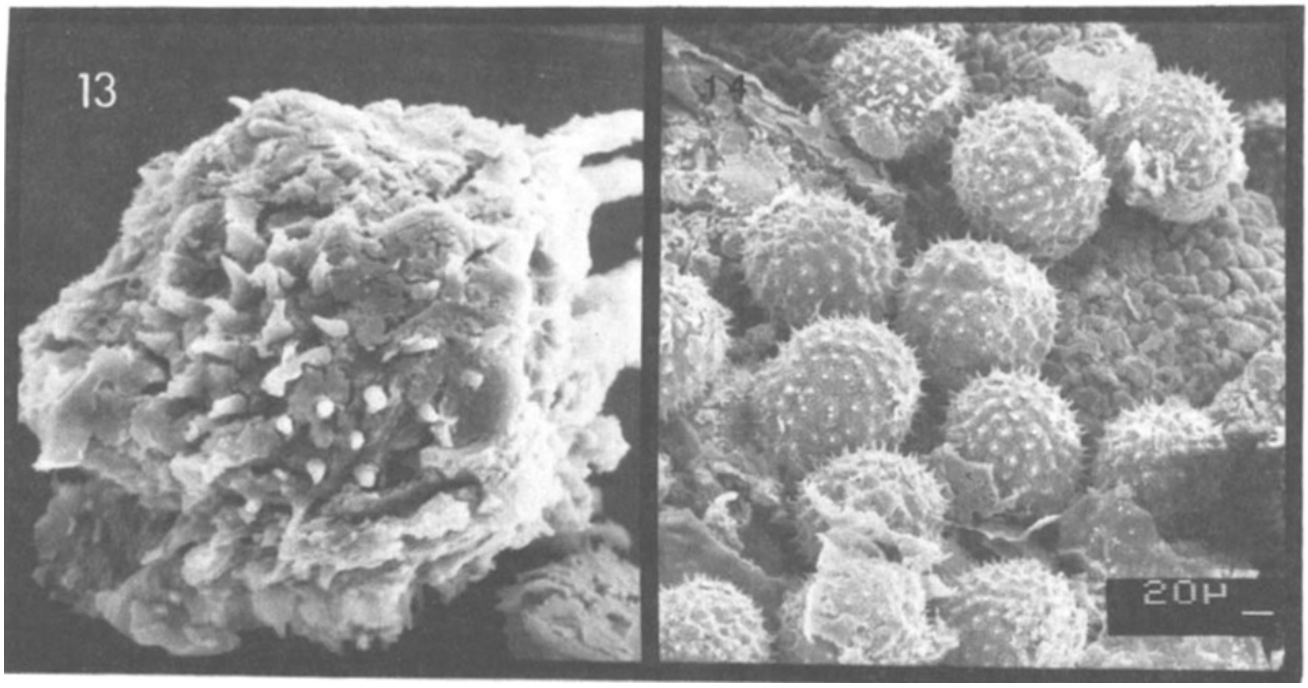

Fig. 13. Scanning electron micrograph of a sterile pollen grain covered with tapetal tissue (×1,000). Fig. 14. Scanning electron micrograph of pollen grains from Coker 201 (×250).

CONCLUSION

Microsporogenesis breakdown in the new dominant male sterile cotton mutant occurred in the post-meiotic stage except for a few degenerating sporogenous cells. This distinguishes the new dominant male sterile from Ms_4. Microsporogenesis was normal through the meiotic stage. Tapetal cells appeared similar to those in anthers from fertile plants and were not considered the source for breakdown of pollen development. Plugs were observed in the germ-pore regions of developing fertile pollen grains when stained with toluidine blue. The plugs were not observed in sterile pollen grains with this particular stain but were observed with PAS. This indicated that there is a difference in plug composition. Germ pores may be involved in nutrient absorption, particularly after exine and intine development. It was theorized that plugs in sterile pollen grains were not as efficient in nutrient absorption, thus contributing to the degeneration of the microspores.

Pollen development in the new dominant male sterile proceeded through exine and intine formation, which distinguishes it from the Ms_7 dominant male sterile. Sterile pollen intines were significantly thicker and stained more intensely than that of fertile pollen grains. Histochemical studies revealed a more dense layer of carbohydrates between the exine and intine of the developing sterile microspores. This unusual intine may contribute to microsporogenesis breakdown in two ways. First, the thicker intine may be produced at the expense of the protoplast, which may require the same nutrients or materials. Second, the very intensely staining layer of carbohydrates lying between the exine and intine may interfere with nutrient absorption if nutrients are absorbed through the pollen walls.

Protein stains revealed disintegrating cytoplasm in sterile microspores. At the time of anthesis, sterile pollen grains were shriveled and contained no cytoplasm. Occasionally, a locule contained one or two viable pollen grains [according to vital-stain testing (21)] along with a number of shriveled, non-viable pollen grains.

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