PHYSIOLOGICAL AND MORPHOLOGICAL STUDIES ON THE CYTOPLASMIC MALE STERILITY OF SOME CROPS

Hiroshi NAKASHIMA

(Laboratory of Industrial Crops, Faculty of Agriculture
Hokkaido University, Sapporo, Japan)

Received February 17, 1978

Contents

I. Introduction

The success of the modern methods of breeding hybrid corn prompted utilization of the hybrid vigor in the breeding of other crops. However, the necessity in many crops of making the crosses by laborious hand procedures prevented its wide adaption. The procedure of making hybrids is greatly facilitated in certain crops by the utilization of male sterile lines. Male sterility has been found in many crops and is generally called pollen sterility. All individuals with male sterility fail to produce male gametophytes

This paper comprises part of a thesis submitted to Hokkaido University in partial fulfiment of the requirements for the degree of Doctor of Agriculture.

although they produce normal functional female gametophytes. Male sterility is caused mainly by hereditary mechanisms of cytoplasm and/or specific genes, but also occasionally by some environmental factor such as temperature and chemical substances. Because of its ease in maintaining male sterility and in restoring the fertility in commercial croping, the so-called "cytoplasmic male sterility" is most useful for the production of hybrid.

The primary object of the present investigations was to clarify the physiological mechanism causing the cytoplasmic male sterility in various crops such as sugar beet, maize and sorghum. Another was to seek out some efficient criteria which would be useful for the pre-anthesis selection of male sterile plants and/or lines. Thus, the author has conducted the physiological and morphological studies on the cytoplasmic male sterile lines in comparison with their maintainers in these crops. These studies were expected to contribute to seeking out the maintainers and restorers, and inducing artificial male sterility in practice. A large number of studies have already been from the point of view of physiology and morphology of pollen, but there is little information available on the development and differentiation of male gametophytes, and particularly in development of anthers. This study is expected to contribute to this field of investigation.

The author would like to acknowledge the continuing guidance and encouragement of Dr. Sadaji HOSOKAWA, Professor of Industrial Crops, Faculty of Agriculture, Hokkaido University. The author also wishes to thank Dr. Man-emon TAKAHASHI, Dr. Yozo OKAZAWA and Dr. Chikahiro TSUDA for their helpful advice during the preparation of this manuscript. The author also would like to express his appreciation to Dr. Yoshiya SHIMAMOTO, Dr. Eishiro SHIKATA and Mr. Hiroyasu FUKUJU for frequent, stimulatory, and helpful discussion. The author must also express his sincere appreciation to the members of the Laboratory of Industrial Crops, Faculty of Agriculture, Hokkaido University and the Sugar Beet Section of Hokkaido Agricultural experiment Station. The cost of the study was partly met by grants from the Hokkaido prefectural goverment and the Japanese Ministry of Education.

II. Literature Review

Since ARCHIMOWITSCH[3] report the male sterility of sugar beet in 1931, many detailed studies and investigations have been conducted.[4,7,58,72,92,93] Many research workers[35,48,84,112] have made efforts to utilize the male sterility for hybrid seed production. The first review of cytoplasmic male sterility was made by EDWARDSON[40] in 1956. Larser and LERSTEIN[73] reviewed much

additional literature and tried to find out exactly when abortion occurs and what happens when it begins. According to these reviews, the predominate causes of cytoplasmic male sterility can be listed as follows, 1) abnormal behavior of the tapetal cell, 2) the structural changes in the stamen filament, 3) callose dissolution, 4) virus, 5) differences of amino acid components in anther, and 6) enzymatic activities.

1) Abnormal behavior of the tapetal cell

According to most literature, the tapetum has been implyed to play an important role as the direct or indirect cause of pollen abortion. The behavior of tapetum in relation to pollen abortion is classified into the following 3 types, a) tapetal behavior is normal but the pollen fails to develop,[18,117] b) tapetal cells do not degenerate normally, and c) lack of tapetum[113]. The chief function of the tapetum has often been said to be the production and transport of enzymes, hormones and nutritive materials which are utilized for microsporegenesis, but its role in exine formation and maintaining a pool of nucleosides for DNA synthesis renewed emphasis[120,121]. Thus it is now presumed that the tapetal abnormality is closely associated with the failure to nourish the microspores.

2) Structural changes in the stamen filament

ROHRBACH[100] showed the structural changes in the stamen filament in male sterile sugar beets, and suggested the pollen abortion resulted from nutritional indeficiency caused by the stamen abnormality. On the other hand, KINOSHITA[72] suggested that the stamen abnormality of this plant was caused by the male sterility. Joppa, MCNEAL and WALSH[69] with *Triticum*, ALAM and SANDAL[1] with sundangrass and IWANAMI and HOSODA[62] with *Brassica* reported that the male sterility resulted from the stamen abnormality. Larser failed to show any structural differences in this organ, between normal and cytoplasmic male sterile sorghum.

3) Callose dissolution

FRANKEL, IZHAR and NITSAN[38], and IZHAR and FRANKEL[64,65] present the evidence that faulty timming of enzymatic digestion of callose causes the microspore abortion in *Petunia*. Acording to this, callase activity changes depending upon pH value, and they suggested that the changes of pH are presumably associated with those of amino acid components. HOSOKAWA, TSUDA and TAKEDA[59,60] presented the particular change of pH value of anther in male sterile sugar beets during its development and the eventual inactivation of some enzymes. WARMKE and OVERMAN[122] found that the callose wall of diard degenerated earlier in the sterile plants.

4) Virus

Because the nature of the cytoplasmic factor has not been obvious and since the behaviour of virus has been similar to those of genes, virus has been extensively investigated in connection with cytoplasmic inheritance. The investigations as to whether virus is responsible for the cytoplasmic factor in cytoplasmic male sterile plant have been carried out by means of grafting, inoculation or thermo treatments. FRANKEL[36,37], EDWARDSON and CORBETT[31], and CORBETT and EDWARDSON[30] reported that male fertile plants grafted on the male sterile petunia showed the male sterility. CECH and POZDENA[13] with *Humulus* and CURTIS[22] with *Beta vulgaris* also demonstrated graft transmissibility of male sterility. CLEIJ[19] with *Beta vulgaris* reported the sterile plant changed to normal plant with thermosock experiments. ATANASOFF[5,6] reviewed numerous studies on cytoplasmic male sterility in viral trasmission. He insisted it should be reconsidered that the cytoplasmic factor was virus in many more plants. OHTA[88,89,90] reported that by means of inoculation and thermotreatment pollen fertility was decreased because of interaction between the cytoplasmic factor and particular virus in *Capsicum*. He speculated that the entities involved in the cells had originated from an exogenous viruses in the course of evolution, followed by the loss of infectionity. In this process, it could have arrived in the state of a plasmatic particle of RNA nature. BREWBAKER[8] suspected that cytoplasmic inclusions were virus particles that survive only in plants with a virus susceptible genotype (ms, ms), and that the virus lived symbiotically in diploid somatic cells, but killing the haploid pollen cells. There are other investigations which insist that cytoplasmic factors are viruses or virus like factors. There are however many experiments that do not support the virus nature[19,72,79,103,107,115,127] EDWARDSON[32] showed inclusions in dense cytoplasmic area in root tip cell of cytoplasmic male sterile maize by electron microscopic technique. He did not believe however that these inclusions could be viruses. Electron microscopic studies of anther tissues have been carried out by DEVRIES and IE[23], and WARMKE and OVERMANN[122]. But they have not found prominent differences in ultrastructure between two types of fertility.

5) Differences in amino acid component

FUKASAWA[39] studied by paper chromatography wheat and maize anthers, relatively large quantity of alanine was present during meiosis and microspore stage in male sterile anthers, while a definite lack of proline was noted in male sterile anther during the formation of microspores. Similar relationships of free amino acid in anther tissue were studied in sugar beets[59,60,100],

sorghum[11] and vegetable crops[45,46] but in *Petunia*[31,65] a large amount of proline was noted in both fertile and sterile anthers.

6) Enyzmatic activities

Various enzymes of many crops such as maize, petunia, sudangrass and *Brassica* have been studied in respect to male sterility[2,15,24,34,41,63,78,123]. Enzymes such as succinic dehydrogenase, amylase, polyphenol oxidase, peroxidase, cytochrome oxidase, acid phosphatase, invertase, phosphatase, catalase, ascorbic acid oxidase and esterase have been reported. In the results of these experiments, peroxidase and polyphenol oxidase show more intense activities in sterile anther, but the remainder showed equal or less intensity in comparison with normal anther.

As mentioned above, it seems likely that some relationship exists between these physiological and morphological abnormalities and male sterility. These abnormalities were also noted much differently among crops. The most common phenomena related with male sterility among various crops were abnormal behavior of tapetum and a lack of proline.

III. Sugar Beet

A. Histochemical Observation of Anther

It is naturally expected that the metabolism of carbohydrate, amino acid, protein, nucleic acid and fat, and enzymatic activities in anther tissues showes a remarkable difference between normal and male sterile types of sugar beets during the development of anthers.

Materials and Methods

Cytoplasmic male sterile line TA-2-CMS and its conterpart 0 type TA-2-0 were used in these experiments. Fresh anthers were smeared on slide glasses. Then respectively under microscope reducing sugar, starch grain and fat in fresh anthers were detected by means of Fehlin's reagent, IKI solution and Sudan III reaction. For the observation of localization of starch grains, flowers of the fertile and sterile plants were fixed in formalin-acetic alcohol (FAA), dehydrated in ethyl-buthyl alcohol series, and embedded in paraffin in the usual manner. Sections were cut to a thickness of 15 μm and were stained with IKI solution and modification of triple stain of Hime *et al.* (Fig. 2)[51]. The degree of the reaction was recorded by the following standard of microscopic observation; 0(−)—4(+++) more definitely presented. Deoxyribonucleic acid (DNA) was stained with Azure A in blue to green, polysaccharides were stained with periodic acid Schiff's (PAS) reaction to red and basic group of protein was stained with Naphtol yellow S to yellow.

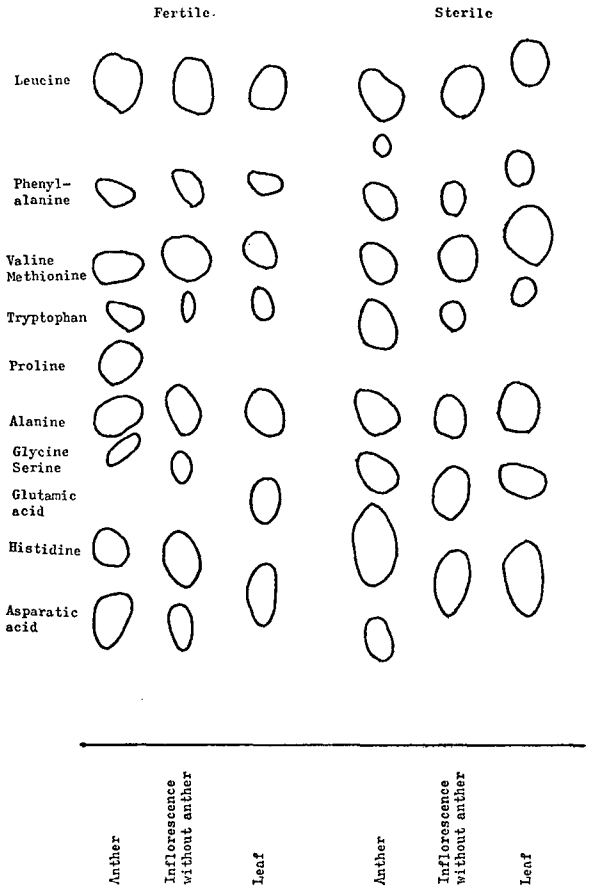


Fig. 1. Tracings of paperchromatograms of amino acids in fertile and sterile sugar beets.

Free amino acids were detected by paper chromatography (Fig. 1).

Flowers of fertile and sterile plants were cut to 26 μm by freezing microtome. Peroxidase and succinic dehydrogenase activities were examined by benzidine and tetrazolium salt methods, respectively (Fig. 3).

Results

Table 1 and 2 showed the reaction of reducing sugar and starch grains, respectively. Table 3 showed the ANOVA of these results. Plate I showes the results of IKI reaction of transverse sections at several stages of anther development. During the early stage of microsporogenesis, many starch grains were observed in endothecium of both fertile and sterile anthers.

Fix the tissues in FAA, dehydrate and infiltrate paraffin
Section the tissues in 15 μm
Mount and sections bring to water
Hydrolyze section N HCl at 60°C for 15 minutes
Wash in cold dist. water

Triple stain

- Stain in Azure A-SO_2 (0.5g azure A in 100 ml of SO_2 water prepared freshly) for 5 minutes
- Rinse 2 times in SO_2 water for 2 minutes
- Wash in water
- Place in periodic acid solution for 5 minutes (0.8g periodic acid with 10 ml of 0.2 M sodium acetate and 90 ml dist. water)
- Rinse in water
- Stain in Schiff's reagent for 5 minutes
- Wash in running tap water for 2 minutes
- Stain in 0.2 % naphtol yellow S in 1 % acetic acid for 2 minutes
- Wash in water

Dehydrate in tertiary butyl alcohol
Clear in xylene and mount in balsam

Fig. 2. The procedure of triple stain for demonstration of DNA, polysaccharides, and the basic proteins.

Benzidine reaction

Section the freeze tissues in 26 μm

Staining for peroxidase

- Place the sections in 0.04 % solution of benzidine in 0.01 M acetate buffer at pH 5.0 for 1 minute at room temperature
- Place the sections in 15 % sodium nitroprusside with 0.06 % H_2O_2
- Place the sections in 9 % nitroprusside in 25 % ethyl alcohol
- Incubate the sections for 1 hour at 4°C

Mount in glycerin-gelatin

Tetrazolium salt method

Section the freeze tissues in 26 μm

Staining for succinic dehydrogenase

- Place the sections in solution composed of 3 ml of 0.1 M sodium succinate, 2.4 ml of 0.1 M Sorensen phosphate buffer at pH 7.6, 0.6 ml of 0.25 % Nitro-BT, and 0.2 ml of 0.1 % phenazine methosulfate
- Incubate the sections in this solution for 15 minutes at 37°C

Mount in glycerin-gelatin

Fig. 3. The procedure for determination of enzymatic activities.

TABLE 1. Reaction of reducing sugar with Fehling's reagent in sugar beet anthers during their developmental stages

Type of fertility	Reaction	Developmental stage of anther				
		PMC	T	MS	P	A
Fertile	0	3	4			
	1	1	4	6		
	2			8	1	
	3				7	6
	ave	0.3	0.5	1.6	2.9	3.0
Sterile	0	2	2	1		
	1	2	4	2		
	2		7	3	3	2
	3				6	4
	ave	0.5	1.4	1.3	2.7	2.7

PMC: Pollen Mother Cell P: Pollen
T: Pollen Tetrad A: Anthesis
MS: Microspore

Reaction 0 (absent) 1, 2, 3 (more definitely presented)

TABLE 2. Starch reaction with IKI in sugar beet anthers during their developmental stages

Type of fertility	Reaction	Developmental stage of anther				
		PMC	T	MS	P	A
Fertile	0					1
	1				4	5
	2			2	4	
	3		2	10		
	4	4	7	3		
	ave	4.0	3.8	3.1	1.5	0.8
Sterile	0					
	1				3	3
	2				4	2
	3		3		3	1
	4	2	8	8		
	ave	4.0	3.7	4.0	2.0	1.2

Symbols are the same as Table 1.

TABLE 3. Analysis of variance for reducing sugar and starch reactions in sugar beet anthers

Source	d. f.	Reducing sugar MS	d. f.	Starch MS
Fertilities (F)	1	4.13**	1	1.88**
Stages (S)	4	18.08**	4	20.57**
FxS	4	3.21**	4	1.47**
Errors	68	0.32	69	0.30

TABLE 4. Histochemical observations of the tissue in fertile and sterile anthers

a) DNA

tissue		Developmental stage of anther PMC	T	MS	P
PMC or Pollen	F	+	−	−	−
	S	+	−	−	
Tapetum	F	−	+	+	
	S	−	+	+	
Transitory tissue	F	−	−	−	−
	S	−	−	−	−
Epidermis	F	−	−	−	−
	S	−	−	−	−
Parenchyma	F	−	−	−	−
	S	−	−	−	−

b) Polysaccharides

tissue		Developmental stage of anther PMC	T	MS	P
PMC or Pollen	S	−	±	±	+
	F	−	±	±	
Tapetum	F	−	−	−	
	S	−	−	−	
Transitory tissue	F	+	+	+	−
	S	+	++	+	±
Epidermis	F	−	−	−	−
	S	−	−	−	−
Parenchyma	F	++	++	++	±
	S	++	++	++	+

TABLE 4. (continued)

c) Proteins

tissue		Developmental stage of anther			
		PMC	T	MS	P
PMC or Pollen	F	+	±	±	
	S	+	±	±	
Tapetum	F	+	++	++	
	S	+	++	++	
Transitorp tissue	F	−	±	±	−
	S	−	±	−	−
Epiderims	F	±	±	±	−
	S	±	±	±	±
Parenchyma	F	−	−	−	−
	S	−	−	−	−

F: fertile −: (absent)
S: sterile ±, +, ++, +++: (more definitely presented)

TABLE 5. Average diameter of anther locules and average radial width of anther walls in sugar beet anthers during their developmental stages (μm)

	Fertile				Sterile			
	PMC	T	MS	P	PMC	T	MS	P
Anther	158.0	213.0	245.0	262.0	149.5	242.8	233.0	212.0
Epidermis	8.0	8.5	9.3	10.8	7.3	8.8	8.8	9.0
Transitory	13.8	15.5	15.0	17.5	12.3	17.0	17.5	18.0
Tapetum	13.3	34.0	22.0	16.0	12.5	37.3	34.5	0.0

Symbols are the same as Table 1.

As the stage of anther advanced, the amount of starch grains of fertile anther decreased gradually. While male sterile anther remained even in anthesis. Tapetal cells disappeare completely in fertile anther, but in contrast, in sterile anthers became abnormal. Plate II showed the results of Sundan III stain. In fertile plants, materials stained with Sudan III were decreased gradually as the stage of anther advanced, but in sterile plants they remained. Table 4 and Plate III showed the results of triple stain. The intensities of reactions were much different among tissues, but no conspicuous difference could be observed between the two types of fertility. Since tapetum was deeply stained, it was presumed that the tissue had strong

TABLE 6. Changes in activities of peroxidase with benzidine reagent in sugar beet anthers during their developmental stages

Type of fertility	Activity	H-19 Developmental stage of anther				TK-76 Developmental stage of anther			
		PMC	T	MS	P	PMC	T	MS	P
Fertile	0	1				9			1
	1	5	1		2	5	4		3
	2	1	10	3	3	4	8	4	6
	3		2	4			6	2	1
	ave	1.0	2.1	2.6	1.6	0.7	2.1	2.3	1.6
Sterile	0					2			
	1	2	1			9			
	2	2	3	1	1	5	13	6	3
	3		5	11	4		1	4	1
	ave	1.5	2.4	3.9	2.8	1.2	2.1	2.4	2.3

Symbols are the same as Table 1.

TABLE 7. Changes in activities of succinic dehydrogenase with tetrazolium salt method during their developmental stages

	Type of fertility	Activity	H-19 Developmental stage of anther				TK-76 Developmental stage of anther			
			PMC	T	MS	P	PMC	T	MS	P
Tapetum	Fertile	0				3				3
		1			2				2	14
		2			2	4			9	3
		3	8	8	5		18	17	5	
		ave	3.0	3.0	2 3	1.1	3.0	3.0	2.2	1.0
	Sterile	0			1	3	2			1
		1	2	4	5	2	12		2	3
		2	1	1	6		4	11	17	1
		3	1	2	1			9	6	
		ave	1.8	1.7	1.5	0.4	1.1	2.5	2.2	1.0
PMC or Pollen	Fertile	0			5	3			10	16
		1				3			1	4
		2	2	4	3		4	14	5	
		3	6	4			14	2		
		ave	2.8	2.5	0.8	0.5	2.8	2.1	0.9	0.2
	Sterile	0			8	4	5		1	4
		1	2	3	3	1	8	2	14	
		2	1	5			5	15	10	
		3	1					2		
		ave	1.8	1.6	0.3	0.2	1.0	2.0	1.4	0.0

Symbols are the asme as Table 1.

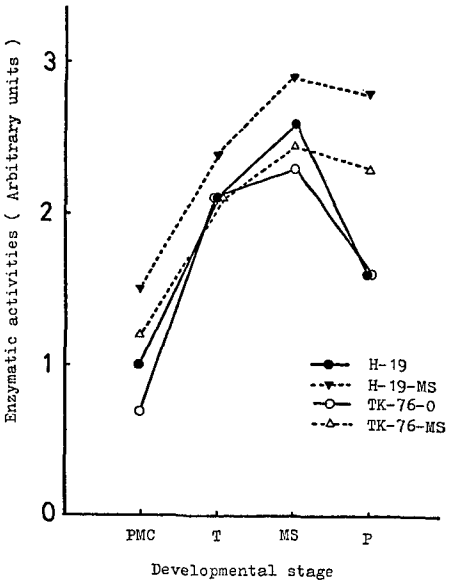


Fig. 4. Peroxidase activities of sugar beet anthers.

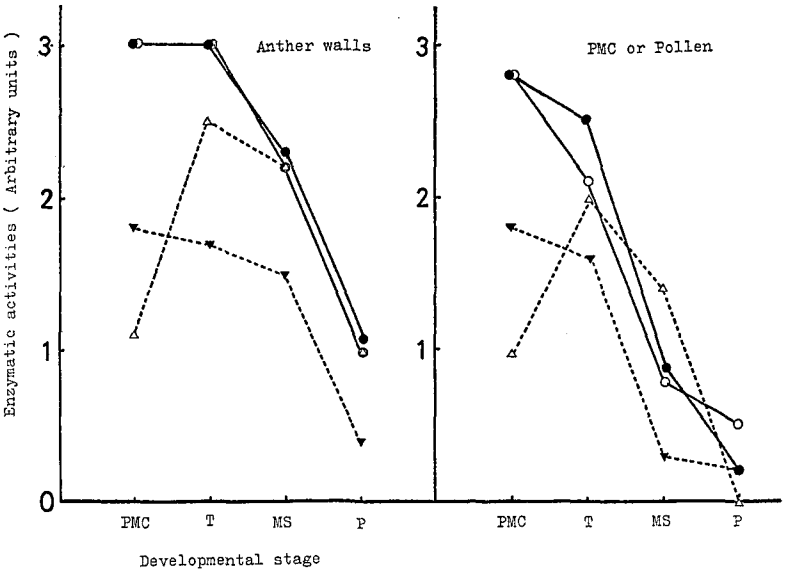


Fig. 5. Succinic dehydrogenase activities of sugar beet anthers. Symbols are the same as Fig. 4.

physiological activities. The tapetal cells were large in size, and the tapetal cytoplasm showed a deep yellow color indicating the existence of abundant proteins. The tapetal nuclei were slightly stained by a greenish blue color responsible for contents of DNA. At the same time, the size of anther and the thickness of anther wall were measured. The results were shown in Table 5.

Data obtained in enzyme experiments were shown in Table 6, 7, Fig. 4, 5, and Plate IV. There were marked increases in peroxidase activities in both fertilities up to microspore stage, and then there was a decrease in intensity in fertile anther, while peroxidase activity in sterile anther remained almost constant. Succinic dehydrogenase activities were decreased gradually or fluctuated irregularly. This activity was much more intense in fertile anthers than in sterile anthers. The localization of peroxidase activities was observed mainly in endothecium and middle layer, but that of succinic dehydrogenase was observed in tapetum, and PMC or tetrad.

B. Experiment by Radioisotope as a Tracer

Materials and Methods

In this experiment, ^{14}C and ^{3}H were used as the tracers. The fates of photosynthates and nucleic acid precursor were traced in both types of fertility by means of radioisotopical technique with ^{14}C and ^{3}H. The materials used were TA-1-CMS, male sterile line and its 0 type TA-1-0. The amount of the translocation of photosynthate and the incorporation of nucleic acid precursor to anther tissue were examined. $^{14}CO_2$ (about 1.5 mCi) was fed

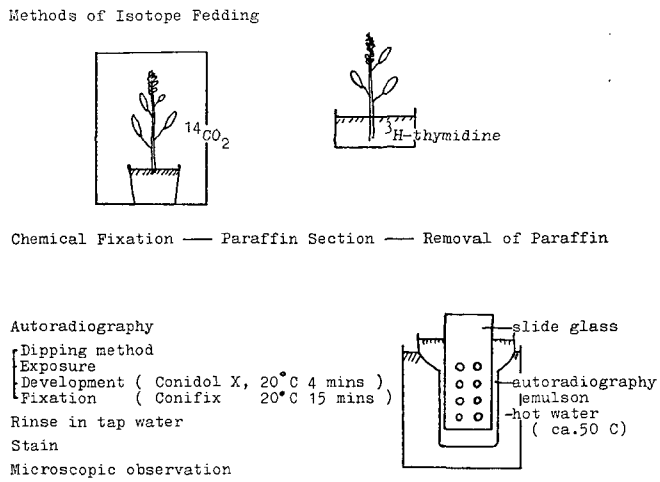


Fig. 6. Procedures of microautoradiography.

to both fertile and male sterile plants, and one day after $^{14}CO_2$ was fed, the anthers in various developmental stages were sampled, dryed and assayed for ^{14}C. Flowers of both fertilities were fixed in FAA, dehydrated in an ethl-butyl alcohol series, and embedded in paraffin in the usual manner. Sections were cut to thickness of 15 μm and microautoradiogram were made by means of dipping method (Fig. 6). In addition to this, ^{3}H-thymidine was applied through the stalk. In this study, annual cytoplasmic male sterile line TA-2-CMS and its 0 type TA-2-0 were used. These inflorescences were cut and put into ^{3}H-thymidine solution. Microautoradiogram was made the same as above.

Results

The amount of translocation of the assimilate at each developmental

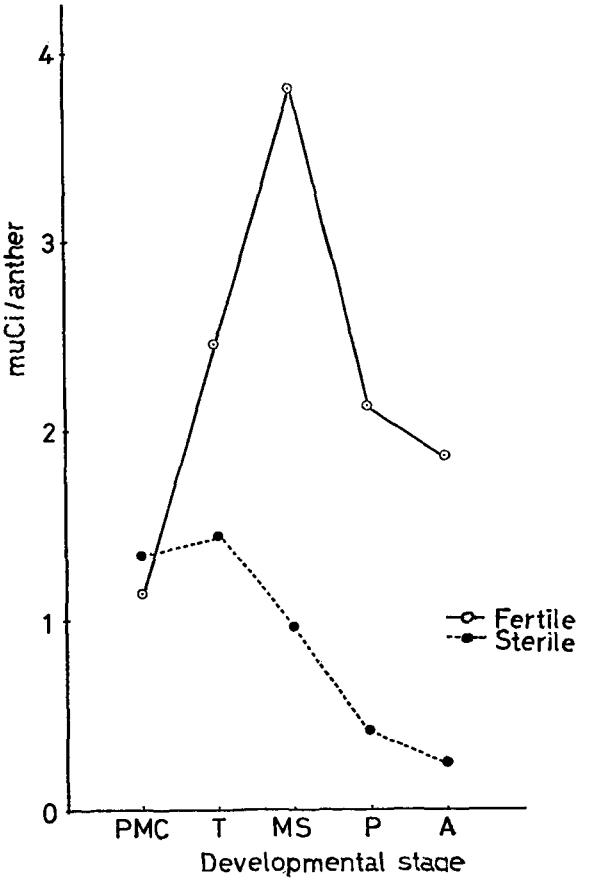


Fig. 7. Amount of ^{14}C incorporated into sugar beets anther.

stage in both fertile and sterile anthers was measured (Fig. 7). The amount of translocation of the assimilate into sterile anthers was less than that of fertile anthers. It was found to be the highest at microspore stage of fertlie anthers. Distribution of the assimilate into various tissues at each developmental stage was shown in Table 8, and the autoradiogram was shown in Plate V-1, 2. The autoradiogram showed that at an early stage of development silver grains were distributed equally over the all tissues in both types of fertility, while at the microspore stage the difference between fertile and sterile anthers became obvious. At the same stage, a large amount of ^{14}C-assimilate existed in fertile anther. The sterile anther showed a conspicuous ^{14}C-assimilate in tapetal cells showing morphological abnormality, but not in microspores. At pollen stage, it was obvious that fertile pollen grains have much more ^{14}C-assimilate than sterile anther in which no ^{14}C-assimilate could be detected. The microautoradiograms of ^{3}H-thymidine experiments were shown in Plate VI. The localization of ^{3}H was detected easily in the nuclei of cambium of seed stalk, peripheral cells of ovary, and anther filament in both types of fertility. The localization of ^{3}H in tapetal cells was detected at tetrad and microspore stages during the development of fertile anther, but could not be detected at any developmental stages of sterile anther. At early stages of anther development there were no clear differences in both types of plant between the amount of translocation of ^{14}C-assimilate and that of the incorporation of ^{3}H-thymidine into the nucleus. However at microspore stage the amount of ^{14}C-assimilate translocated into anther showed

TABLE 8. Distribution of ^{14}C-compound in anther tissue of fertile and sterile sugar beets

tissue		Developmental stage of anther			
		PMC	T	MS	P
PMC or Pollen	F	++	++	+	+
	S	++	++	±	
Tapetum	F	++	++	++	
	S	++	++	+	
Anther wall	F	++	++	++	±
	S	++	++	++	−
Parenchyma	F	++	++	++	+
	S	++	++	+	±

Symbols are the same as Table 4.

quite a difference between both types of fertility (Fig. 7). Results obtained by autoradiography did not show such a clear-cut difference (Table 8). This is because the amount of ^{14}C-assimilate measured was obtained from all ^{14}C in whole anther tissue, but microautoradiographic measurements only showed the results of materials which do not melt away by a serial method. Although many silver grains were observed in microspore of fertile plant, in sterile plant no silver grain was found. As the author previously recognized the remaining of starch grains in anther wall of sterile plant, it was presumed that the supply of assimilate from anther wall to microspores were quite obstructed. This obstruction was closely associated with the ab normal metabolic activities of tapetal cells. Though a small amount of translocates though filament were observed in sterile anther at microspore stage, it quite decreased at pollen stage, and at this stage the anther shape was flat. This showed that the metabolic activities of sterile anther were almost inhibited so that anther did not act as sink. The author assumed that abnormal features of anther filament was due to the results of sterility, but not the cause. Though tapetal cells had already disappeared at pollen stage, many silver grains were found in fertile pollen. It was estimated that the assimilates were translocated into pollen grains without the mediation of tapetum. Moreover ^{3}H-thymidine was translocated to the anther filament in both types of fertility, but incorporation of ^{3}H-thymidine into tapetal cell nucleus were observed only in fertile anther. However, based on the histochemical observations, there also many nuclei in sterile tapetal cells. It suggested that even in sterile tapetal cells DNA synthesis occured. From these observations, the differences of ^{3}H-thymidine incorporation into tapetal nuclei were caused by the decreased translocation of thymidine or less activities of DNA synthesis in sterile anther. It was presumed that abnormal DNA synthesis brought about the disturbance of genetical information and resulted in microspore degeneration.

C. Electron Microscopic Experiment

The author attempted to examine the morphological differences of tapetal cell organella between fertile and sterile plants. At the same time, since there have been a few reports that cytoplasmic male sterility is controlled by virus, the author expected to obtain some evidence whether such is the case.

Materials and Methods

Anther tissues of cytoplasmic male sterile sugar beets, TA-1-CMS, and those of corresponding 0 type, TA-1-0, were observed under microscope.

Anthers of various developmental stages were fixed with 2% glutaladehyde and 1% osmic acid. Samples were dehydrated in an ethyl-propylen oxide series, and embedded in SPURR's[109] low biscosity embedding media. Ultra thin sections were stained with uranyl acetate and lead citrate, and observed under electron microscope.

Results

Electron microscopic photograms of anther tissues were shown in Plate VII. Both fertile and sterile tapetal cell of PMC stage (Plate VII-1-1) have nuclei (N), mitochondria (M), plastids (P), ribosome (R) vacuoles (V) and so on. At this stage, no clear difference was observed. As the stage advanced to tetrad (Plate VII-1-2), organelles assumed to pro UBISCH bodies[119] observed at inner tapetal cell walls of anther locule side. Electron dense materilas (d m) were also observed between tapetum and endothecium, between tapetal walls, and in anther locule. It was presumed that the materials were closely related with degeneration of middle layer. At this stage, endoplasmic reticulum (ER) were developed and ribosome (R) were scattered in crowd. Male sterile tapetal cells were closely similar to fertile ones. After that (Plate VII-1-3), tapetal cell walls and cristea of mitochondria almost disappeared in sterile anther. A clear-cut difference between fertile and sterile anther was observed. In fertile plants of microspore stage (Plate VII-2-1) callose wall, tapetal cell wall and electron dense materials had already disappeared, and sporopollenin was deposited at pollen exine, but pollen intine was not formed. In fertile tapetal cells of this stage, many UBISCH bodies were developed, few plastids and many mitochondria were observed. In sterile tapetal cells of microspore stage, UBISCH bodies were not observed in the tapetal cytoplasm, and mitochondria had already disappeared. As the anther advanced (Plate VII-2-2), in fertile anther, tapetal cells were collapsed, tapetal mitochondria were released in anther locule, and mircospore formed exine and intine. On the other hand, in sterile anther, tapetal mitochondria were collapsed, plastids were seen in anther locule, and microspore had poor exine and no intine. Furthermore, peculiar granular substances (○) were gathered at germ pore of fertile microspores, but not in sterile microspores. From these results, the process of degeneration of tapetal cell was inhibited in sterile anther. It was presumed that male sterility of sugar beets inherited cytoplasmically, was closely related to the abnormal behavior of UBISCH bodies and mitochondria.

IV. Maize

A. Histochemical Experiments

Materials and Methods

Changes of carbohydrate, components of amino acid and activities of enzymes in anther tissues were studied. Cytoplasmic male sterile maize $W23^T$ and its maintainer W23 were used in carbohydrate and amino acid experiments. Electrophoretic studies of various enzymes were made using the acrylamid. Three maize hybrids, WF9×W22, having normal cytoplasm, $WF9^T \times W22$ having T type cytoplasm and $WF9^C \times W22$ having C type cytoplasm, were used in this experiment.

Results

Carbohydrates and amino acids were studied by the same methods as those for sugar beets. Reducing sugars in anther tissues increased gradually till the microspore stage, and then maintained its level or tended to decreased until anthesis (Table 9). Starch grains in fertile anthers increased till the tetrad stage, and then decreased till microspore stage, and after that, they

TABLE 9. Reaction of reducing sugar with Fehling's reagent in maize anthers during their developmental stages

Type of fertility	Recation	Developmental stage of anther							
		PMC		T		MS		P	
		I	II	I	II	I	II	I	II
Fertile	0		1						
	1	3	5						
	2	6	2		4			1	
	3	6	2	3	3	6		2	7
	4			8	3	32	20	12	11
	ave	2.2	1.5	3.7	2.9	3.8	4.0	3.7	3.6
Sterile	0	5		2					
	1	2	6	4	1	1		4	1
	2	2	4	3	3	9	4	4	1
	3			8	3	13	9	6	7
	4			4	3		7	1	11
	ave	0.8	1.4	2.4	2.8	2.5	3.2	2.3	3.4

I: 1966
II: 1967
Symbols are the same as Table 1.

TABLE 10. Starch reaction with IKI in maize anthers during their developmental stages

Type of fertility	Reaction	Developmental stage of anther							
		PMC		T		MS		P	
		I	II	I	II	I	II	I	II
Fertile	0						6		
	1		6				20		
	2	11	14	3	5	5	25		
	3	5	1	3	15	14	8		2
	4			9	20	19	1	16	28
	ave	2.3	1.8	3.4	3.4	3.4	1.6	4.0	3.9
Sterile	0		1				2	10	16
	1	1	6			1	11	6	32
	2	11	3	2	18	16	23		2
	3	1		6	28	36	4		
	4			12	8	7			
	ave	2.0	1.2	3.5	2.8	2.8	1.7	0.4	0.7

Symbols are the same as Table 9.

increased again at pollen stage. In sterile anther they increased till tetrad stage and then decreased rapidly (Table 10). The behavior of starch grains in maize was different from that of sugar beets. The localization and changes of starch grains in anther tissues were shown in Plate VIII. At early stage of anther development, a large amount of starch grains were found mainly in endothecium and conective. As anther developed, the amount of starch grains disappeared in anther walls of both types of fertility, but fertile pollem grains showed a prominent IKI reaction. Paper chromatography of free amino acids in anthers showed that the nearly matured anthers in fertile plants had the proline, while sterile anther did not have this amino acid. Enzymes such as glutamic dehydrogenase, esterase, malic dehydrogenase, acid phosphatase and peroxidase were examined by electrophoresis (Fig. 8 and Plate IX). Enzymatic banding pattern of esterase and acid phosphatase were obviously different among three hybrids. For glutamic dehydrogenase, enzymatic banding pattern was not obvious, but there seemed to be differences among hybrids. Activity of this enzyme was the weakest in C type. In contrast to this, peroxidase activities more intense in sterile anther than in fertile anther, especially for C type of sterility.

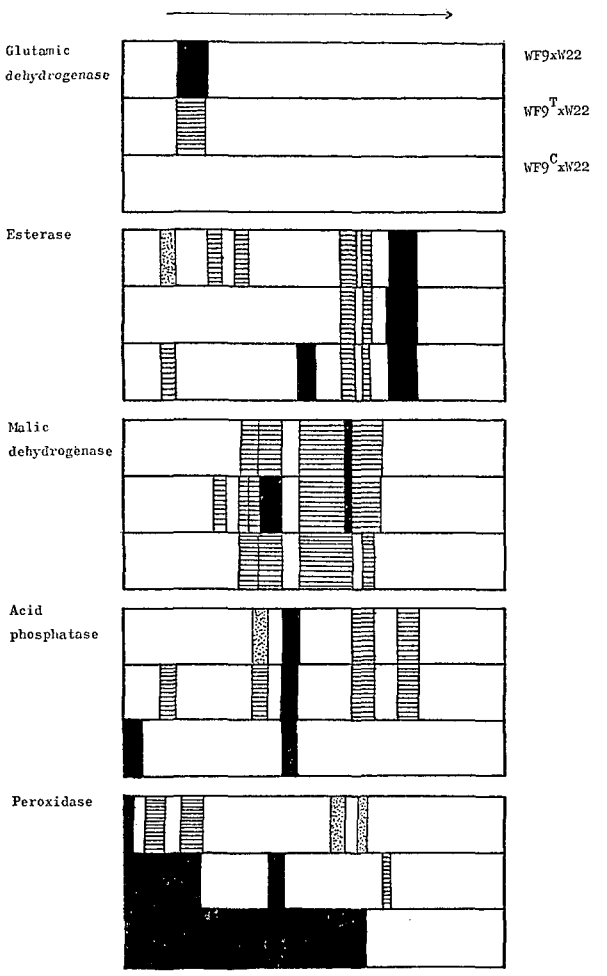


Fig. 8. Electrophoreic banding pattern of isozymes of various enzymes in crude homogenates prepared from maize anthers having different cytoplasms.

B. Respirations of Anther and Distribution of ^{14}C-assimilate in Plant Organs

Previous experiments suggested that metabolic activities were quite decreased in male sterile anther. This experiment was carried out to find what pathways in metabolics were inhibited.

Materials and Methods

For the study of the respiration of anther, W23^{T}, cytoplasmic male

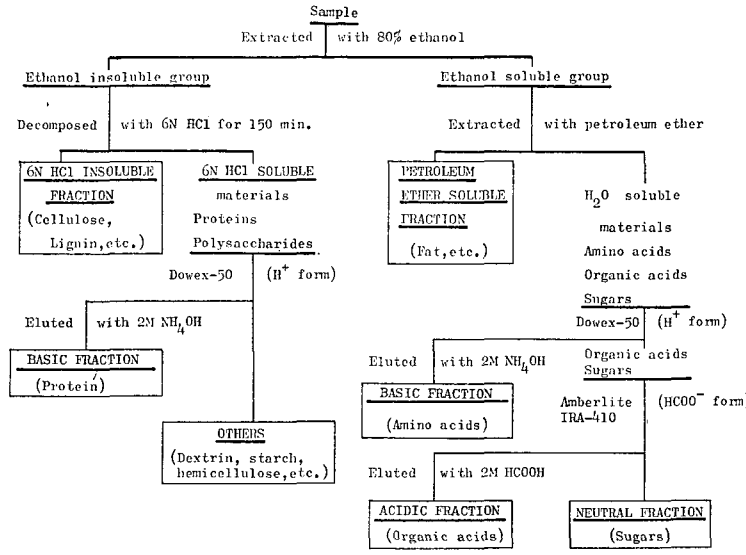


Fig. 9. Scheme for the isolation of ^{14}C-labeled components[63].

sterile line having T type cytoplasm, and W23, maintainer, were used in this experiment. Anthers at three developmental stages such as early microspore, late microspore and pollen stages were collected. Respiration rate was measured by using Warburg's manometolic method at 30°C with 100 anthers. In another experiment to find the efficiency of the distribution of ^{14}C-assimilate in plant organs, $W23^T$, $W375B^T$ and their maintainer W23 and W375B were used. At about the microspore stage, inflorescence of two types were cut and put in water. One day after $^{14}CO_2$ was fed, various organs were sampled, dryed, ground, and assayed for ^{14}C. The scheme for the isolation of ^{14}C-labeled components were shown in Fig. 9[63]. Basic fraction of both ethanol soluble and insoluble groups of anthers in W23 lines were fractionated by paper chromatography. Nutral fractions of ethanol soluble group of anther, spikelet and rachis of W375B lines were also fractionated by paper chromatography. After developing, autoradiography were made using X ray film.

Results

Until microspore stage no difference in amount of respiration in anther between fertile and sterile plants was found, but as anther development proceeds, a clear differece was detected; indicating that sterile anther showed decreased respiration (Fig. 10). The amount of respiration in sterile anthers was about 1/3 of that in fertile anthers. The amount of translocation of

^{14}C-assimilates and the distribution percentages of each fraction were shown in Table 11. At about microspore stage, there were larger amounts of ^{14}C-assimilate in fertile anthers than in sterile anthers. The distribution percentages of the primary metabolite such as sugars, amino acids and organic acids fractions were about 40% and 60% for fertile and sterile anther respectively. On the other hand, those of the secondary metabolites such as alcohol insoluble fractions, fat and others were about 60% and 40% for fertile and sterile anthers respectively. The distribution percentages of sugars and amino acids were larger, but those of organic acids tended to be smaller in sterile anthers than in fertile anthers. Furthermore, in sterile anthers, ethanol insoluble and 6N HCl insoluble fraction (cell wall fraction) showed high distribution percentages, but proteins and fats fractions showed lower values. In spikelet (without anthers), there were clerar differences in amount of translocation of ^{14}C-assimilate between both fertilities. The distribution percentages in spikelet were not similar to those of anthers. In rachis, there was no clear differece in amount between both fertilities. The amount of translocation of ^{14}C-assimilate and distribution percentages were shown in Table 12. The amount of translocation increased in the following order, leaf brade<leaf sheath<internode, this tendency was the same in both types of fertility. The paper chromatographic analyses of amino acids and amino acid residues in anthers were shown in Table 13. As reported previously, proline was not detected in sterile anthers, but proline residue was found in anthers of both fertilities. The number of ^{14}C-labeled amino acid was 6 in fertile anther and 2 in sterile anther. The number of ^{14}C-labeled amino acid residues was 8 in fertile anther and 0 in sterile anther. Autoradiography of sugar fraction was shown in Plate X. It was detected that main components of sugars were sucrose, glucose and fractose. Maltose was exist

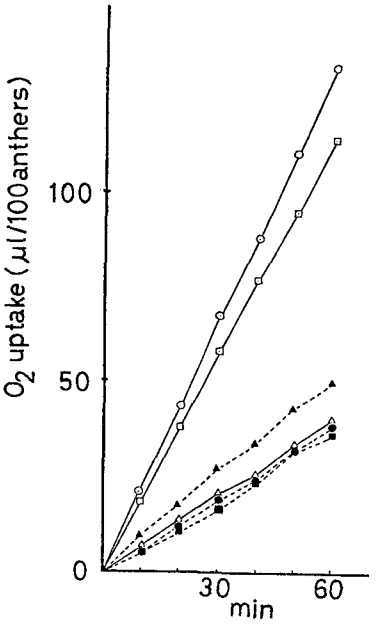


Fig. 10. Respiration of maize anthea.

—○— fertile pollen
—□— microspore
—△— young microspore
···●··· sterile pollen
···■··· microspore
···▲··· young microspore

only in fertile anther, but was not detected in pollen grains of maize. The incorporation of ^{14}C into fat fraction was observed in fertile anther, but not in sterile anther. The translocation of ^{14}C-assimilate per dry weight of each organ in fertile plants was increased in the following, anther>spikelet>rachis, but in those of sterile plants the reverse relationship was observed. It was

TABLE 11. The distribution of ^{14}C incorporated into fertile and sterile anthers, spikelets and rachis

	Anther		Anther		Spikelet		Rachis	
	W23	W23^T	W375B	W375B^T	W375B	W375B^T	W375B	W375B^T
Total (mμCi/mg. dry. wt.)	51.3	3.6	55.7	4.8	21.4	9.4	11.3	15.5
Ethanol sol.	69.2(%)	69.4	68.4	75.0	62.1	57.0	60.2	71.5
Neutral Fraction	18.7	30.6	10.1	31.3	30.4	33.3	39.0	54.2
Acidic Fraction	11.7	5.6	11.3	8.3	8.9	6.5	6.2	7.1
Basic Fraction	8.4	22.0	16.0	20.8	8.9	8.6	5.3	5.2
Petrolium Ether Fraction	9.0	0.0	4.1	0.0	0.4	0.0	0.0	0.0
Others	21.4	11.2	26.9	14.6	13.5	8.6	9.7	4.5
Ethanol insol.	30.8	30.6	31.6	25.0	37.9	43.0	39.8	29.0
6N HCl sol.								
Basic Fraction	9.0	2.8	11.9	4.2	3.7	2.2	1.8	1.3
Others	19.9	22.2	15.4	12.5	17.8	16.1	19.4	12.2
6N HCl insol.	1.9	5.6	4.3	8.3	16.4	24.7	18.6	15.5

TABLE 12. The distribution of ^{14}C incorporated into fertile and sterile maize plant

	1st Leaf Blade		1st Leaf Sheath		1st Internode	
	W375B	W375B^T	W375B	W375B^T	W375B	W375B^T
Total (mμCi/mg. dry. wt.)	5.1	8.4	34.1	57.0	43.4	83.5
Ethanol sol.	58.8(%)	65.5	32.0	47.5	40.1	41.7
Neutral Fraction	13.7	37.0	17.3	16.8	17.1	15.3
Acidic Fraction	3.0	8.3	3.0	5.6	3.9	4.9
Basci Fraction	4.7	11.9	4.7	9.3	10.1	11.4
Petrolium Ether Fraction	—	—	—	—	—	—
Others	25.5	8.3	7.0	15.8	9.0	10.1
Ethanol insol.	41.2	34.5	68.0	52.5	59.9	58.3
6N HCl sol.						
Basic Fraction	6.0	6.0	3.5	3.2	2.5	3.8
Others	17.6	16.6	21.1	21.6	13.8	22.3
6N HCl insol.	17.6	11.9	43.4	27.7	43.6	32.2

Table 12. (Continued)

	2nd Leaf Blade		2nd Leaf Sheath		2nd Internode	
	W375B	W375B^T	W375B	W375B^T	W375B	W375B^T
Total (mμCi/mg. dry. wt.)	11.8	12.4	23.8	36.9	83.3	121.7
Ethanol sol.	53.4(%)	49.2	41.6	37.7	36.9	53.2
Neutral Fraction	29.7	16.1	21.4	17.9	4.8	13.1
Acidic Fraction	9.3	9.7	5.0	3.5	3.1	6.2
Basic Fraction	12.7	12.9	7.2	6.3	24.5	20.3
Petrolium Ether Fraction	—	—	—	—	—	—
Others	1.7	10.5	8.0	10.0	4.5	13.6
Ethanol insol.	46.6	50.8	58.4	62.3	63.1	46.8
6N HCl sol.						
Basic Fraction	11.9	11.3	5.5	5.4	7.2	6.3
Others	16.9	20.2	15.1	20.3	31.0	27.4
6N HCl insol.	17.8	19.3	37.8	36.6	24.9	13.1

TABLE 13. The detection of amino acids and amino acids residues

	Non-radiocative				Radioactive			
	Free Amino acid		Fraction of Protein		Free Amino acid		Fraction of Protein	
	W23	W23^T	W23	W23^T	W23	W23^T	W23	W23^T
Leucine	+	+	##	##	±	−	#	−
Phenylalanine	±	+	#	#	−	−	#	−
Valine; Methionine	+	#	##	##	+	−	+	−
Tryptophan	±	+	+	+	−	−	+	−
Proline	#	−	+	+	+	−	−	−
Alanine	##	#	##	##	#	#	#	−
Glycine; Serine	+	#	+	+	#	−	+	−
Glutamic acid	+	+	+	+	+	+	−	−
Histidine	±	+	+	+	−	−	+	−
Aspartic acid	±	+	+	+	−	−	+	−

Spmbols are the same as Table 4.

considered that the obstruction of translocation of nutrient into sterile anther tissue followed the abnormal behavior of tapetum and abortion of pollen. From this study, the translocation of assimilate from rachis to spikelet was also obstructed. It is well known that the translocation of assimilate is obviously large in amount in young tissues and organs being under development and differetiation. From the point of view of the relationship between sink and source, it was presumed that no difference was found in the photo-

synthetic potentiality of leaves of both types of plant, but the amount of translocation of ^{14}C-assimilate into the flower parts was much different between both types of fertility. Since the tassel of maize did not have female organ, these phenomena demonstrated the capacity of anther to accept the photosynthetic products. From the chemical constituents of anthers it was speculated that in fertile anther the second metabolic products were easily synthesized from the primary metabolic products, but in sterile anther this potenciality was obstructed. Though the amount of translocation of ^{14}C-assimilate is remarkably different between the two types, there were many problems to compare only distribution percentages of constituents. In comparison to fertile anther, it was found that in sterile anther there were high distribution percentages in sugars and amino acids similar to polysaccharides, and low distribution percentages in proteins and organic acids fractions. Because distribution percentage of amino acid was high, but protein distribution percentage was low, and that of orgainc acids which was closely associated with mitochondrial fraction was low, it suggested that these phenomena demonstrated the obstruction of protein synthesis and low respiration in sterile anther. Furthermore, since there were few ^{14}C-labeled amino acids and no ^{14}C-labeled amino acid residues in sterile anther this also suggested that, in this stage protein synthesis was completely obstructed. The organs except anther and rachis showed no common tendency in distribution percentages between organs and between fertilities. From the above mentioned data, the following points can be speculated; at the microspore stage of sterile maize various metabolic pathways in anther are obstructed, and associated with this little amount of assimilation products is translocated to flower parts.

V. Sorghum

In this chapter, sorghum, autogamous plant, was studied. Cytoplasmic male sterility of sorghum was actually used in plant breeding.

A. Changes of Carbohydrates and Amino Acids in anther tissues

Materials and Methods

In this study, Combine Kafir 60 (A-line), cytoplasmic male sterile line and Combine Kafir 60 (B-line), its maintainer, were used. Changes of quantity of reducing sugars and starch grains during mircosporogenesis were examined with the same methods as used in sugar beets and maize reported previously. Free amino acids in anther tissues were also examined by paper chromatography.

Results

The results of reducing sugar examination are shown in Table 14. Until microspore stage of anther development, reducing sugars were increased in

TABLE 14. Reaction of reducing sugar with Fehling's reagent in sorghum anthers during their developmental stages

Type of fertility	Reaction	Developmental stage of anther							
		PMC		T		MS		P	
		I	II	I	II	I	II	I	II
Fertile	0	10	6	2	4				
	1	5	14	8	12	1	2	1	2
	2		17	3	20	4	10	5	15
	3		2	1	3	10	20	16	2
	4						7	9	
	ave	0.3	1.4	1.2	1.6	2.6	2.9	3.1	2.0
Sterile	0	9	9	5					
	1	5	7	10	8		1		5
	2	1	13	1	11	7	10	8	9
	3		5		18	8	16	11	6
	4				1		11	12	
	ave	0.5	1.5	0.8	2.3	2.5	3.0	3.1	2.1

Symbols are the same as Table 9.

TABLE 15. Starch reaction with IKI in sorghum anthers during their developmental stages

Type of fertility	Reaction	Developmental stage of anther							
		PMC		T		MS		P	
		I	II	I	II	I	II	I	II
Fertile	0								
	1					2	14		
	2			2	13	7	24		
	3	2	13	13	18	6	2	2	
	4	13	27		9			27	20
	ave	3.9	3.7	2.9	2.9	2.3	1.7	3.9	4.0
Sterile	0						4	1	9
	1	1		2	6		18	13	11
	2			4	27	8	18	18	
	3	8	16	9	7	6			
	4	7	24			1			
	ave	3.3	3.6	2.5	2.0	2.5	1.4	1.5	0.6

Symbols are the same as Table 9.

reaction in accordance with the anther development. Starch grains detected by IKI in whole anthers tended in both types to decrease until microspore stage, as shown in Table 15. In sterile anthers, reaction of IKI continued to decrease, but in fertile anther, it increased as well as in maize. This is because of accumulation of starch in pollen grains. The photograms of changes and localization of starch grains in anther tissues were shown in Plate XI. At early stages of anther development, a large amount of starch grains were seen in endothecium and connective in both types of plant. As anther development proceeded, starch reactions in anther wall disappeared in both types of plant. The results of free amino acids by paper chromatography showed that proline could be detected only in fertile anther at pollen stage. Microspores were covered with callose wall in fertile plant, but not in sterile plant.

VI. General Discussion

Genetic male sterile plants have normally functioning female organs. Namely, male sterile plants undergo the normal macrosporogenesis and the abnormal microsporogenesis. It is apporopriate to conclude that stamen, anthers and filaments, showed abnormal behavior. The author observed the differences between fertile and cytoplasmic male sterile plants, such as sugar beets, maize and sorghum, from the point of view of morphology and physiology. The author attempted to discuss the following male sterile factors.

1) Abnormal behavior of the tapetal cell

In all crops examined in this study, abnormal behavior of tapetum was accompanied with male sterility. Under unfavorable conditions of environment such as temperature, light, moisture, gametocide and irradiation, male sterility was often brought about, with which the abnormal behavior of tapetum was accompanied. Thus it can be concluded that the most common phenomena in male sterility are tapetal abnormality, although according to TOKUMASU[118], and CHE *et al*[18]. a few species such as *Pelargonium* and rice plant do not show the abnormal tapetum. However, up to the present, the role of tapetum in microsporogenesis has not been satisfactorily clarified. The chief function of tapetum has been supposed to be the production and transport of enzymes, food materials, and growth substances for developing pollen mother cells and pollen grains. There are two types of tapetum ;[77,120] a) Secretary or Glandular tapetum ; the tapetal cell remain intact and gradully absorbed *in situ*. b) Amoeboid or Plasmodial tapetum ; the tapetal cell walls break down and the tapetal protoplasts invade the central portion of anther sac and fuse to form a composite mass of tapetal periplasmodium in which

developing young microspores are bathed. Sugar beets, maize and sorghum studied here have the secretary tapetum, and both tapetal cells of fertile and sterile of these plants collapse in different way. This collapse is an abnormal phenomenon for cell development, so it is difficult to explain the cause of male sterility to the collapse of tapetal cells. Physiological and morphological differences during the collapse of tapetum in both fertile and sterile plants must be compared in order to clarify the cause of male sterility. From the data in Table 5, it is apparent that tapetal cells in sterile anther show the collapse of tapetal cells but not the hypertrophy. From the results of enzymatic activities, succinic dehydrogenase activities are remarkable in tapetal cells and its activities are more intense in fertile than in sterile anther. Based on the results of distribution of ^{14}C-assimilate in anther tissues, a fairly large amount of ^{14}C-assimilate is translocated in abnormal tapetum of sterile plants (Plate V-2). Examination of the ultrastructure of tapetal cells by electron microscope showed that UBISCH bodies did not develop and mitochondria became abnormal in tapetal cells of sterile anthers. It is presumed that these phenomena in abnormal tapetal cells are responsible for pollen sterility. According to ECHLIN[29], UBISCH bodies are speroidal structure in anther of many genera of angiosperms, both monocotyledons and dicotyledons, and some gymnosperms. They occur in large numbers on the walls of the tapetal cells, especially those lining the anther loculus. The walls of the coating of UBISCH bodies apparently consist of sporopollenin the main constituent of the mature microspore exine[9], and a material of considerable durability and characteristics resistance to acetolysis. The development and function of UBISCH body is not obvious, but it has been supposed to be formed during the collapse of tapetal cells. Exine of microspore in sterile anther is not completely formed (Plate VII-2). In sugar beets, that the UBISCH bodies did not develop in sterile anther was closely associated with male sterility. However this phenomenon has not been found in wheat[23], sorghum[91,122] and capsicum[56] Mitochondrion which is one of the important components of cell showed abnormal features in cells of sterile anther. In sugar beets, normal mitochondria were not found in sterile tapetal cells after microspore stage. This seems to be responsible for the decrease in oxygen uptake in the sterile anther of maize (Fig. 10).

2) Structural changes of the stamen filament

In the study of translocation of ^{14}C-assimilate into the anther tissues, the amount of traslocation was less in sterile anther than in fertile anther (Fig. 7). In the observation of microautoradiography, many silver grains which show the existence of ^{14}C were observed in pollen tetrad and in anther walls

of both types of fertility till microspore stage, but in microspore itself, less silver grains were found in sterile plant than in fertile plant (Plate V-1, 2). It suggested that sterile anther did not act as sink. Thus it was presumed that this decrease of translocation was not caused by the abnormal bascular bundle system in sterile filament. This presumption was supported by the facts that translocation of ^{14}C-assimilate into spikelet of sterile plant was markedly decreased in amount, and incorporation of ^{3}H-thymidine was found in filament of sterile plant. On the base of these experiments, it is concluded that the abnormality of filament was the result, but not the cause of pollen sterility.

3) Callose dissolution

As far as the present study of sorghum is concerned, fertile microspores were covered with callose wall, but not in sterile microspores as in petunia[64]. This phenomenon was not found in sugar beets and maize. Furthermore, in morphological observation of sugar beets anther under electron microscopic level, there was no difference in existence of callose wall between two types of fertility. It was concluded that the relationships between callose wall of microspore and male sterility differ from species to species.

4) Virus

Electron microscopic observations by EDWARDSON[32] showed that root tip cells of cytoplasmic male sterile corn had the inclusions and similar inclusions were seen in tapetal cells of sterile and maintainer lines. According to BRBWBAKER[82], 'an intriguing possibility emerges from electron microscopic observations of unique cytoplasmic inclusions in cytoplasmically sterile plants. It is supported that these inclusions are virus particles that survive only in plants with a virus-susceptible genotype. The virus lives symbiotically in diploid somatic cells.' According to his theory, complete male sterile type can not be expected to appeare in F_2 generation, but in sugar beets, complete male sterile types were found in that generation. Furthermore in the present study of sugar beets, virus or inclusion could not be detected in anther tissues, with electron microscopy. FUKASAWA and NISHIYAMA[43] reported the differences in histone of young spikes between fertile and sterile types of wheat. And ALAM and SANDLAL[2] reported the total protein and the basic protein of anther by electrophoresis. Evidence from their study for basic protein in male sterile sudangrass suggested that histone might be repressing the regulator gene(s). This could cause the failure of the synthesis of some specific protein(s) and subsequently resulting in pollen degeneration. In the present ^{3}H-thymidine fedding experiment, silver grains due to

^{3}H were detected in fertile tapetal nuclei, but not in sterile ones. This may suggest less translocation of ^{3}H-thymidine or timing lag of synthesis of nucleic acid. This difference may induce the disturbance of genetic information, and result in the physiological and morphological abnormalities associated with male sterility. The abnormal behavior of tapetal cell was closely related with that of mitochondria (Plate VII-1). Evidence of the genetic continuity of the mitochondria comes from observation of poky in *Nurospora*, and petite in yeast. SHAH and LEVINGS[104,105] reported that the buoyant densities and molar % GC of the chloroplast DNAs and mitochondrial DNAs were identical for the normal and cytoplasmic male sterile maize hybrids. They concluded that the techniques used in their investigation had limited resolution, therefore, if the differences between the two cytoplasms were due to minute alternations in their DNAs, such mutation would remain undetected. Cytoplasmic factors controlling male sterility were investigated exclusively, but the evidence for genetic factors was not clear. There are other investigations by means of grafting, inculation or thermo treatment which insist that cytoplasmic factors are viruses or virus like factors. Conversely there are many experiments that do not support the virus nature. In this present experiment, the author could not support the virus nature.

5) Differences in amino acid compornent, especially the existence of proline

In many investigations, proline at pollen stage was not dected in sterile anther. In the studies on sugar beets, maize and sorghum, similar results were obtained. From this, it was easily presumed that amino acid constituents were different between both types of fertility, and amino acid metabolism was disturbed in sterile plant. Paper chromatographic investigation of ^{14}C-assimilate into maize anther revealed 6 ^{14}C-labeled amino acids in fertile in contrast to 2 in male sterile, and 8 ^{14}C-labeled amino acid residues in fertile in contrast to 0 in male sterile. Proline residue was detected in even in sterile anther. HOSOKAWA *et al.*[59,60] reported that proline was not detected in both types of fertility by pollen stages, and was detected only in fertile anther at pollen stage and anthesis. From this, it was concluded that deficiency of proline in sterile anther was the result but not the cause of sterility.

6) Enzymatic activities

It was found that peroxidase activities were more intense in sterile anther than in fertile anther at the later stage of development in sugar beets and maize. On the other hand, succinic dehydrogenase activities were more intense in fertile sugar beets anther than in sterile anther, and malate dehy-

drogenase and glutamic dehydrogenase activities were more intene in fertile maize anther than in sterile anther. These decreased enzymatic activities were supposed to be closely associated with the abnormality of mitochondria.

From the point of view of metabolism, primary metabolisms such as photosynthesis, glucolysis, TCA cycle and amino acid synthesis might be considered at first. Photosynthesis is mainly carried out in the leaves. Plant height, plant weight and yielding ability are similar between both types of fertility, so no difference in photosynthetic potential can be expected between both types. From the investigation of translocation of ^{14}C-assimilate of maize, in male sterile organs except for anther and spikelet, there were large amount of translocate in comparison with fertile organs. From the point of view of primary metabolism, it was presumed that male sterility was closely related with the disturbance of TCA cycle, because of less distribution percentages of organic acid fraction of ^{14}C-assimilate, low respiratory activity, morphological abnormality of mitochondria and less enzymatic activities such as succinic dehydrogenase, malic dehydrogenase and glutanic dehydrogenase. It was easily presumed that this abnormality of TCA cycle was closely associated with the disturbance of glycolysis and amino acid metabolic products, such as cellulose, lignin, starch, polysaccharide and nucleic acid, and it appeared that there was not such a prominent difference between fertile and sterile plants. For protein and fat, more distribution percentages of ^{14}C-assimilate were detected in fertile plants than in sterile plants. Further more, decolorization of male sterile anther suggested less synthesis of carotinoid. It was noticed that clear differences between both types were mainly observed in protein and fat fractions which were derived from amino acids and organis acids. From histochemical observation, semi quantity of starch grains, polysaccharides, proteins, fats and nucleic acids were found to be similar in both types at early stage of developments. These facts suggested that the metabolic pathways in male sterile anthers were not perfectly obstracted but metabolic activities were decreased at late stage of developments. Starch grains and fats which remained in later developing stage of sterile anther suggested that synthate was not used by further metabolic pathways.

To summarize, it was concluded that cytoplasmic male sterility was closely associated with the abnormal behaviors of tapetal cells, and abnormal behavior of tapetal cells were originaly related with abnormal TCA cycle in mitochondria.

Summary

Male sterility found in many crops such as maize, sugar beet, onion, sorghum and wheat etc. has been put to practical use for hybrid seed production and genetical studies of this phenomenon have also been conducted by many research workers. However the physiological mechanism of the cytoplasmic male stertility of the plant has not been elucidated satisfactorily, and there remain numerous unexplained problems. In order to clarify the physiological causes responsible for cytoplasmic male sterility, the author conducted physiological and morphological studies on the phenomenon mainly on sugar beet, maize and sorghum.

The results obtained up to the present can be summarized as follows:

A) Sugar Beet

1) Reducing sugars in fresh anthers smeared on slide glasses were observed using Fehling's reagent under a light microscope. It was noted that in both types of fertility, the amount of reducing sugar increased graduallly as the stages of anther developed.

Starch grains in fresh anthers smeared on slide glasses were observed by means of IKI solution under a light microscope. In earlier developmental stages of anthers, a large amount of starch grains was observed in both types of fertility, and as a result of histochemical studies a large amount of starch grains was also found in the endothecium and parenchyma. At the pollen stage, starch grains were not observed in fertile anther tissues, but starch grains were seen in sterile anther tissues.

Lipids in fresh anther smeared on slide glasses were detected by means of Sundan III stain method under a light microscope. It was also noted that lipids were detected at the pollen stage of sterile anthers, but were not detected in fertile anthers.

Examinations of paper chromatography of free amino acids showed that nearly all mature fertile anthers contained proline, however it was shown that male sterile plants did not have this amino acids.

2) The diameter of anther sac, thickness of epidermis, transitory tissues and tapetum were measured. Each measurement was significantly different among the developmental stages of anthers, and the measurements of tissues were significantly different between both types of fertility except for that of the epidermis.

Applying the histochemical method of triple stains for DNA, polysaccharides and proteins, the following results were obtained. The reaction intensities of DNA, polysaccharides and proteins were apporoximately the

same in both types of fertility. At the mature pollent stage, reaction of polysaccharides in fertile plants completely disappeared, on the other hand, those of sterile plants still remained in the endothecium.

3) Flowers of various developmental stage of types of fertility were cut to a thickness of 26 μm by using a freezing microtome. Histochemical methods were used to estimate the enzymatic activities and to observe the localizations of enzymes.

Peroxidase activities were observed in the endothecium and the middle layer in both types of fertility. The enzymatic activites of both types of anther showed an increase in intensities with the progress of anther development while the enzymatic activities of sterile anthers showed a higher intensity than those of fertile anthers at late stages of development.

Succinic dehydrogenase activities were observed mainly at the tapetum and pollen mother cells or tetrads, and the enzymatic activities of fertile anthers were generally more intense than those of sterile anthers.

Acid phosphatase activities were observed in all anther tissues. The enzymatic activities of both types of fertility were not different from each other.

4) Applying the tracer method for photosynthetic products using $^{14}CO_2$, incorporation of ^{14}C into fresh anthers were measured. ^{14}C showed a higher incorporation into fertile anthers than in sterile anthers at the later developmental stages of the anthers. With the microautoradiographic method, abundant ^{14}C-compounds were observed in anther tissues of both types of fertility at the tetrad stage. At the microspore stage, hardly any ^{14}C-compounds were observed in the microspore of sterile plants, but on the other hand, abundant ^{14}C-compounds were found in those of fertile plants. The amount of ^{14}C-incorporation into normal tapetum, abnormal tapetum and pseudopodium like incursion showed difference in paraffin sectioning.

5) Applying the microautoradiographic method, incorporation of ^{3}H-thymidine into the anther was examined. ^{3}H was incroporated into the floral axis, and mainly located in the cambial zone of vascular bundle. At floral parts, ^{3}H was located at the peripheral zones of ovaries and the filaments in both types of fertility. However, ^{3}H was incorporated into the tapetal cells of fertile anthers, but they were not incorporated into those of sterile anthers.

6) Anther tissues of cytoplasmic male sterile sugar beets and those of corresponding 0-type sugar beets were observed under an electron microscope. In this case clear differences were observed between fertile and sterile anthers. At the tetrad stage, mitochondria of sterile tapetal cells became abnormal,

and the cristea of mitochondria were found to be degenerated. At the microspore stage, UBISCH bodies of fertile anthers were seen at the surface of inner tapetal cells, but they were not observed in sterile anthers. Other differences were also observed in the behavior of lipid-like materials. Numerous lipid-like materials were presented in the sterile tapetal cells at the microspore stage. At the late microspore stage, many lipid-like-droplets were observed along the outer tapetal cell membranes in fertile anthers, but they were not observed in sterile anthers. As regards the microscpore itself, peculiar unknown substances were observed at the pores of pollen in fertile microspore, but they were not observed in sterile microspores. Exine was found in the sterile microspore, but intine was not found in it.

B) Maize

7) Reducing sugars and starch grains in fresh maize anthers were detected by the same methods as used in sugar beet experiments. The amount of reducing sugars increased gradually as the stage of anther developed, and significant differences were found between fertile and sterile plants during microsporogenesis. A large amount of starch grains was observed and were located in the endothecium and parenchyma as a result of histochemical experiments. As the stages of anther developed, a gradual increase was followed by a decrease, but a large amount of starch grains remained at anthesis in the fertile pollen grains. On the other hand, male sterile plants left no traces of starch in anther tissues at anthesis.

As a result of examination of paper chromatography of free amino acids nearly all mature anthers showed proline, which could not be found in other parts of the flowers or leaves. In sterile plants, proline was not detected ever in anthers at anthesis.

8) Three hybrids of maize, which have different cytoplasms responsible for male sterility, were used for the study of the enzyme. Esterase, acid phosphatase, glutamic dehydrogenase, malic dehydrogenase and peroxidase were tested with acrylamide gel disc electrophoresis method. In each zymogram, some differences were found between the development stage of anthers and among cytoplasms. Peroxidase showed more intensity and number of bands in sterile anthers than in fertile anthers. Glutamic dehydrogenase showed only one band for all materials, but activities were highly intense in fertile anthers than in sterile anthers. With special regard to other enzymes, clear differences were not found, but activities and number of bands tended to be predominant in fertile anthers.

9) The respiration activities in sterile anthers were lower than those in fertile anthers. Using ^{14}C tracer methods, the following results were ob-

tained on the distributions of ^{14}C-assimilates. At the microspore stage, the translocation of photosynthates into the anthers and spikelet was obstructed in sterile plants. In comparison with the fertile anthers, sterile anthers showed a low distribution ratio in the acidic fraction, but a high distribution ratio was seen in the neutral and the basic fractions. In the fertile anthers, one unidentified sugar sport was detected. A remarkable differnce between fertile and sterile plants was also detected in ^{14}C amino acid residues. ^{14}C-assimilates are incorporated into the lipid fraction in the fertile anthers, but not in the sterile anthers.

C) Sorghum

10) Reducing sugars and starch grains in fresh anthers were observed by the same methods as used in sugar beet and maize experiments. The amount of reducing sugars increased gradually as the stage of anther developed, and significant differences were found between fertile and sterile plants during microsporogenesis. A large amount of starch grains was observed and they were located in the endothecium and parenchyma as result of histochemical experiments. As the stage of anther developed, they gradually decreased in sterile anther, but they kept remained in fertile pollen grain. On the other hand, sterile plants left no trace of starch in auther tissues at anthesis.

The results of the observations described above may pernit us to draw the following conclusions:

The essential physiological cause of pollen abortion due to the cytoplasmic male sterility is the insufficient supply of the carbohydrates and other nutrients to developing microspores. This is based on the various abnormalities observed in the behavior of the developing tapetum which is presumed to play an important role for the nutrient supply to the developing microspores. In other words, the organelles such as mitochondrion and the UBISCH body could not be observed or showed an abnormal appearence in the tapetum of male sterilities, and some evidence of the inactivation or obstruction in some physiological synthetic and respiratory processes in the tapetum of male sterilities were found.

Literature Cited

1. ALAM, S. and SANDAL, P. C.: Cyto-histological investigations of pollen abortion in male-sterile sudangrass, *Crop. Sci.*, **7**: 587–589. 1967
2. ALAM, S. and SANDAL, P. C: Electrophoretic analyses of anther proteins for male-fertile and male sterile sudangrass, *Sorghum vulgare var. sudanese* (Piper), *Crop Sci.*, **9**: 157–159. 1969

3. ARCHIMOWITSCH, A.: The problem of inbreeding in sugar beets, *Byull. Byelolserkovskoi Selektsionnoi Stansii*, 1931
4. ARTSCHWAGER, E.: Pollen degeneration in male-sterile sugar beets, with special reference to the tapetal plasmodium, *J. Agr. Res.*, **75**: 191–197. 1947
5. ATANASOFF, D.: Viruses and cytoplamic heredity, *Z. Pflanzenezüchtg.*, **51**: 197–214. 1964
6. ATANASOFF, D.: The viral nature of cytoplasmic male sterility in plants, *Phytopath. Z.*, **70**: 306–322. 1971
7. BOSEMARK, N. O.: Studies on cytoplasmic male sterility in sugar beet, Report of a IIRB joint study, *J. Internatl. Inst. Sugar Beet Res.*, **5**: 232–251. 1972
8. BREWBAKER, J. L.: Agricultural Genetics p. 42–45. Prentice-Hall, Inc., Englewood Cliffs, Jersey, U. S. A., 1964
9. BROOKS, J. and SHAW G.: Recent developments in the chemistry, biochemistry, geochemistry and post-tetrad ontogeny of sporopollenins derived from pollen and spore exines, In: Pollen; development and physiology (Ed.) J. Heslop-Harrison p. 99–144. Butherworths London, 1971
10. BROOKS, J. S. and BROOKS M. H.: Pollen abortion in relation to cytoplasmic-genetic male sterility in Sorghum, *Crop Sci.*, **7**: 47–50. 1967
11. BROOKS, M. H.: Comparative analyses of some of the free amino acids in anthers of fertile and genetic cytoplasmic male sterile sorghum, Genet., **47**: 1629–1638. 1962
12. BROOKS, M. H., BROOKS, J. S. and CHIEN, L.: The anther tapetum in cytoplasmic-genetic male sterile sorghum, *Am. J. Bot.*, **53**: 902–908. 1966
13. CECH, M. and POZDENA, J.: Untersuchungen über die infektiöse Sterilität des Hopfens, *Phytopath. Z.*, **44**: 273–281. 1962
14. CHAUHAN, S. V. S. and SINGH, S. P.: Pollen abortion in male-sterile hexaploid wheat ('Norin') having *Aegilops ovata* L. cytoplasm, *Crop Sci.*, **6**: 532–535. 1966
15. CHAUHAN, S. V. S. and SINGH, S. P.: Studies on pollen abortion in *Cucumis melo* L., *Agra Uuiv. J. Res. Sci.*, **17**: 11–22. 1968
16. CHILDERS, W. E. and MCLENNAN, H. A.: Inheritance studies of a completely male-sterile character in *Medicago sativa* L., *Can. J. Genet. Cytol.*, **2**: 57–65. 1960
17. CHRISTENSEN, J. E., HORNER, H. T. Jr. and LERSTEN, N. R.: Pollen wall and tapetal orbiclar wall development in *Sorghum bicolor* (Gramineae), *Am. J. Bot.*, **59**: 43–58. 1972
18. CHE, Y. E., LI, Y. S., SHINJYO, C. and LI, H. W.: Hybrid rice breeding III. Cyto-histological investigation of pollen degeneration in anther of cytoplasmic male-sterile plants, *Jap. J. Genet.*, **47**: 179–183. 1972
19. CLEIJ, G.: Influencing of the cytoplasmic male sterility and fertility in beets, *Euphytica*, **16**: 23–28. 1967
20. CORBETT, M. K. and EDWARDSON, J. R.: Inter-graft transmission of cytoplasmic male sterility, *Nature*, **201**: 847–848. 1964
21. CORTESSI, H. A.: Investigations made into male sterile beets, *Euphytica*, **16**: 425–432. 1967
22. CURTIS, G. J.: Graft-transmission of male sterility in sugar beet (*Beta vulgaris L.*), *Euphytica*, **16**: 419–424. 1967

23. DEVRIES, A. Ph. and IE, T. S.: Electron-microscopy on anther tissue and pollen of male sterile and fertile wheat (*Triticum aestivum* L.), *Euphytica*, **19**: 103-120. 1970
24. DMITRIEVA, A. N. and KHAVZHINSKAIA, O. E.: Physiological-biochemical differences between fertlie forms of corn and their analogues with male cytoplasmic sterility, *Trudy Ukrains. Nauch. Instit. Rasteinovodstva. Selek. Genetiki*, **7**: 147-153. 1962
25. DUBEY, D. K. and SINGH, S. P.: Mechanism of pollen abortion in three male sterile lines of flax (*Linum usitatissimum* L.), *Crop Sci.*, **5**: 121-124. 1965
26. DUDLEY. J. W.: Effects of sodium 2, 3-dichloroisobutyrate, a selective gamatocide, on sugar beets, J. Am. Soc. Sugar Beet Technol., **11**: 25-36. 1960
27. DUVICK, D. N.: Cytoplasmic pollen sterility in corn, *Adv. Genet.*, **13**: 1-56. 1965
28. ECHLIN, P.: Pollen, *Sci. Am.*, **218**: 80-90. 1968
29. ECHLIN, P.: The role of the tapetum during microsporogenesis of angioperms, In: Pollen; development and physiology (Ed.) J. Heslop-Harrison p. 41-61. Butterworths London, 1971
30. EDWARDSON, J. R.: Cytoplasmic male-sterility, *Bot. Rev.*, **22**: 696-738. 1956
31. EDWARDSON, J. R. and CORBETT, M. K.: Asexual transmission of cytoplasmic male sterility, *Proc. Nat. Acad. Sci.*, **47**: 390-396. 1961
32. EDWARDSON, J. R.: Cytoplasmic differences in T-type cytoplasmic male sterile corn and its maintainer, *Am. J. Bot.*, **49**: 184-187. 1962
33. EDWARDSON, J. R.: Cytoplasmic male sterility, *Bot. Rev.*, **36**: 341-420. 1970
34. ERICKSON, J. R.: Biochemical investigations of cytoplasmic male sterility in spring wheat (*Triticum aestivum* L.), Ph. D. Thesis North Dakota State Univ. Fargo. 1967
35. FILION, W. G. and CHRISTIE, B. R.: The mechanism of male sterility in clone of orchardgrass (*Dactylis glomerata* L.), *Crop Sci.*, **6**: 345-347. 1966
36. FRANKEL, R.: Graft-induced transmission to progeny of cytoplasmic male sterility in petunia, *Sci.*, **124**: 684-685. 1956
37. FRANKEL, R.: Further evidence of graft induced tranission to progeny of cytoplasmic male sterility in petunia, *Cenet.*, **47**: 641-646. 1962
38. FRANKEL, R., IZHAR, S. and NITSAN, J.: Timing of callase activity and cytoplasmic male sterility in petunia, *Biochem. Genet.*, **3**: 451-455. 1962
39. FUKASAWA, H.: On the free amino acids in anthers of male-sterile wheat and maize, *Jap. J. Genet.*, **29**: 135-137. 1954
40. FUKASAWA, H., MITO, K. and FUJIWARA, M.: Preventive effect of sugars on the pollen degeneration of wheat plant, *Bot. Mag. Tokyo*, **70**: 251-257. 1957
41. FUKASAWA, H.: The reduction of triphenyltetrazolium chloride by dehydrogenase in cytoplasmic male-sterile wheat (Japanese), *Nucleus and Cytoplasm*, **2**: 32-35. 1961
42. FUKASAWA, H.: Biochemical mechanism of pollen abortion and other alteration in cytoplasmic male-sterile wheat, *Seiken Ziho*, **13**: 107-111. 1962
43. FUKASAWA, H. and NISHIYAMA, K.: Disc electrophoresis of histone fractions isolated from wheat germ and young spikes (Japanese), *Nucleus* and *Cytoplasm*, **10**: 24-29. 1967

44. FUKASAWA, H.: Abnormal increase of amide nitrogen in cytoplasmic male-sterile wheat, *Jap. J. Genet.*, **43**: 299-304. 1968
45. FUJISHITA, N.: Cytological, histological and physiological studies on the pollen degeneration I. On the free amino acids with reference to pollen degenration in male sterile vegetable crops (Japanses with English Summary), *Jour. Jap. Soc. Hort. Sci.*, **33**: 133-139. 1964
46. FUJISHITA, N.: Cytological, histological and Physiological Studies on the Pollen degeneration II. On the free amino acids with reference to pollen degeneration caused by low temperature in fruit vegetable crops (Japanese with English Summary), *Jour. Jap. Soc. Hort. Sci.*, **34**: 113-120. 1965
47. FUKUJU, H.: The effect of temperature on the activity of rice grain in recieving the sucrose-^{14}C (Japanese), *Rep. Tohoku Branch Crop Sci. Soc. Jap.*, **10**: 15-16. 1968
48. HARVEY, P. H., LEVINGS, C. S. III and WERNSMAN, E. A.: The role of extra-chromosomal inheritance in plant breeding, *Adv. Agron.*, **24**: 1-27. 1972
49. HAYWARD, M. D. and MANTHRIRATNA, M. A. P. P.: Pollen development and variation in genus *Lolium*. I. Pollen size and tapetal relationships in male-fertiles and male-steriles, Z. *Pflanzenzüchtg.*, **67**: 131-144. 1972
50. HESLOP-HARRISON, J.: Origin of exine, *Nature*, **195**: 1069-1071. 1962
51. HIMES, M. and MORIBER, L.: A triple stain for deoxyribonucleicacid, polysaccharides and proteins, *Stain Technol.*, **31**: 67-70. 1956
52. HOEFERT, L. L.: Ultrastructure of *Beta* pollen. I. Cytoplasmic constituents, *Am. J. Bot.*, **56**: 363-368. 1969
53. HOEFERT, L. L: Fine structure of sperm cells in pollen grains of *Beta*, *Protoplasma*, **68**: 237-240. 1969
54. HOEFERT, L. L.: Ultrastructure of tapetal cell ontogeny in *Beta*, *Protoplasma*, **73**: 397-406, 1971
55. HOGABOAM, G. J.: Factors in influencing phenotypic expression of cytoplasmic male sterility in the sugar beet (*Beta vulgaris* L.), *J. Am. Soc. Sugar Beet Technol.*, **9**: 457-465. 1957
56. HORNER, Jr. H. T. and ROGERS, M. A.: A comparative light and electron microscopic study of microsporogenesis in male-fertile and cytoplasmic male-sterile pepper (*Capsicum annuum*), *Can. J. Bot.*, **52**: 435-441. 1974
57. HORNSEY, K. G.: Attempted pollen-transmission of cytoplasmic male sterility and the spontaneous occurrence of male sterility in 0-type lines of sugar beet (*Beta vulgaris* L.), *Theoret. Appl. Genet.*, **43**: 31-34. 1973
58. HOSOKAWA, S., TAKEDA, T., ŌTANI, Y. and IKEHATA, M.: Cyto-histological studies on male sterility of sugar beet, with special reference to pollen degeneration and tapetal plasomdium (Japanese with English Summary), *Jap. J. Breed.*, **4**: 196-202. 1954
59. HOSOKAWA, S., TSUDA, Ch. and TAKEDA, T.: Histochemical studies on the cytoplasmic male-sterility of sugar beets, with special reference to the hydrogen-ion concentration, carbohydrates and amino acids in anthers (Japanese with English Summary), *Jap. J. Breed.*, **13**: 117-124. 1963
60. HOSOKAWA, S. and TSUDA, Ch.: Histochemical studies on the cytoplasmic male-

sterility of sugar beets (Difference in some histochemical properties between Mendelian male-sterile and normal sugar beets) (Japanese with English Summary) *Bull. Sugar Beet Res.* Suppl., **4**: 1-12. 1965

61. IWANAMI, Y.: Physiological studies of pollen, *J. Yokohama Municipal Univ. Series C-34* (Biol. 13) No. 116. 1959
62. IWASAKI, F. and HOSODA, T.: Studies on development of floral organs in male sterile Rape (*Brassica napus* L.) with morphological and histochemical methods (Japanese with English Summary), *Jap. J. Breed.*, **20**: 282-286. 1970
63. IWASAKI, F.: The characteristics of isozyme in male sterile rape (*Brassica napus* L.) (Japanese with English Summary), *Jap. J. Breed.*, **22**: 274-276. 1972
64. IZHAR, S. and FRANKEL, R.: Mechanism of male sterility in petunia. The relationship between PH, callase activity in the anthers and the breakdown of the microsporogenesis, *Theoret. Appl. Genet.*, **41**: 104-108. 1971
65. IZHAR, S. and FRANKEL, R.: Mechanism of male sterility in *Petunia* II. Free amino acids in male fertile and male sterile anthers during microsporogenesis, *Theoret. Appl. Genet.*, **43**: 13-17. 1973
66. JENSEN, W. A.: Botanical histochemistry. W. H. Freeman and Company 1972
67. JINKS, J. L.: Extrachromosomal inheritance p. 40-42. Prentice-Hall, Inc. Englewood Cliffs, New Jersey, 1964
68. JOHRI, B. M. and VASIL, I. K.: Physiology of pollen, *Bot. Rev*, **27**: 325-381. 1961
69. JOPPA, L. F., MCNEAL, F. H. and WELSH, J. R.: Pollen and anther development in cytoplasmic male sterile wheat, (*Triticum aestivum* L.), *Crop Sci.*, **6**: 296-297, 1966
70. KAUL, C. L. and SINGH, S. P.: Studies in male-sterile barley. II. Pollen abortion, *Crop Sci*, **6**: 539-541. 1966
71. KHOO, U. and STINSON, H. T. Jr.: Free amino acid differences between cytoplasmic male sterile and normal fertile anthers, *Proc. Nat. Acad. Sci.*, **43**: 603-607. 1957
72. KINOSHITA. T.: Genetical studies on the male sterility of sugar beets (*Beta vulgaris* L.) and its related species, *J. Fac. Agr. Hokkaido Univ.*, **56**: 435-541. 1971
73. LARSER, K. D. and LERSTEN, N. R.: Anatomy and cytology of microsporogenesis in cytoplasmic male sterile angiosperms, *Bot. Rev.*, **38**: 425-454. 1972
74. LEDBETTER, M. C. and PORTER, K. P.: Introduction to the fine structure of plant cells. Springer-Verlag Berlin, Heidelberg, New York 1970
75. LINSKENS, H. F.: Physiologische Untersuchungs zur Reifeteilung. II, *Acta Bot. Neerl.*, **7**: 61-68. 1958
76. LINSKENS, H. F.: Pollen physiology, *Ann. Rev. Plant Physiol.*, **15**: 255-270. 1964
77. MAHESHWARI, P.: An introduction to the embryology of angiosperms. p. 28-53 McGrawHill, New York 1950
78. MARSALEK, L.: A study of some physiological and biochemical processes in pollen-fertile and sterile genotypes of maize, *Acta. Univ. Agr. Fac. Agron. Orno*, **19**: 695-702. 1971
79. MEER, Q. P. VAN DER and VAN BENNEKOM J. L.: Failure of graft transmission

of male-sterilizing substance in onion (*Allium cepa* L.), *Euphytica*, **19**: 430-432. 1970

80. MEPHAM, R. H. and LANE, G. R.: Exine and role of the tapetum in pollen development, *Nature*, **219**: 961-962. 1968
81. MEYER, V. G.: Some effects of genes cytoplasm, and environment on male sterility of cotton (*Gossypium*), *Crop Sci.*, **9**: 237-242. 1969
82. MURAKAMI, K., KAWAGUCHI, K. and MIZUSHIMA, U.: Histochemical anomalities in anther tissues of rice plant, *Oryza sativa* L., under artificial low temperature conditions with reference to the intervarietal difference in cold resistance (Japanese with English Summary), *Jap. J. Breed.*, **8**: 119-127. 1958
83. NAKASHIMA, H. and HOSOKAWA S.: Studies on histological features of male sterility in sunflower (*Helianthus annuus* L.), *Proc. Crop Sci. Soc. Japan*, **43**: 475-481. 1974
84. NISHI, S. and HIRAOKA, T.: Studies on F_1 hybrid of vegetable crops I. Studies on the utilization of male sterility on F_1 seed production (part 1) Histological studies on the degenerative process of male sterility of some vegetable crops (Japanese with English Summary) *Bull. Nat. Inst. Agr. Sci. Ser.*, E, **6**: 1-41. 1958
85. NISHIYAMA, I.: Male sterility caused by cooling treatment at the meiotic stage in rice plants. IV. Resipratory activity of anthers following cooling treatment at the meiotic stage, *Proc. Crop Sci. Soc. Japan*, **39**: 65-70. 1970
86. OHTA, Y.: Grafting and cytoplasmic male sterility in *Capsicum*, *Seiken Ziho*, **12**: 35-39. 1961
87. OHTA, Y.: Cytoplasmic inheritance and virus (Japanese), *Nucleus* and *Cytoplasm* **9**: 34-39. 1966
88. OHTA, Y.: Effect of thermoshock and virus inoculation on cytoplasmic male sterility in *Capsicum* and *Petunia*, *Ann. Rep. Nat. Inst. Genet. Jap.*, **18**: 26. 1968
89. OHTA, Y.: Effect of virus inoculation and thermoshock treatment on cytoplasmic male sterility in *Capsicum*, *Seiken Ziho*, **20**: 63-67. 1968
90. OHTA, Y.: Cytoplasmic male sterility and virus infection in *Capsicum annuum* L., *Jap. J. Genet.*, **45**: 277-283. 1970
91. OVERMAN, M. A. and WARMKE, H. E.: Cytoplasmic male sterility in sorghum. II. Tapetal behavior in fertile and sterile anthers, *J. Hered.*, **63**: 226-234. 1972
92. OWEN, F. V.: Cytoplasmically inherited male-sterility in sugar beets, *J. Agr. Res.*, **71**: 423-440. 1945
93. OWEN, F. V.: The sugar beet breeding problem of establishing male sterile populations for hybrid purposes, *Proc. Am. Soc. Sugar Beet Technical Genet. Meeting*, **5**: 156-161. 1950
94. PATON, J. B.: Pollen and pollen enzymes, *Am. J. Bot.*, **8**: 471-501. 1921
95. PRING, D. R.: Maize mitochondria: Cytochromes of fertile and cytoplasmic male-sterile lines, *Plant Physiol.*, **55**: 203-206. 1975
96. PRITCHARD, A. J. and HUTTON, E. M.: Anther and pollen development in male-sterile *Phaseolus atropurpureus*, *J. Hered.*, **63**: 280-282. 1972
97. QUINBY, J. R.: Effects of male-sterile-inducing cytoplasm in sorghum hybrids, *Crop Sci.*, **10**: 614. 1970

98. RHOADES, M. M.: Cytoplasmic inheritance in *Zea mays*, Sci., **73**: 340–341. 1931
99. ROBARDS, A. W.: Electron microscopy and plant ultrastructure, McGRAW-HILL London, 1970
100. ROHRBACH, U.: Beitrage zum Problem der Pollen sterilität bei *Beta vulgaris* L. I. Untersuchungen über die ontogenese des Phanotyps, *Z. Pflanzenzüchtg.*, **53**: 105–124. 1965
101. ROWLEY, J. R.: Ubisch body development in *Poa annua*, *Grana Palynol.*, **4**: 25–36. 1963
102. ROWLEY, J. R.: Formation of the pore in pollen of *Poa annua*, In: Pollen physiology and fertilization (Ed.) H. F. LINSKENS p. 59–69 North-Holland Publishing Company Amsterdam 1964
103. SAND, S. A.: Autonomy of cytoplasmic male sterility in grafted scions of tabacco, *Sci.*, **131**: 665. 1960
104. SHAH, D. M. and LEVINGS, C. S. III.: Characterization of chloroplast and nuclear DNAs of *Zea mays* L., *Crops Sci.*, **13**: 709–713. 1973
105. SHAH, D. M. and LEVINGS, C. S. III: Mitochondrial DNA from maize hybrids with normal and Texas cytoplasms, *Crop Sci.*, **14**: 852–853. 1974
106. SHIGA, T. and BABA, S.: Cytoplasmic male sterility in oil seed rape, *Brassica napus* L., and its utilization to breeding (Japanese with English Summary), *Jap. J. Breed.*, **23**: 187–197. 1973
107. SHUMWAY, L. K. and BAUMAN, L. F.: The effect of hot water treatment, X-ray irradiation and mesocotyle grafting of cytoplasmic male sterility of maize, *Crop Sci.*, **6**: 341–342. 1966
108. SINGH, S. P. and HADLEY, H. H.: Pollen abortion in cytoplasmic male sterile sorghum, *Crop Sci.*, **1**: 430–432. 1961
109. SPURR, A. R.: A low-viscosity epoxy resin embedding medium for electron microscopy, *J. Ultrastructure Res.*, **26**: 31–43. 1969
110. STEPHENS, J. C. and HOLLAND, R. F.: Cytoplasmic male-sterility for hybrid sorghum seed production, *Agron. J.*, **46**: 20–23. 1954
111. SUTO, C.: Cytogenetical studies of male sterility in maize, *Bull. Nat. Inst. Agr. Sci.*, **5**: 127–175. 1955
112. TATE, S.: Genic analysis on cytoplasmic male sterility in maize; nature of causal genes and their side effect on expression of some agronomic characters (Japanese with English Summary), *Hokkaido Pref. Agr. Exp. Sta. Rep.* 13 1966
113. TATEBE, T.: Cytological studies on pollen degeneration in male sterile onions (Japanese with English Summary), *Jour. Jap. Soc. Hort. Sci.*, **21**: 73–75. 1952
114. THEURER, J. C: Linkage tests of mendelian male sterility and other genetic characters in sugarbeets, *Beta vulgaris* L., *Crop Sci.*, 8: 698–701. 1968
115. THEURER, J. C., HECKER, R. J. and OTTLEY, E. H.: Attempted graft transmission of cytaplasmic male sterility in sugar beets (*Beta vulgaris* L.), *J. Am. Soc. Sugar Beet Technol.*, **14**: 695–703. 1968
116. TOGASAWA Y., KATSUMATA, T., ISURUGI, Y. and TAKAHASHI, M.: Biochemical studies on pollen II. sugar substances of pollen of *Zea mays* L. var *indurata* Bail., *Pinus thunbergii* Parl., *Lilium loncifolium* Thunb. and *Cucurbita maxima* Ouch.

(Japanese with English Summary), *Jour. Fac. Agr. Iwate Univ.*, **6**: 6-10. 1962

117. TOKUMASU, S.: Histological studies on pollen degeneration in male-sterile radish (Japanese with English Summary), *Jap. J. Breed.*, **6**: 249-254. 1957
118. TOKUMASU, S.: Comparison of anther and pollen development between male-sterile diploids and male-fertile tetraploids in *Pelargonium roseum* (Japanese with English Symmary), *Jap. J. Breed.* **20**: 211-218. 1970
119. UBISCH, G. V.: Zur Entvicklungsgeschichte der Anthern, *Planta*, **3**: 490-495. 1927
120. VASIL, I. K.: Some aspects of the physiology of anthers. In: Plant tissue culture (Ed.) P. R. WHITE and A. G. GROVE p. 341-356. MrCutrhan Berkeley, 1965
121. VASIL, I. K.: Physiology and cytology of anther development, *Biol. Rev.*, **42**: 327-373. 1967
122. WARMKE, H. E. and OVERMAN, M. A.: Cytoplasmic male sterility in sorghum. I. Callose behavior in fertile and sterile anthers, *J. Hered.*, **63**: 103-108. 1972
123. WHITED, D. A.: Biochemical and histochemical properties associated with genetic male-sterility at Ms locus in barley, *Hordeum vulgare* L. emed. Lam.. Ph. D. Thesis North Dakota, State Univ., Fargo, 1967
124. WIT, F.: Chemically induced male sterility, a new tool in plant breeding?, *Euphytica*, **9**: 1-9. 1960
125. YANG, H. J. and NAKAGAWA, S.: The critical period of degeneration of tapetum and pollen grains in Satsuma orange (*Citrus unshiu Marc.*), *J. Jap. Soc. Hort. Sci.*, **38**: 137-143. 1969
126. ZENKTELER, M.: Microsporogenesis and tapetal development in normal and male-sterile carrots (*Daucus carota*), *Am. J. Bot.*, **49**: 341-348. 1962
127. ZEVEN, A. C.: Transfer and inactivation of male sterility and sources of restorer genes in wheat, *Euphytica*, **16**: 183-189. 1967

Explantion of Plate

Plate I

Starch reactionin anther tissues of fertile (left) and sterile (right) sugar beet. (× 150)

a. pollen mother cell stage
b. tetrad
c. microspore
d. pollen

A large amount of starch grains (S) were observed in both fertile and sterile plants (a, b, c). Abnormal tapetum can be seen in the sterile anther (c-right). At mature pollen stage (d), starch grains completely disappeared in fertile plant. On the other hand, those of sterile anther (d-right) still remained in the endothecium.

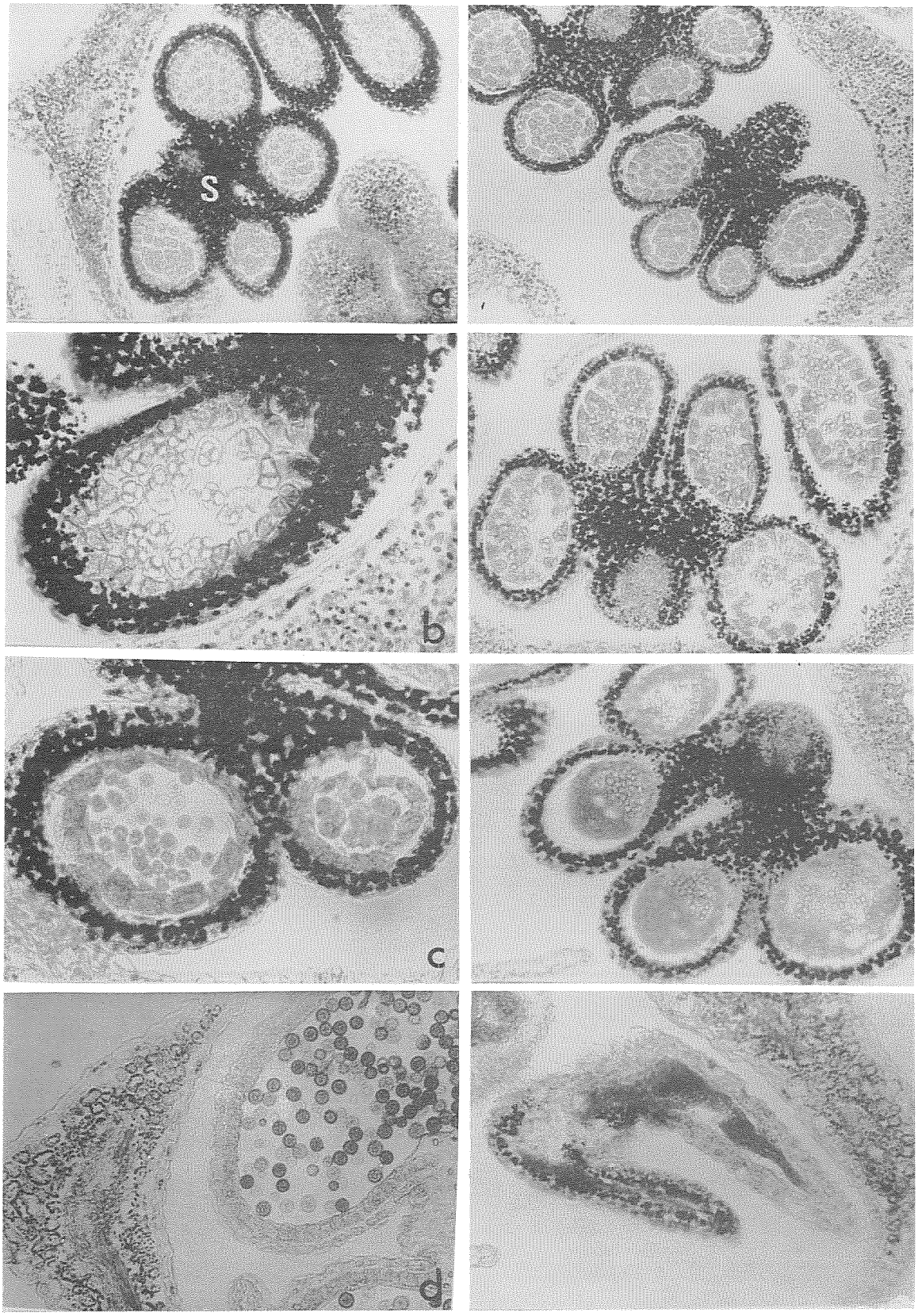

S
a
b
c
d

Plate II

Sundan III stain for demonstration of lipid. Fertile (left) and sterile (right) sugar beet. (× 300)

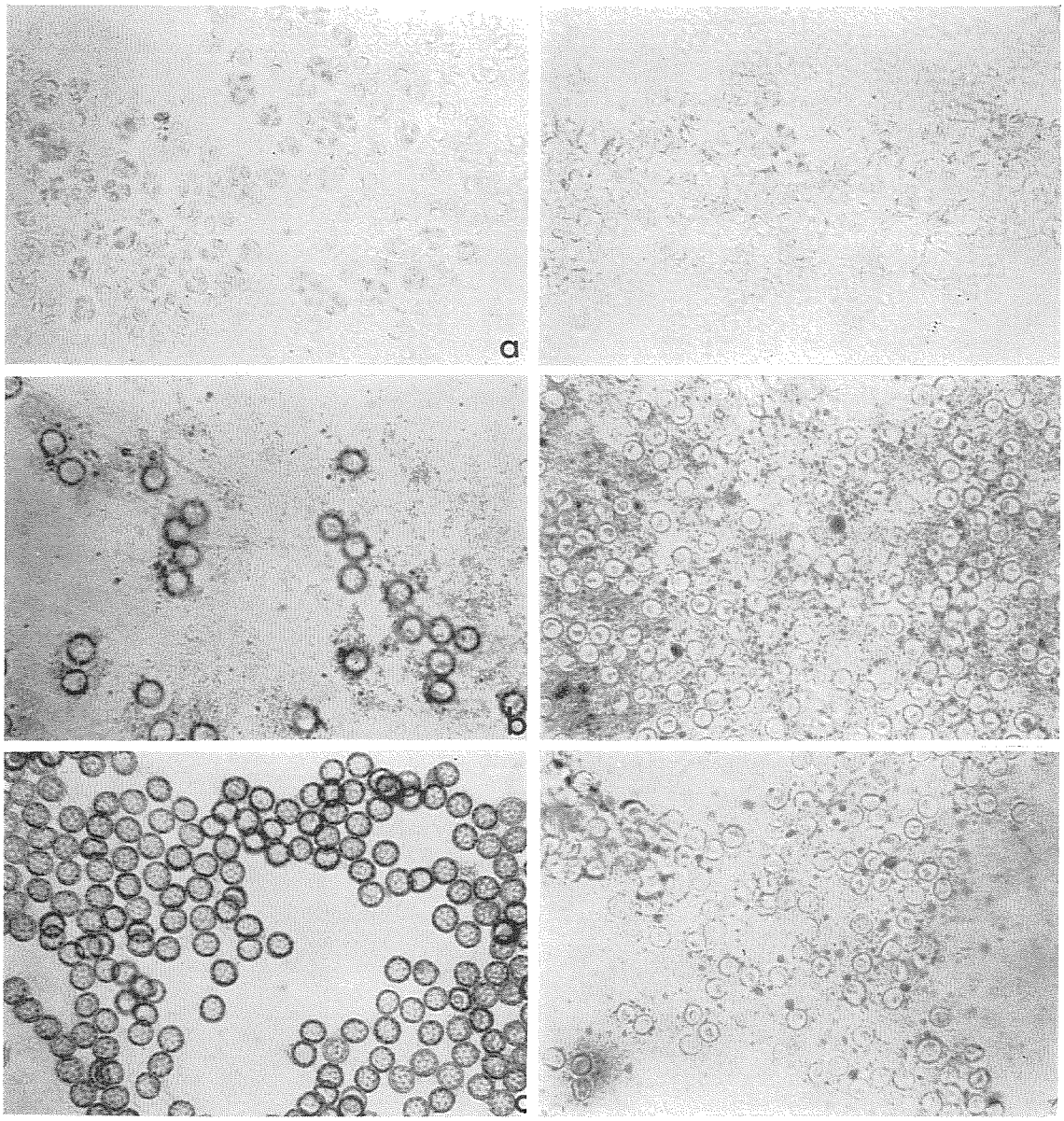

a
b
c

Plate III

Triple stained microphotograph of anther tissues in fertile (left) and sterile (right) sugar beet. (× 400)

a. pollen mother cell stage

Tapetal cytoplasm showed yellow, indicating the existence of protein. Red colored particles were observed at the endothecium and the parenchyma, indicating the existence of polysaccharides.

b. tetrad stage

Tapetal cells were larger in size, and the tapetal cytoplasm showed deep yellew. Tapetal cell nuclei were slightly stained a greenish yellow responsible for contents of DNA.

c. microspore stage

The tetrads were released into the anther locules and grew as microspore. Tapetal cell became disintegrated.

d. pollen stage

Reaction of polysaccharides in fertile anther (left) completely disappeared, but that in sterile anther was still in the endothecium.

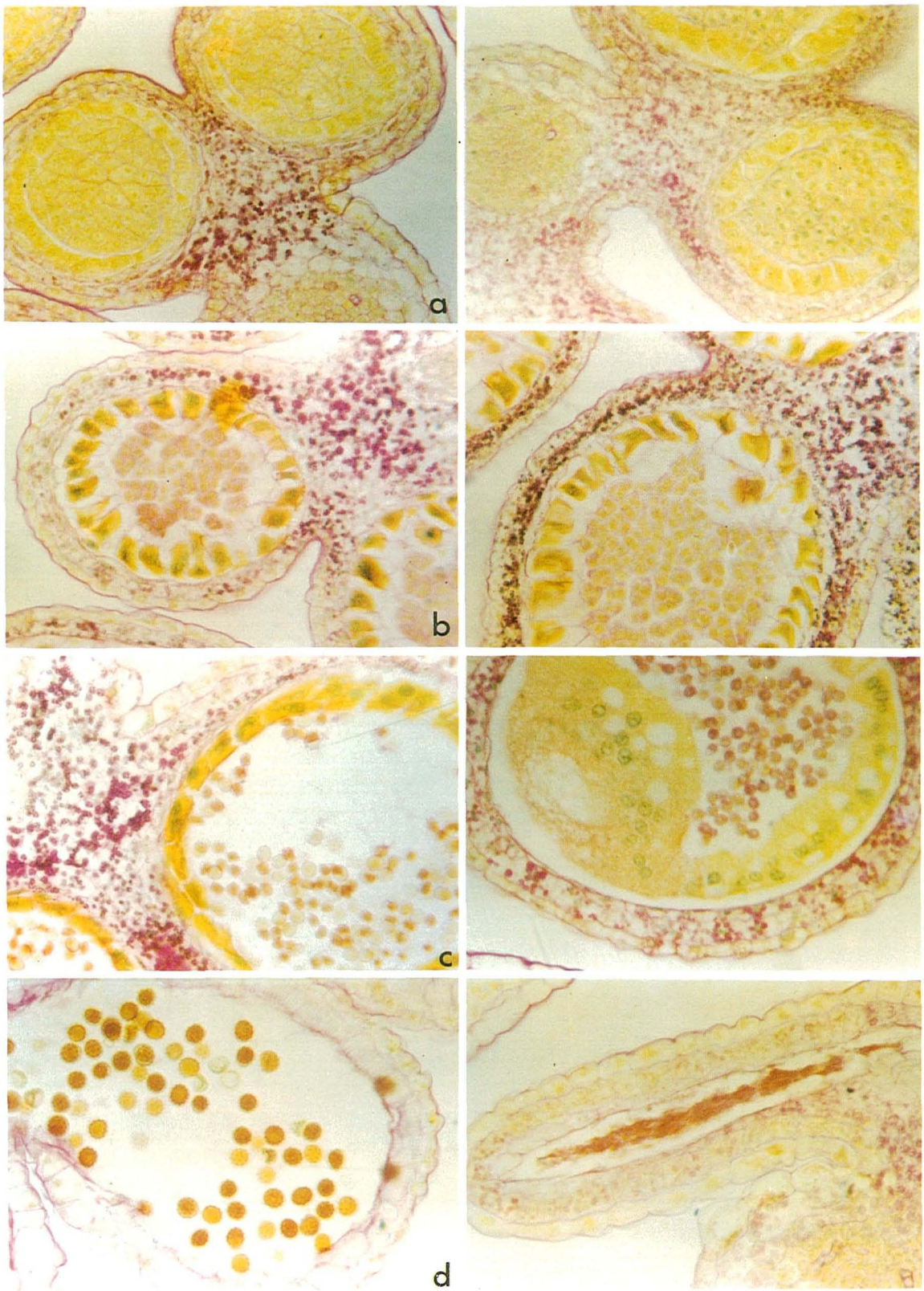

a
b
c
d

Plate IV

Peroxidase (a, b) and succinic dehydrogenase (c, d) activities of anther tissues in fertile (left) and sterile (right) sugar beet. (× 300)

Peroxidase activities were observed in the endothecium and the middle layer in both fertile and sterile plants. The enzymatic activities of sterile anthers (b-right) were more intense than those of fertile anther (b-left) at the late stage of development.

Succinic dehydrogenase activities were mainly observed in the tapetum and pollen mother cells or tetrads. The enzymatic activites of fertile anther were generally more intense than those of sterile anthers.

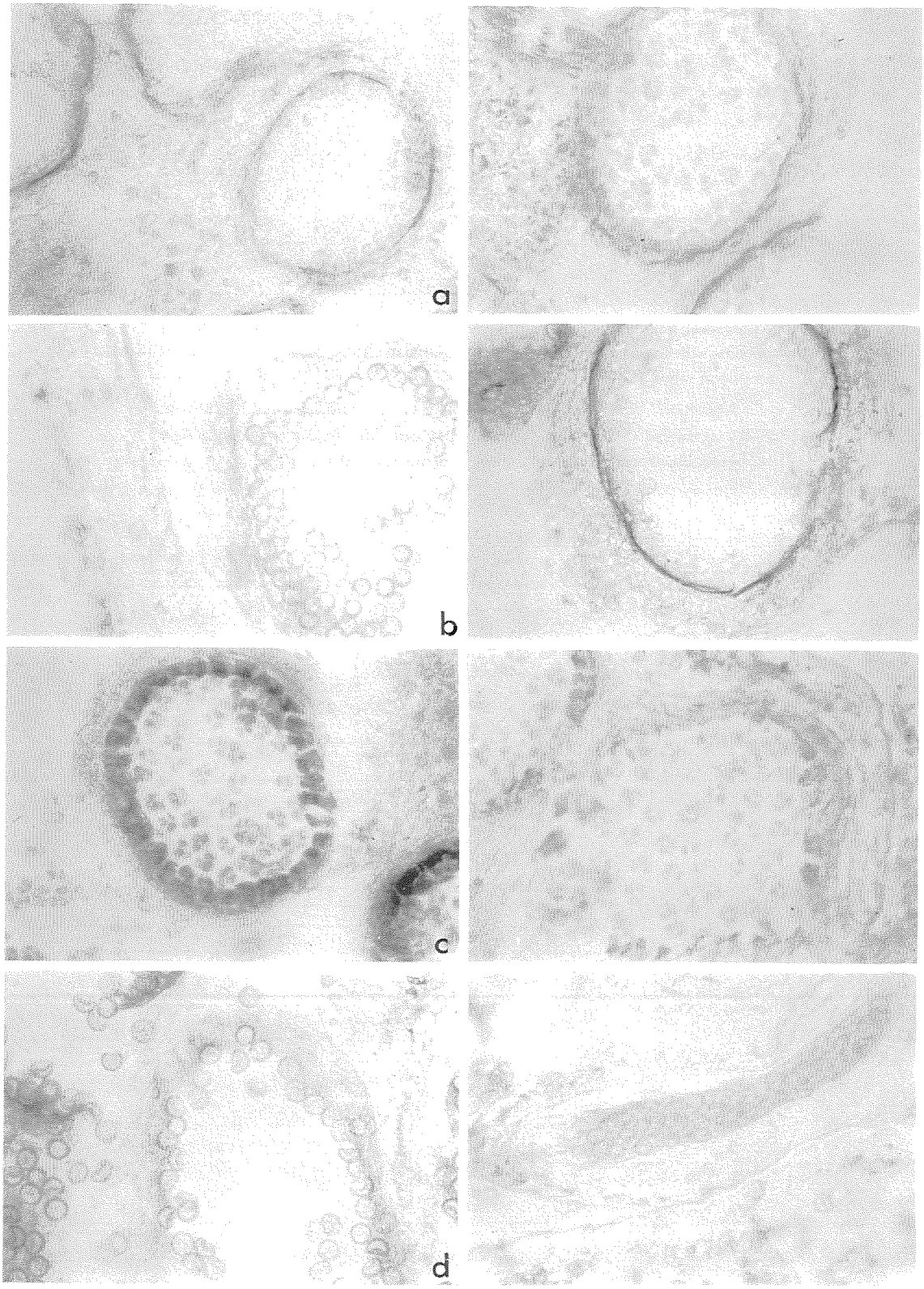

a
b
c
d

Plate V-1

Autoradiograph of fertile anthers of sugar beet labeled with ^{14}C by photosynthesis. (× 200) Forcal level of tissues (left) and forcal level of emulsion (*right*)

Abundant ^{14}C-compound were observed in the anther tissues at PMC (a), tetrad (b) and microspore (c) stages. At pollen stage (d), little ^{14}C were observed at pollen grains and anther walls.

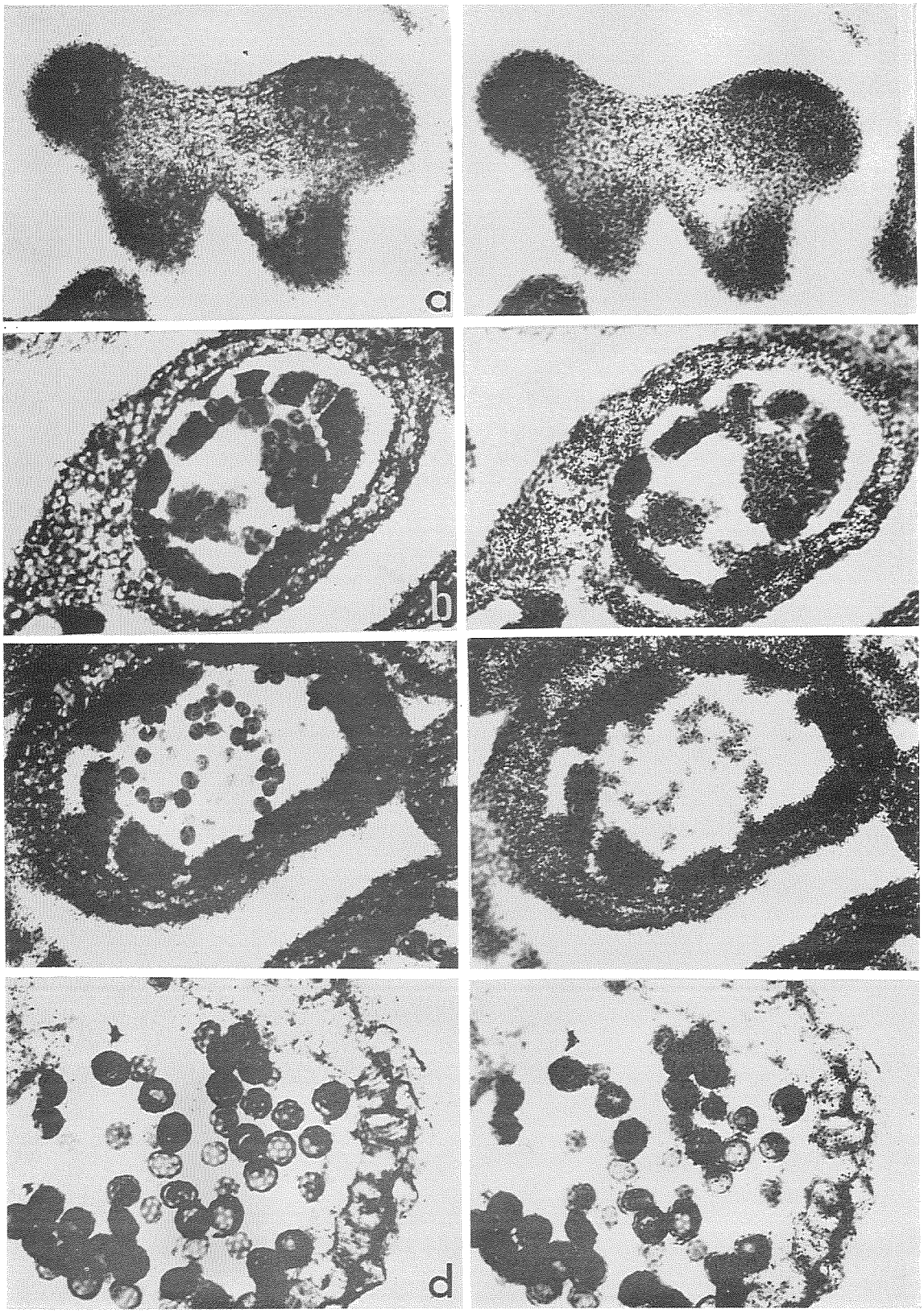

a
b
d

Plate V-2

Autoradiograph of sterile anthers of sugar beet labeled with ^{14}C by photosynthesis. (× 200)

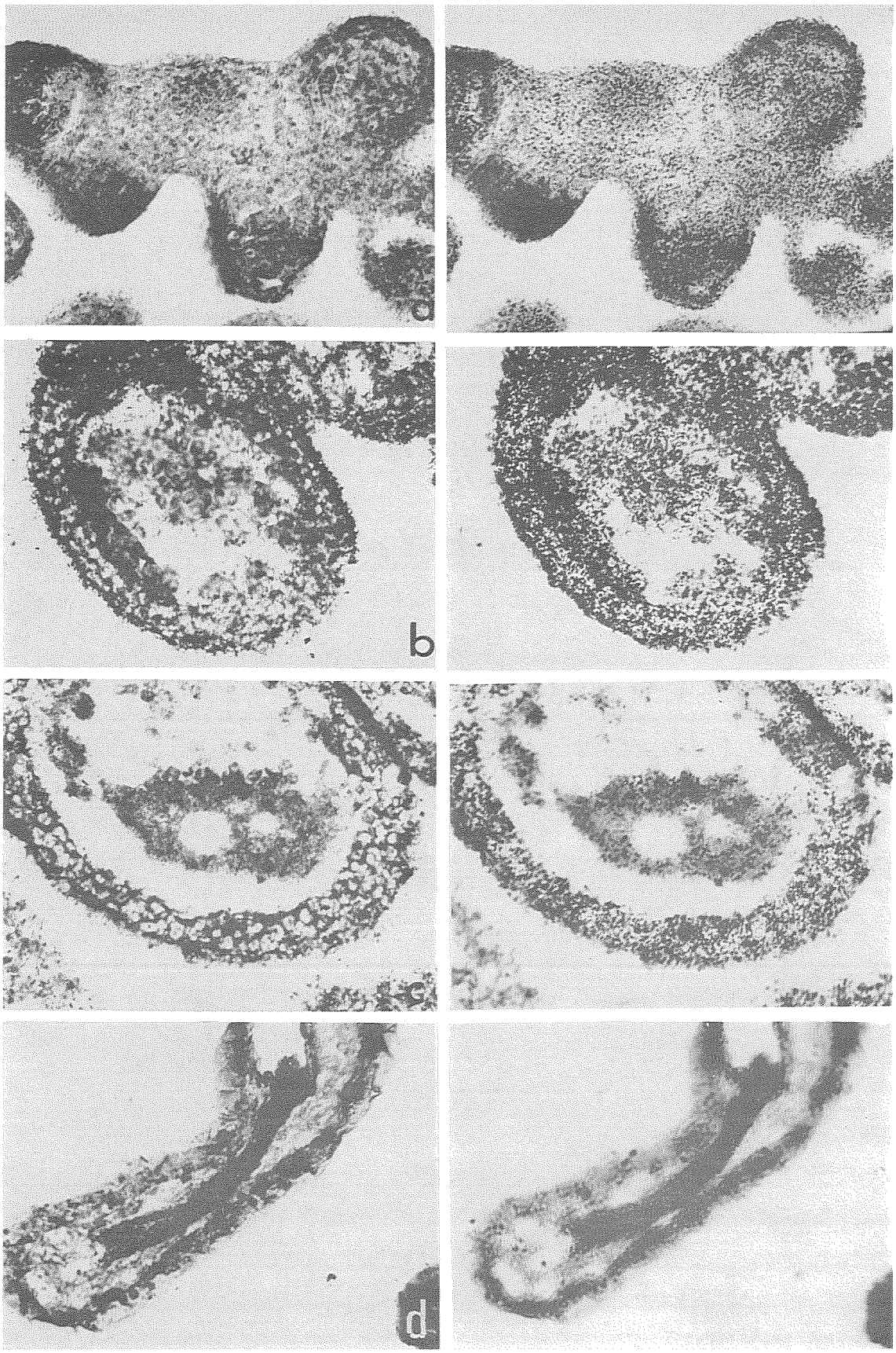

a
b
c
d

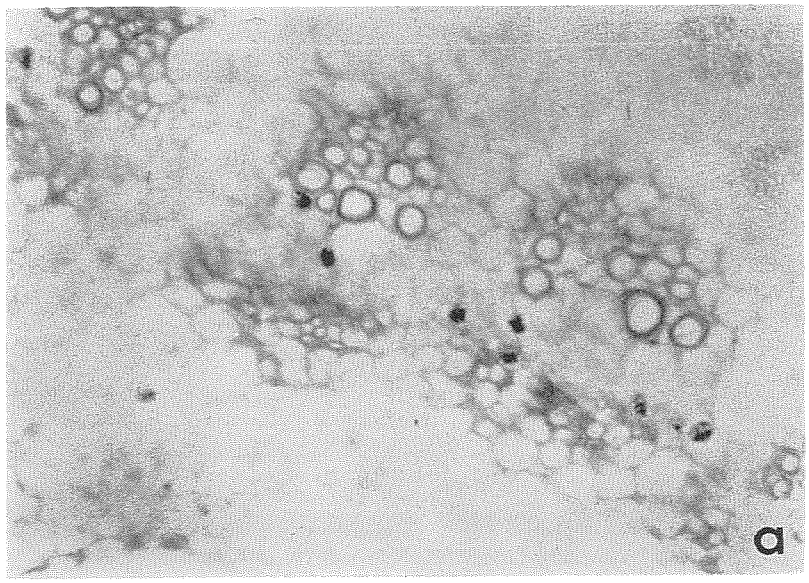
Plate VI

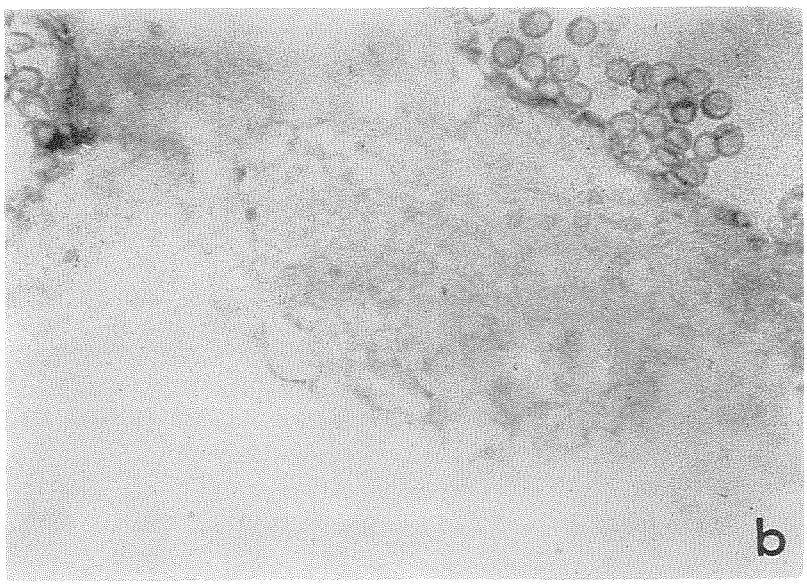
Autoradiograph of anther tissues in sugar beet labeled with ^{3}H-thymidine.

a. ^{3}H were labeled at cambium of both fertile (a) and sterile flower stalk. (× 300)

b. ^{3}H were labeled at filament of both fertile (b) and sterile plants. (× 300)

c. ^{3}H were only labeled at tapetal cell of fertile plants. (× 300)


a


b

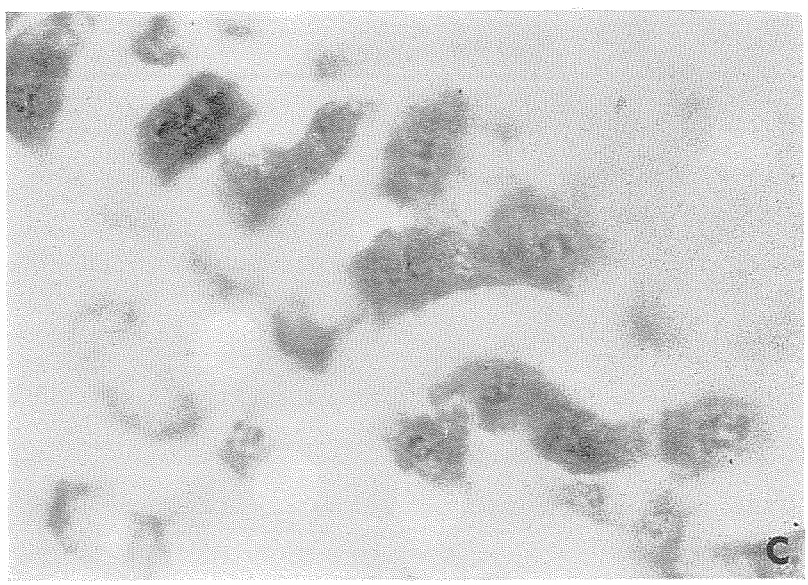

c

Plate VII-1

Electron microphotograph of anther tissues in fertile (left) and sterile (right) sugar beet.

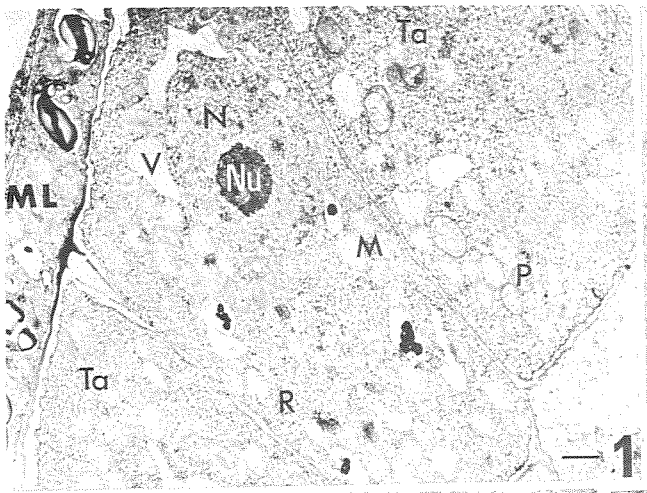
Abbreviation and symbols. The scale bars represent 1 μm.

C: Callose, CW: Cell Wall, E: Exine, IN: Intine, M: Mitochondrion, ML: Middle Layer, N: Nucleus, Nu: Nucleolus, P: Plastid, PG: Pollen Grain, R: Ribosome, S: Starch, T: Tetrad, Ta: Tapetum, U: Ubisch Body, V: Vacuole, dm: dense material, ld: lipid like droplet, lm: lipid like material, pU: pro Ubisch body, O: peculia substance.

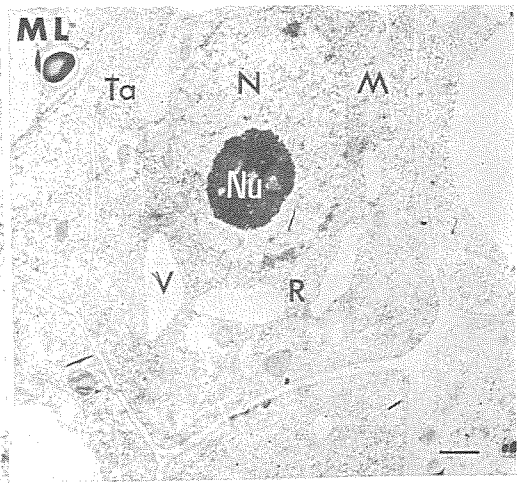
1. Each tapetal cell has one nucleus (N) with prominent nucleolus (Nu), mitochondria (M) plastids (P), Ribosomes (R) and fairly large vacuoles (V). No clear difference can be found between fertile (left) and sterile (right) tapetal cells.

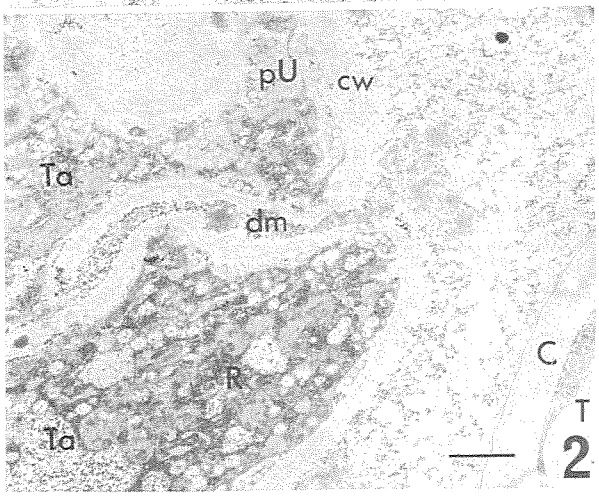
2. Many pro Ubisch bodies (pU) are observed in the inner tapetal cell wall (CW) for both fertile (left) and sterile (right) anthers.

3. The exine of microspore deposited sporopollenin. The thick tapetal cell walls have disappeared and pro Ubisch bodies develop into Ubisch bodies (U) coated with sporopollenin. (left) The tapetal cell walls and pro Ubisch bodies have disappeared. The mitochondria are not observed, but many lipid like materials are observed in the tapetal cytoplasm.

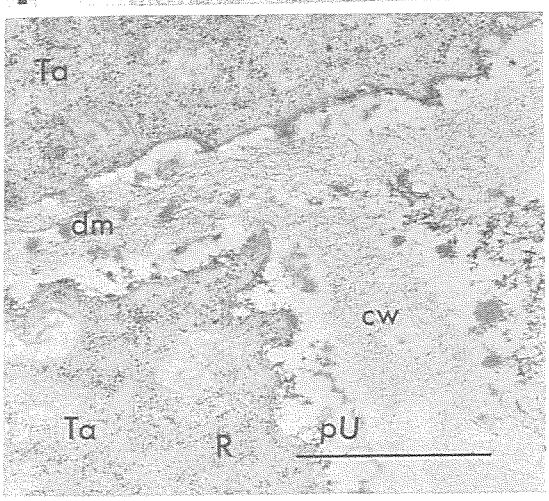

Ta
N
V
Nu
ML
M
P
Ta
R

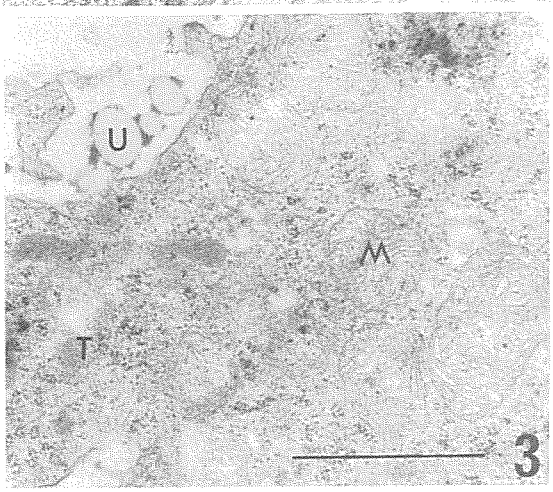
1


ML
Ta
N
M
Nu
V
R


pU
cw
Ta
dm
C
R
T
Ta

2


Ta
dm
cw
Ta
R
pU


U
M
T

3

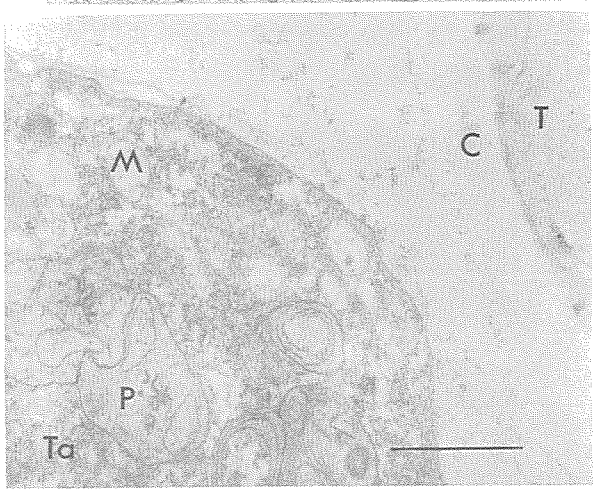
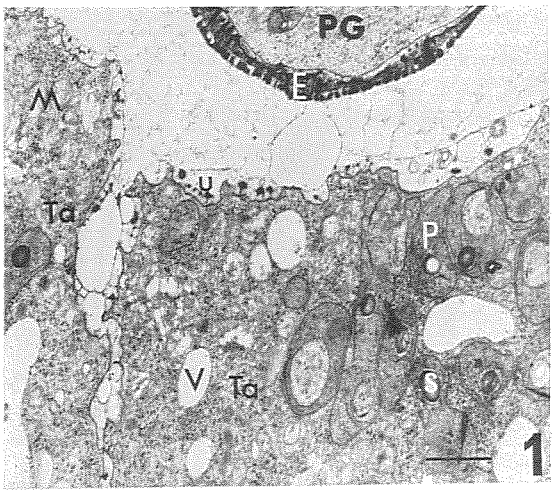

T
C
M
P
Ta

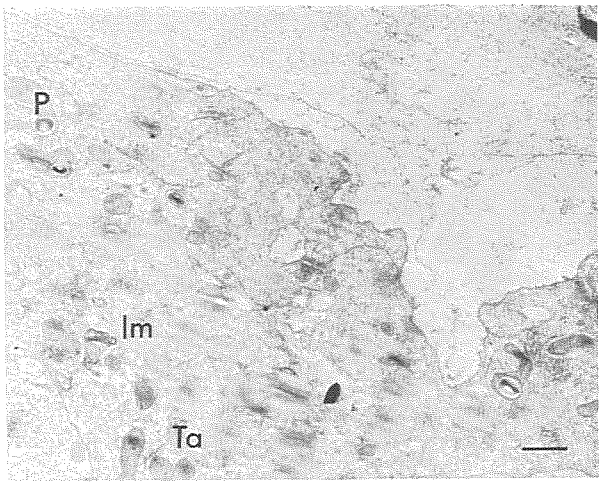
Plate VII-2

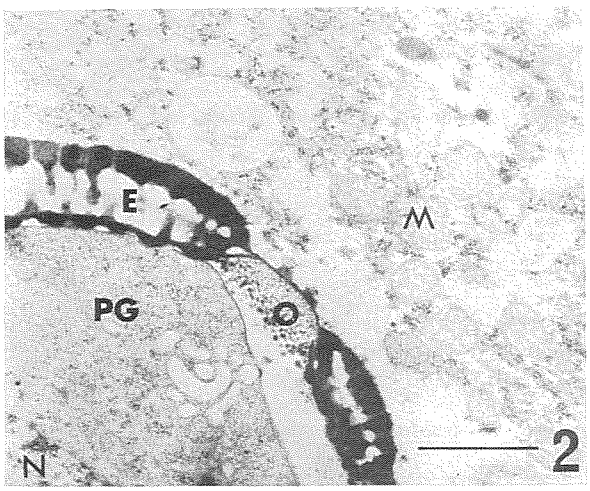
1. The mitochondria (M) and the Ubisch bodies (U) are conspicuous in fertile tapetum (left). Since the cristae of mitochondria (M) have degenerated, typical mitochondria are not observed. (right)

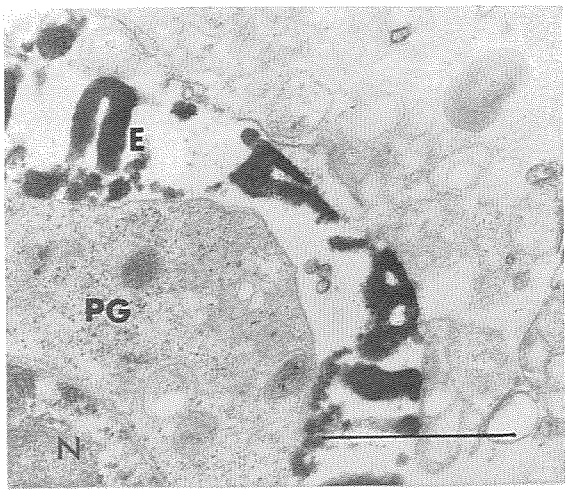
2. The tapetal cell membrance has deteriorated and mitochondria (M) are seen in the anther cavity. The peculiar substanlces (O) are observed at the pore of pollen grain (PG). (left) The tapetal cell has degenerated and remnants of tapetal cytoplasm exist in the anther cavity, but cell organelles are not readily recognizable. (right)

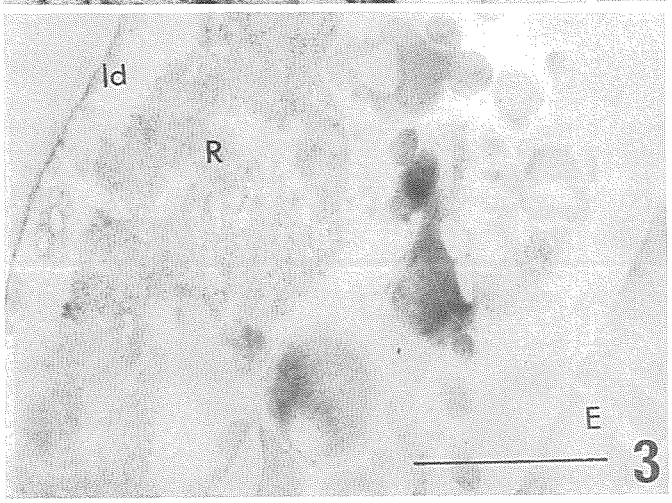
3. The lipid like droplets (ld) are seen along the outer tapetal membrane. (left) Organelles of sterile microspore begin to degenerate and exine (E) does not develop fully. (right)


PG
E
M
Ta
U
P
V
Ta
S
1


P
Im
Ta


E
M
PG
O
N
2


E
PG
N


Id
R
E
3

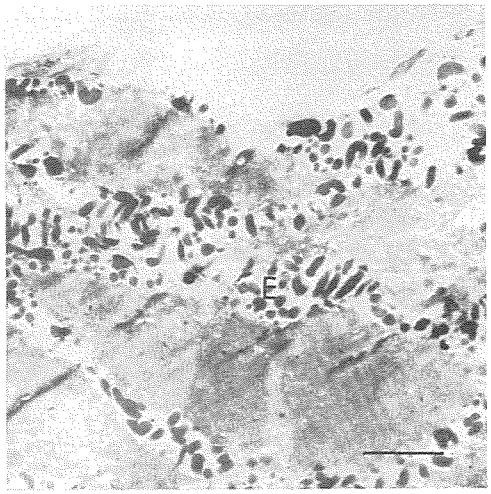

E

Plate VIII

Starch reaction in anther tissues of fertile (left) and sterile (right) maize. (× 150)

At early stages of anther development (a, b, c), a large amount of starch grains was observed in the both fertile and sterile plants. They were found to be located in the endothecium and parenchyma. At pollen stage of anther development (d), starch grains were located in fertile pollen grains, but in sterile anther no trace of them were left in anther tissues.

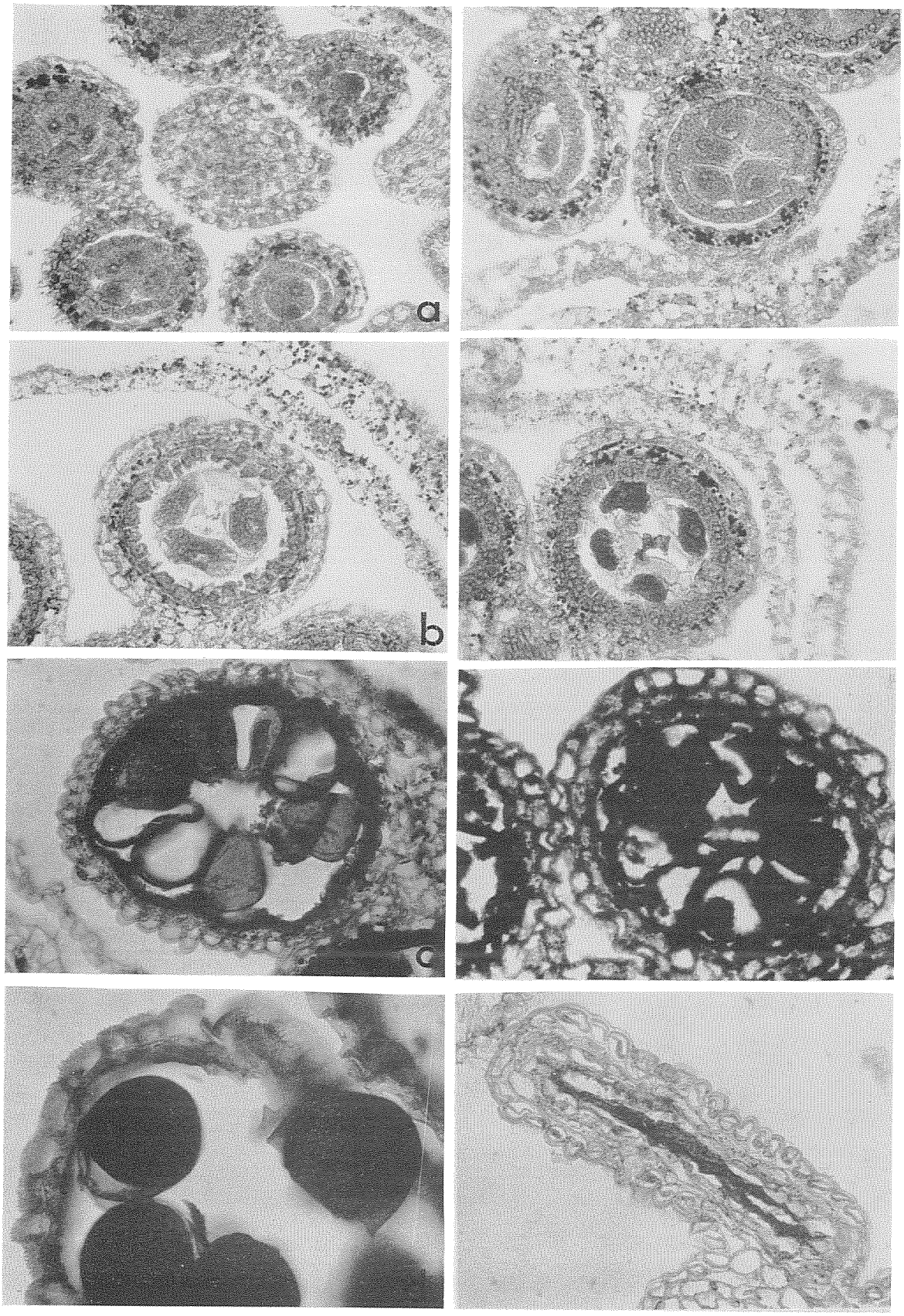

a
b
c

Plate IX

Electrophoreic banding patterns of isozymes of various enzymes in crude homogenates prepared from maize anther having different cytoplasm.

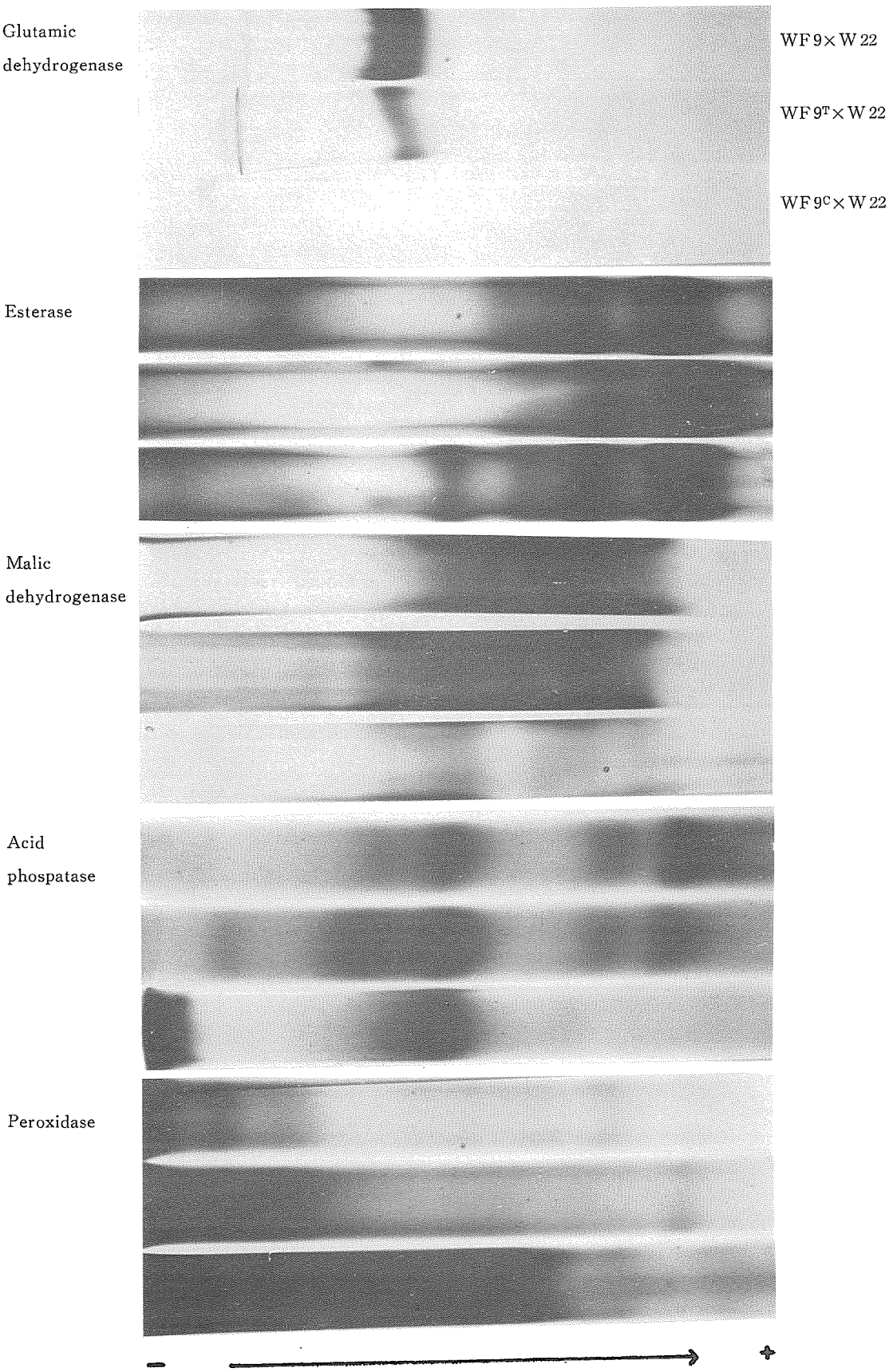

Glutamic dehydrogenase
WF 9×W 22
WF 9T×W 22
WF 9C×W 22
Esterase
Malic dehydrogenase
Acid phospatase
Peroxidase
−
+

Plate X

Autoradiograph of sugars extracted from maize.

W 375 B (fertile) and W 375 B^T (sterile)

FR: Fructose, GL: Glucose, SU: Sucrose

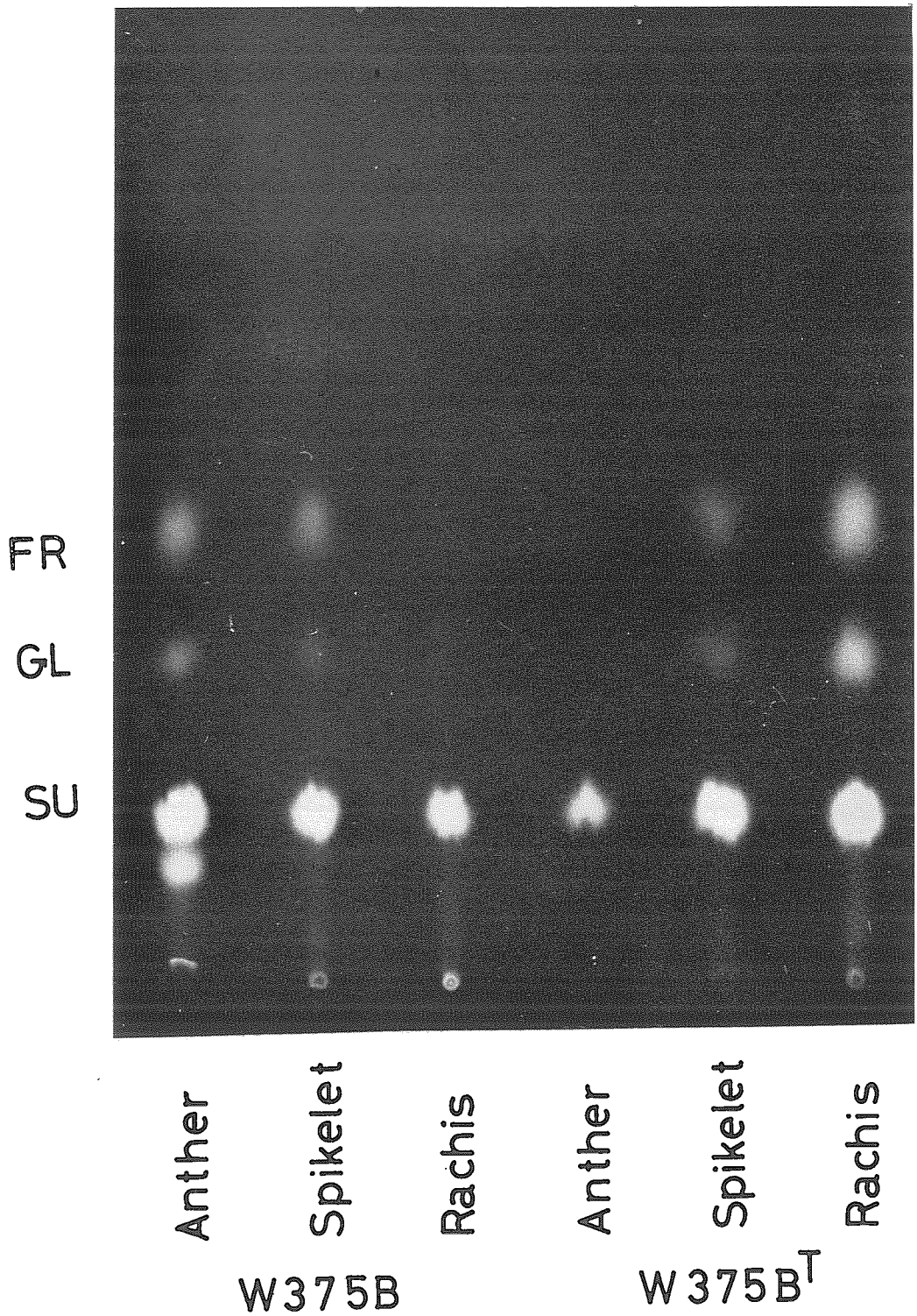
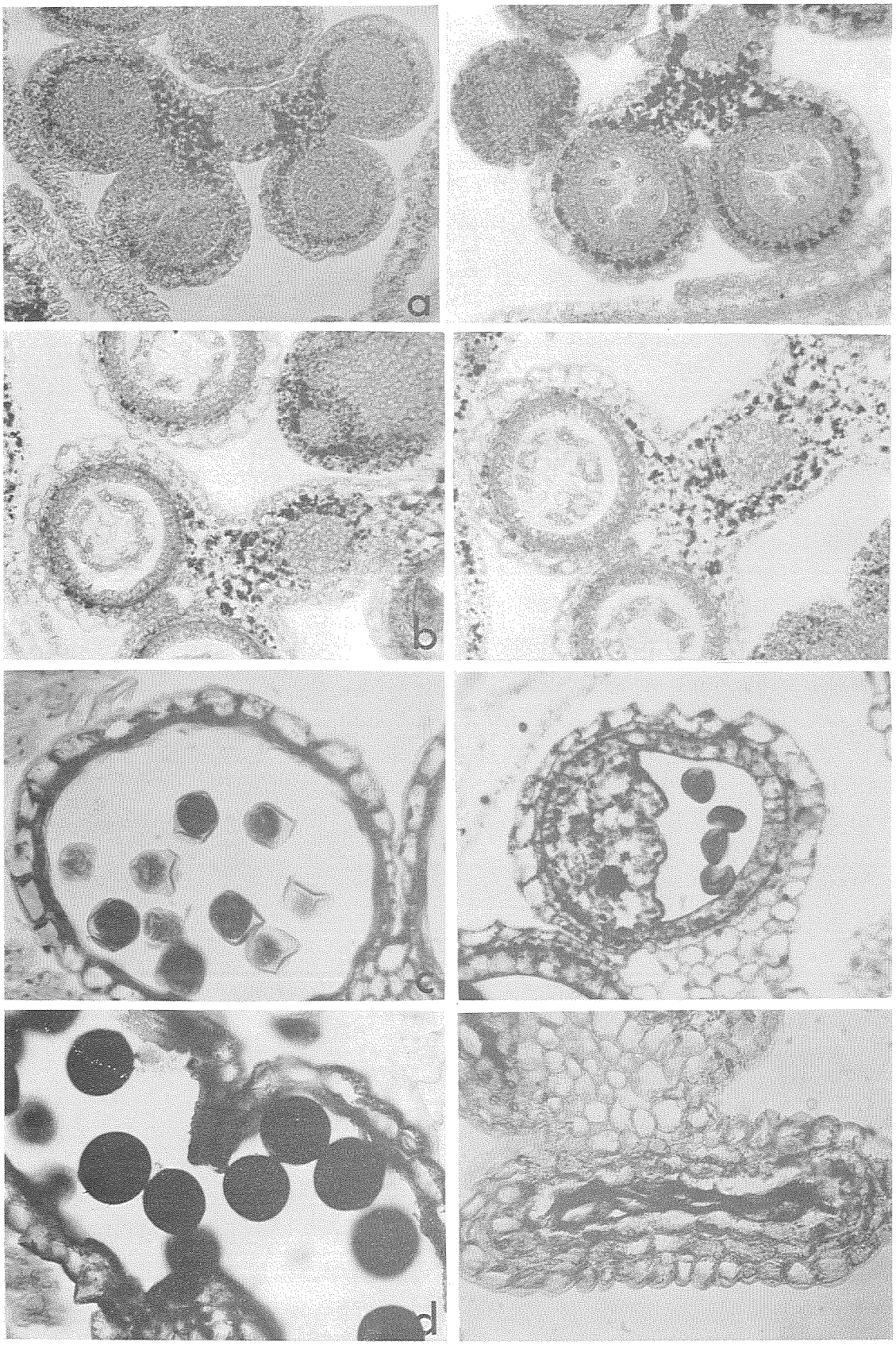

FR
GL
SU
Anther
Spikelet
Rachis
Anther
Spikelet
Rachis
W375B
W375B^T

Plate XI

Starch reaction in anther tissues of fertile (left) and sterile (right) sorghum. (× 150)

At early stages of anther development (a, b), a large amount of starch grains was observed in both fertile and sterile plants. They were found to be located in the endothecium and parenchyma. In fertile anther, starch grains were located in pollen grain (d-left). Sterile lines left no traces of them in anther tissues. (d-right)


a
b
c
d