MORPHOLOGICAL AND HISTOCHEMICAL STUDIES ON POLLEN DEGENERATION IN CYTOPLASMIC MALE-STERILE SUGAR BEET (*BETA VULGARIS* L. VAR. *SACCHARIFERA*)

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Introduction

ARCHIMOWITSCH[2] was the first to describe the phenomenon of male sterility in sugar beets. According to OWEN[14], sterility in sugar beets was controlled by the interaction of two genes *X* and *Z* in sterile cytoplasm *S*. In addition to this, a monofactorial and a poly-hybrid mode of inheritance has been reported in a diploid male-sterile sugar beet (*Beta vulgaris* L. var. *saccharifera*)[4,17]. The present investigation deals with morphological and histochemical studies on pollen degeneration in this diploid male-sterile line.

Materials and Methods

Flower buds of male-fertile and cytoplasmic male-sterile *Beta vulgaris* L. var. *saccharifera* were collected from plants grown at the Exerimental Farm, Plant Breeding Institute, Faculty of Agriculture, Hokkaido University, Sapporo, Japan and were fixed in 80% acetone. These were dehydrated, cleared and microtomed at 7-16 μ by usual methods.

For morphological studies, the sections were stained with Heidenhain's iron-alum haematoxylin. Squashes of anthers were made in 0.5% acetocarmine.

For histochemical localization of total carbohydrates of insoluble polysaccharides (PAS test), total proteins (Ninhydrin Schiff's reaction), histones (Alkaline-fast green test) and DNA (Feuglen reaction) as described by JENESEN[9] were followed.

Experimental Results

1. Morphological

Anther development and Microsporogenesis : Development of an anther

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till sporogenous mass stage is similar in both MF and CMS plants. However, in subsequent stages the behaviour of anther wall layers, microspores and anther connective deviates from normal course. In the following paragraphs the description is limited to CMS line where it differs from MF line.

The epidermal cells in the anthers of CMS plants become irregular in outline (Fig. 1). The cells in endothecial layer, elongate tangentially and fail to develop fibrous bands (Figs. 4, 5). The middle layers degenerate along with tapetal cells (Fig. 4).

The tapetal cells at sporogenous mass stage are filled with dark stained cytoplasm and degenerated nuclei. In a limited number of anthers, the sporogenous cells degenerate prior to the onset of meiosis (Fig. 1). This is followed by the degeneration of tapetal cells (Fig. 2). On the other hand, tapetal cells in most of the anthers, remain intact till vacuolate pollen stage. Pollen mother cells (PMCs) in such anthers undergo normal meiosis. The microspores in the tetrads remain cemented to make an irregular mass (Fig. 3). On liberation, the microspores increase in size but their wall differentiates only into a rudimentary exine. The pollen grains possess scanty degenerated protoplast. At this stage, the tapetal cell walls break down and their protoplasmic contents migrate into anther sac to make a pseudoperiplasmodium (Fig. 4). Because of this, the pollen grains become over-crowded (Fig. 5). Finally, the dark stained plasmodial fragments and pollen grains degenerate (Fig. 6).

The pro-cambial strand in the anther connective fails to differentiate (Fig. 7). However, at vacuolate microspore stage, two to four thin walled xylem elements enclosed by degenerated phloem appear. The parenchyma cells in the connective region are irregular and possess large, hyaline intracellular inclusions (Figs. 7, 8).

2. Histochemical

Table 1 gives evaluation of histochemical reactions observed during various stages of anther development. Anther development is divided into five stages. The sporogenous cells were initially evident and the last was near anthesis when pollen grains become engorged with reserves. The intensity of various reactions is divided arbitrarily into four parts viz. low or slight (*), moderate (**), high or intense (***) and highest or most intense (****).

Localization of total carbohydrates of insoluble polysaccarides (PAS reaction): At sporogenous stage (*a*), all the parts of an anther of MF plants show slight PAS reaction except epidermis, the cells of which are moderately stained. In the subsequent stages (*b* & *c*), the intensity of the reaction gradually increases. On microspore tetrad formation (stage *c*), cuticle shows

most intense, while intense reaction shown by the tapetal cells is marked in their radial and inner tangential walls. Endothecial cells, however, show only moderate reaction. At stage *d*, the intensity of the reaction remains more or less the same as in the preceding stage with a few exceptions. The endothecial cells show an increase from moderate to intense. At vacuolate pollen grain stage, the intensity of the reaction is maintained in all the parts except middle layers, tapetum and pollen grains. The middle layers and tapetum degenerate at this stage while the engorged pollen grains show most intense PAS reaction (Fig. 10).

On the other hand, the intensity of PAS reaction in the anthers of CMS plants either remains low or moderate in all the stages and falls short as compared to those of MF anthers (Figs. 9, 11).

Accumulation of starch is observed in different parts of an anther of both MF and CMS plants (Fig. 9). It's presence is shown in Table 1 as S.

Starch appears in the connective parencyhma and all the wall layers except epidermis in both MF and CMS anthers (Fig. 9, 11). However, in the anthers of MF plants. starch disappears from tapetal cells at early tetrad stage, while in endothecium, middle layers and connective parenchyma cells starch persists till vacuolate microspore stage. In PMCs also starch accumulates prior to meiotic division but disappears just at the onset of meiosis. The tetrads and vacuolate microspores are devoid of starch, but engorged pollen grains show highest concentration of starch (Fig. 10). On the other hand, in the anthers of CMS plants, starch persists in all the wall layers except epidermis till stage *e*, but the vacuolate pollen grains are devoid of starch (Fig. 11).

DNA, Histones and Total Protein: The distribution patterns of these substances run more or less parallel. At stage *a*, the concentration of DNA, histones and total protein is low in most parts of an anther of MF plants. However, in tapetal and sporogenous cells it was high and moderate, respectively. During meiotic division (stage *b*), the concentration of these substances increase significantly in different parts of an anther. In the tapetal cells, the increase is maximum as is apparent by most intense staining reactions. The PMCs also show intense concentrations. At stage *c*, the tapetal cells show a slight decline in the protein content, but the concentrations of DNA and histones reach their maximum. However, there is a reduction in the concentrations of all these substances in the young microspores enclosed within the common mother wall. During the period of microspores released from the callose wall, their rapid growth and vacuolation (stage *d*), the concentrations increase steadly. The tapetal cells at this stage show high con-

TABLE 1. Evaluation of PAS, Feulgen, Alkaline fast-green, and Ninhydrin-Schiff's reactions in the anthers of MF & CMS plants of *Beta vulgaris* at different

Reaction	Stages of development	Strain	Cuticle	Epidermis	Endothecium	Middle layers
PAS	*a*	MF		++	+	+
		CMS		+	+	+
	b	MF		+++	++S	++S
		CMS		++	+	+S
	c	MF		+++	++S	++
		CMS		++	+S	+
	d	MF	+++	+++	++	++
		CMS		++	+S	+S
	e	MF	+++	+++	++	
		CMS		++	++S	++S
DNA & Histone	*a*	MF		+	+	+
		CMS		+	+	+
	b	MF		++	++	++
		CMS		+	+	+
	c	MF		++	++	++
		CMS		+	+	+
	d	MF		++	++	+
		CMS		+	+	+
	e	MF		++	++	
		CMS		+	+	
Proteins	*a*	MF		+	+	+
		CMS		+	+	+
	b	MF		+	++	++
		CMS		+	+	+
	c	MF		+	++	+
		CMS		+	+	+
	d	MF		+	+++	+
		CMS		+	+	+
	e	MF		+	++	+
		CMS		+	+	+

stages of development (+ Low or slight, ++ Moderate, +++ High or intense, ++++ Highest or most intense and S Presence of starch)

Tapetum	Spore mother cells	PMCs	Tetrad	Micro-spore	Pollen grains	Connective
+	+					++
+	+					+
++ S		++				++ S
++		+				+
+++			+++			++ S
++ S			++			+ S
+				++		++ S
+ S				++		++ S
					+++	+++
					++ S	++
+++	++					++
+	+					+
+++		+++				++
+		+				+
+++			+++			++
++			+			+
++				++		+
+				+		+
					+++	+
+					+	+
+++	++					++
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centrations and the other parts exhibit only slight or moderate reactions. Vacuolate pollen grains in maturity (stage *e*) show a further increase in DNA, histones and total protein. The tapetal cells almost disintegrate at this stage and their remaining fragments show only low or slight reactions. At the time of anthesis, the concentration of these substances further increase in pollen grains ready to shed (Figs. 12, 13, 15).

On the other hand, the concentrations of these substances in various parts of an anther since beginning is low in CMS plants. In subsequent stages, these substances also fail to show any appreciable increase as compared to those of MF anthers. The plasmodial mass of tapetum, mature pollen grains and other parts of a mature anther exhibit either low or moderate reactions (Figs. 14, 16).

Discussion

1. Morphological:

The foregoing account of anther development in *Beta vulgaris* L. var. *saccharifera* indicates that pollen abortion in CMS plant is associated with abnormal formation of pseudoperiplasmodium. Similar observations have been recorded in other CMS strains of *Beta vulgaris* as well as in other CMS plants by several investigators (see KINOSHITA 1971 and LASER and LERSTEN 1972). ARTSCHWAGER[3] was first to suggest that the activity of tapetal plasmodium in the anthers of CMS sugar beets is hypermetabolic and is unable to benefit the developing microspores. According to him, the causative factor in the development of periplasmodium in sterile anthers may either be deficiency of food and nutrient reserves or may be due to a sudden release of metabolic waste products stored in large vacuoles. In the opinion of the present author's, the first possibility seems to be more appropriate as is evident by the low staining capacity of highly vacuolated plasmodial mass and their degenerated nuclei. This clearly reflects on their deficiency of nutrients.

This is further supported by the fact that the vascular differentiation in the anther connective of CMS anthers is much inhibited and parenchyma cells in this region showed signs of degeneration. Therefore, in all probability, the protoplasmic fragments of the tapetal cells wander among the microspores in quest of food meterial to leave them finally as non-viable and degenerated. Vascular deficiency in the anthers of CMS wheat and sugar beets have also been reported[10,11,16].

The present investigation reveals another notable feature. In the connective parenchyma of CMS anthers, hyaline intra-cellular granules develop.

Similar granules have also been observed in CMS as well as in chemically induced male-sterile plants of *Capsicum annuum, Datura alba* and *Solanum melongena*[6]. It is interesting to note that sugar beet plants infected by beet mosaic virus also show the presence of such granules[15]. No inference can, however, be drawn from this, excepting that formation of these granules in CMS anthers may either be a result of depleted vascular supply or may be due to deposition of substances left unutilized during the course of pollen development.

2. Histochemical:

The description on the localization of total carbohydrates of insoluble polysaccharides in the anthers of MF and CMS plants indicated inconspicous PAS reaction in the latter. These observations lend support to the findings of DE FOSSARD[7] in the anthers of *Chenopodium rubrum*. According to him, the development of endothecium is controlled by tapetum and only after the complete degeneration of tapetum, fibrous bands appear in the cells of this layer. In the presently studied material of CMS plants, the tapetal cells remain intact and seems to inhibit the formation of thickenings in the endothecium.

The present observations on the accumulation of starch grains in various parts of anthers of both MF and CMS plants are in line with those of HOSOKAWA *et al.*[8] on CMS sugar beets. They have observed high starch contents in both sterile and fertile anthers. However, according to them, the contents decreased and finally disappeared from MF anthers with age. On the other hand, in CMS anthers, starch grains persisted throughout development.

The present account on localization of DNA, histones and total protein in the anthers of MF and CMS plants indicates that the anthers of CMS plants are deficient of these substances. Similar observations have also been recorded in sorghum[1,5] and onion[18]. The deficiency of these vital substances serving as a limiting factor for the development of viable pollen grains in all probability is caused the inhibition of vascular supply. This in the opinion of the present authors, blocks the biochemical pathway for the synthesis of these substances. This results in the abnormal behaviour of tapetum and finally in the abortion of pollen. This is further corroborated by the histochemical and microautoradiographic studies of NAKASHIMA and HOSOKAWA[13]. They studied the changes of DNA, polysaccharides and proteins as well as translocation of photosynthetic products in the anthers of CMS sugar beets. On the basis of their observations they have concluded that insufficient supply of carbohydrates and other essential nutrients from the

tapetum to microspores was connected with the abnormality of tapetum and finally the male sterility in sugar beets.

Summary

1. The present investigation deals with the morphological and histochemical studies in cytoplasmic male-sterile plants of *Beta vulgaris* L. var. *saccharifera*.

2. Abnormal tapetal periplasmodium formation and vascular deficiency was found associated with pollen degeneration in the anthers of CMS plants.

3. Connective parenchyma cells in the anthers of CMS plants showed the presence of hyaline intracellular granules. These may either be due to vascular depletion or a result of deposition of substances left unutilized during pollen development.

4. Histochemical studies have indicated inconspicous PAS reaction and marked deficiency of DNA, histones and total protein in the anthers of CMS plants.

5. It is concluded from these studies that due to inhibition of vascular differentiation biochemical pathways for DNA, histones and proteins synthesis in CMS anthers might have been blocked. In all probability, this resulted in abnormal behaviour of tapetum and consequently pollen degeneration.

Acknowledgements

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Legend for Plate I

Figs. 1–8. Transverse part of anthers of CMS *Beta vulgaris*.

Fig. 1. Degeneration of PMCs and tapetal cells. 280×.

Fig. 2. Degenerated mass of PMCs and tapetal cells. 210×.

Fig. 3. Microspore tetrads in mass. 280×.

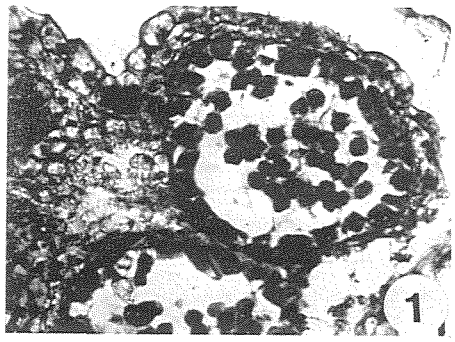
Fig. 4. Degeneration of pollen grains and tapetum. 280×.

Fig. 5. Degeneration of pollen grains, tapetum and middle layers. 280×.

Fig. 6. Mature anthers with degenerated mass. Note vascular inhibition. 80×.

Fig. 7. Anther connective with pro-cambial strand and the presence of hyaline granules in the parenchyma. 210×.

Fig. 8. Connective parenchyma with hyaline granules in mature anthers. 210×.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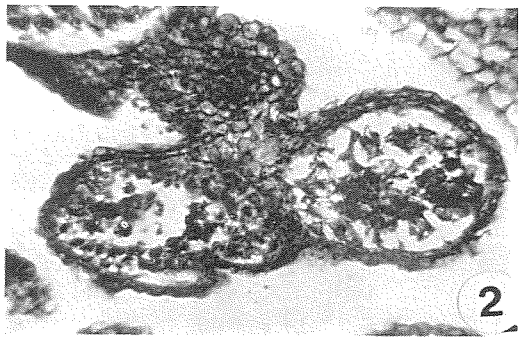
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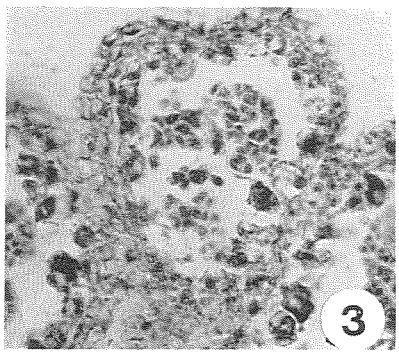
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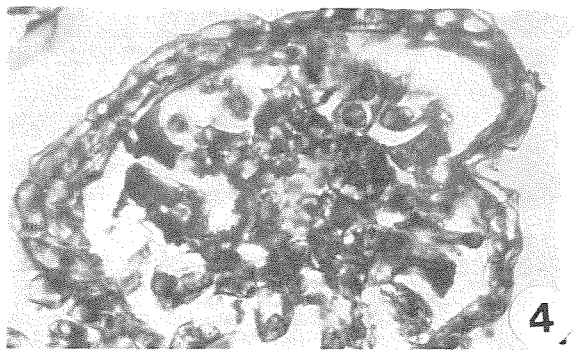
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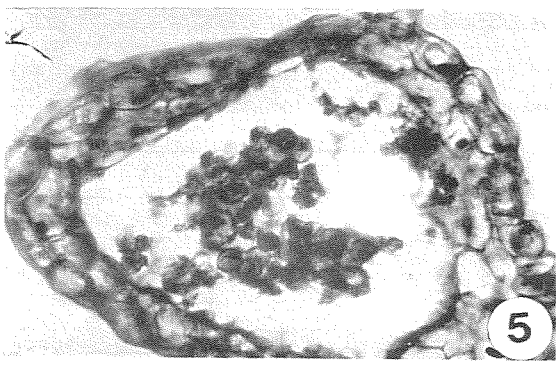
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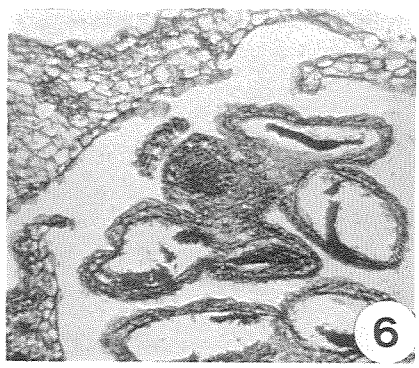
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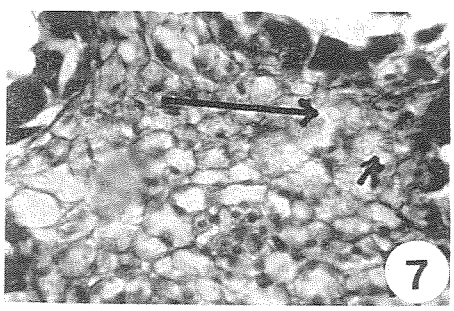
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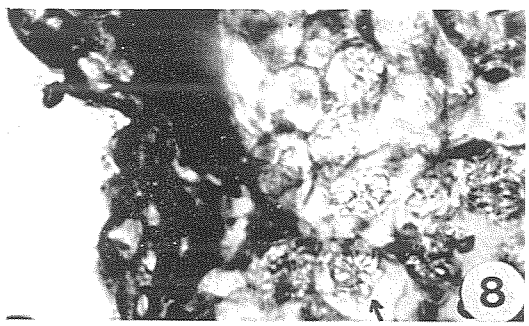
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Figs. 9-16. Histochemical localization in the anthers of MF and CMS *Beta vulgaris*.

Fig. 9. PAS reaction in the anthers of CMS plants at sporogenous tissue stage. 120×.

Fig. 10. PAS reaction in the anthers of MF plants. 120×.

Fig. 11. PAS reaction in the anthers of CMS plants. Note the presence of starch grains in connective region. Tapetal plasmodium and pollen grains are poorly PAS positive. 210×.

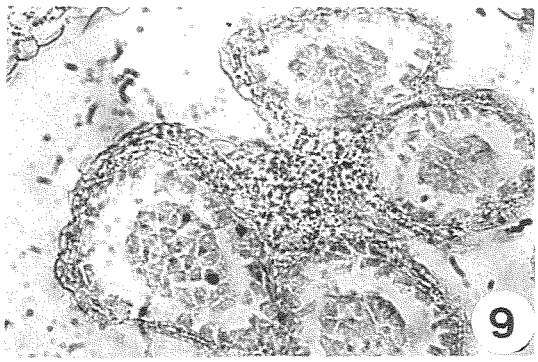
Fig. 12. Localization of proteins in the anthers of MF plant, at pollen grain stage. 210×.

Fig. 13. DNA localization in mature anthers of MF plants. 280×.

Fig. 14. Protein localization in mature anthers of CMS plants. 210×.

Fig. 15. Histone localization in mature anthers of MF plants. 210×.

Fig. 16. Histone localization in mature anthers of CMS plants. 80×.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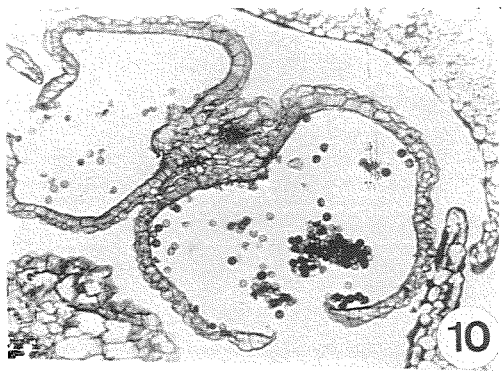
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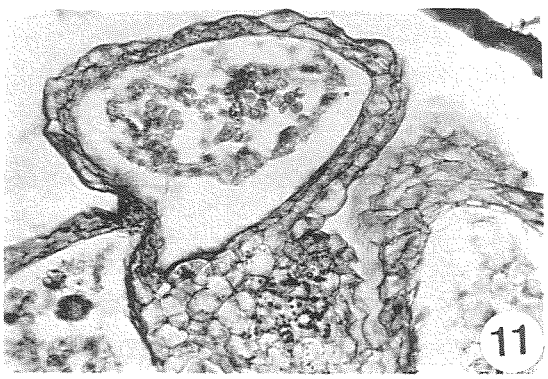
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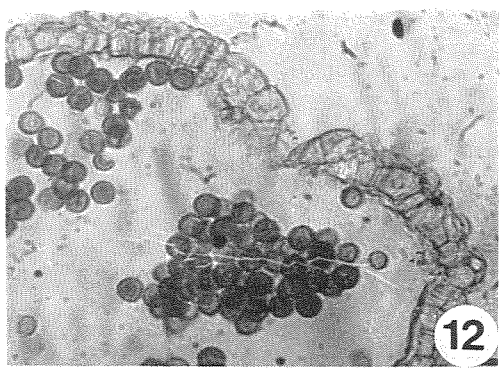
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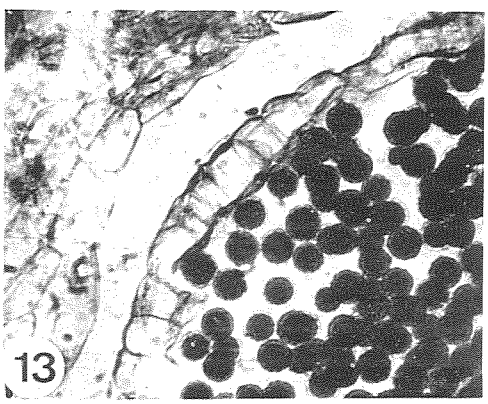
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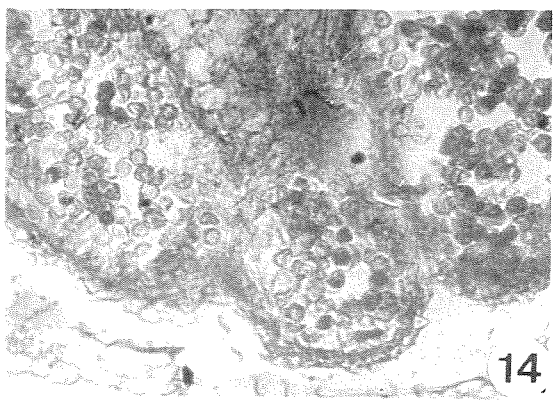
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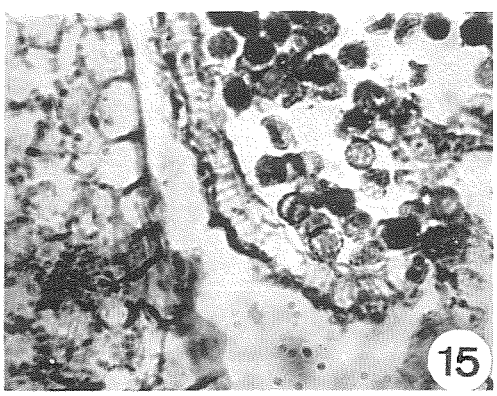
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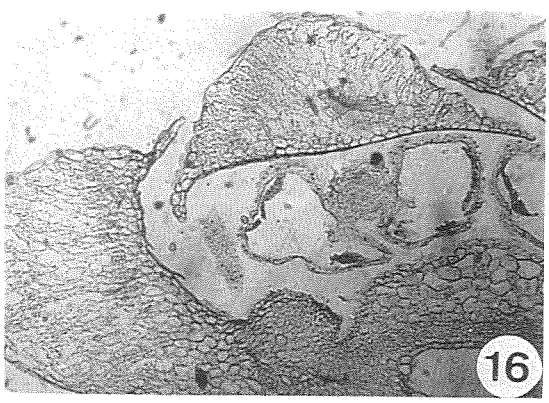
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