Histopathological Examination of Pelargonium Infected With Tomato Ringspot Virus

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ABSTRACT

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An anatomical study was carried out on tomato ringspot virus (TomRSV)-infected *Pelargonium* × *hortorum* 'Nittany Lion Red' to determine the relationship between macroscopic and microscopic symptoms and to determine the relationship between microscopic symptoms and male sterility in TomRSV-infected plants. Leaves, stems, roots, florets; individual anthers; and shoot, root, and floral meristems were examined. No anatomical changes were detected in vegetative tissues using light microscopy. However, pollen grain abortion, abnormal ovules, aborted ovules, and generalized tissue disintegration were detected in TomRSV-infected florets. Many of the symptoms seen in anthers and pollen grains resembled those of genetic male sterility in *P.* × *hortorum*.

Additional key words: floret abortion, seed propagation, integuments, tapetum.

Tomato ringspot virus (TomRSV) infects commercial geraniums, *Pelargonium* × *hortorum* Bailey. Although the symptoms induced by TomRSV in geranium have been studied (13), there have been no reports concerning the histopathology of symptom expression in infected plants.

Tomato ringspot virus was first reported in tobacco seedlings by Price (12) in 1936. In 1966, Teliz et al. (14) found that Tom RSV obtained from the American Type Culture Collection was transmitted by the nematode *Xiphinema americanum* Cobb. Seed transmission of TomRSV in soybean was reported by Kahn (8) and pollen transmission of TomRSV in *Pelargonium* × *hortorum* was reported by Scarborough (13).

Kemp (9) described symptoms of TomRSV-infected seedlings of *P.* × *hortorum* 'Nittany Lion Red' as stunted leaves with yellow or chlorotic spots and ringspots. Two months after infection, stunting remained, but new growth was symptomless. Scarborough (13) studied the symptoms of *P.* × *hortorum* cultivar Nittany Lion Red infected with TomRSV. Data obtained from observations of seedlings and cuttings showed that the major symptoms were interference with flowering and seed production. Floret abortion increased significantly and anther abortion occurred more frequently in infected plants. The viability of the pollen from TomRSV-infected plants was significantly reduced compared to that of their healthy counterparts. In addition, infected plants that were self-pollinated yielded fewer seed per pollination and the seed that were harvested exhibited lower germination.

Dale and Rogers (4) attributed male sterility in *P.* × *hortorum* to two recessive genes, ms_1 and ms_2. However, several of the lines that were test-crossed did not fit an identifiable test ratio and genes for male sterility other than ms_1 and ms_2 may have been responsible. In addition, male-sterile lines became male-fertile when environmental conditions varied.

Cohan (2) reported that genetic male sterility in geraniums resulted in degenerate anthers in several diploid inbred lines. The lines were classified into three groups based on phenotypic expression in which the presence or absence of aborted anthers equaled male-fertile or male-sterile, respectively, and a mixture of fertile and aborted anthers equaled partially fertile lines. In tests under different temperature regimes (16-30 C), reactions for the three classes varied. Male-fertile plants at low temperatures frequently were reclassified as either partially fertile or male-sterile at higher temperatures. The sterile anthers in partially fertile and male-sterile lines failed to dehisce and the microspores were degenerate.

With the discovery of genetically true breeding lines, seed propagation has become increasingly important in the geranium industry. However, male sterility causes a reduction in seed production and is an economic drawback to this method of propagation. Plant breeders have not been able to elucidate clearly the genetic basis for male sterility; macroscopically, symptoms of genetic male sterility and those due to TomRSV are similar. This study was initiated to determine if the microscopic symptoms of TomRSV infection are similar to those described for genetic male sterility. A histopathological examination was made of vegetative tissues, leaves, stems, roots, root and shoot meristems, and reproductive tissues (floral meristems, floret buds, and anthers) during a period of 19 weeks to determine the anatomical changes associated with TomRSV infection.

MATERIALS AND METHODS

Pelargonium × *hortorum* cultivar Nittany Lion Red

seeds (Park Seed Wholesale Inc., Greenwood, S. C.) were scarified by gently scraping away a small portion of the testa to expose the cotyledon (3). Seed then were planted in a steam-treated mixture of soil, peat, and perlite (1:1:1, v/v) supplemented with superphosphate (2.4×10^3 g/m^3), gypsum (1.2×10^3 g/m^3), magnesium sulfate (8.0×10^2 g/m^3), dolomitic limestone (1.6×10^3 g/m^3), and potassium nitrate (4.0×10^2 g/m^3) additives. Plants were fertilized daily with a solution containing Peters' 15-15-15 Geranium Special (15g/liter) (Robert B. Peters Co. Inc., Allentown, Pa.), potassium nitrate (0.25 g/liter), magnesium sulfate (0.25 g/liter), and sodium borate (0.005 g/liter) through a plastic-tube irrigation system. To promote growth and flowering, the geraniums received continuous illumination from 40 W Gro-Lux lamps (GTE Sylvania Inc., Denvers, Mass.) suspended approximately 1.5 m above the greenhouse bench.

The strain of TomRSV obtained from A. F. Ross, Department of Plant Pathology, Cornell University, Ithaca, New York, was propagated in *Cucumis sativus* L. 'Improved Chicago Pickling', purified, and positively identified by Ouchterlony gel double diffusion (11). Leaves or geranium seedlings at the four- to six-leaf stage were dusted with Carborundum and mechanically inoculated with TomRSV-infected cucumber cotyledons ground (1:1, w/v) in 0.05 M phosphate buffer, pH 7.2.

Sampling began 4 weeks after the geranium seedlings were inoculated. Samples of leaves, stems, roots, and shoot and root apical meristems were collected weekly from three infected and two healthy plants. Portions of sampled leaves, stems, the base of meristems (a 1-mm slice approximately 1-2 mm below the meristematic dome), roots, and root tips were indexed to verify the presence of virus. Tissue samples were ground (1:1, w/v) in 0.05 M phosphate buffer containing 4% polyethylene glycol 6000 (PEG) and mechanically inoculated onto Carborundum-dusted primary leaves of *Vigna sinensis* (Torner) Savi 'Big Boy'.

To determine any histological changes associated with reproductive tissues, healthy and infected vegetative and floral meristems, healthy and aborted floret buds, and healthy and aborted anthers were collected and indexed on a weekly basis for 15 weeks. Floret buds and anthers were indexed by removing a portion of the inflorescence peduncle and indexing as described above. It was assumed that the infection was systemic throughout the inflorescence if the index was positive.

Samples were fixed in formalin-acetic acid-alcohol (7) and dehydrated in a tertiary butyl alcohol series (7). Anthers and floret buds were aspirated in the fixative and throughout the dehydration to remove air. Paraplast (Sherwood Medical Industries, St. Louis, Mo.) was used for infiltration and embedding. Before sectioning, stem and shoot apical meristem samples were softened in a 10% glycerine solution containing 1% sodium lauryl sulfate (Dreft) for 24 and 29 hours respectively (1). All samples were placed in ice water to chill before cutting. A rotary microtome was used to cut serial sections 10 μm thick. The sections were then mounted and stained. Johansen's Modified Quadruple Stain was used for leaves, stems, roots, anthers, and florets (7) and Harris' Hematoxylin and Orange G was used to stain shoot and root meristems (7). Histochemical tests included tests for starch (I-KI on florets, stems, leaves), callose (lacmoid method on florets), gum (phloroglucinol method on florets), cellulose (zinc-chlor-iodide reaction and I-KI and H_2SO_4 method on florets), and suberin (Sudan IV test on florets) (6, 7). In addition, polarized light was used to detect starch and cellulose in florets, floral and vegetative apical meristems, leaves, stems, roots, and root meristems. The tissues were examined on a Leitz Ortholux research microscope and photomicrographs were taken with Kodak Plus X Pan film on a Leitz Aristophot camera with a 10.2 × 12.7-cm (4 × 5-inch) Graflex back.

RESULTS

Throughout the study, a total of 40 control plants and 60 TomRSV-infected plants were indexed. All indices of control plants were negative whereas all indices of TomRSV-inoculated plants were positive.

Vegetative tissues.—Macroscopic symptoms on geranium leaves first were observed 7 days after inoculation with TomRSV. The symptoms varied from chlorotic flecks on younger leaves to chlorotic ringspots on older leaves. Approximately 17-21 days after inoculation, leaf symptoms faded and young expanding leaves were symptomless.

A microscopic examination of noninfected control tissues of *P.* × *hortorum* cultivar Nittany Lion Red revealed the anatomical structure of a typical dicotyledonous plant. The youngest fully expanded leaves are composed of an upper epidermis, palisade layer, spongy mesophyll, and lower epidermis. Transverse and longitudinal sections of stems, taken one to two nodes below the growing shoot, show that the epidermis and cortex form the outer tissues and a cortical starch-laden sheath of cells surrounds the vascular cylinder. Collateral vascular strands with sieve tube members and lignified xylem vessel elements are separated by interfascicular tissue. The pith tissue fills the center of the stem and the cells contain abundant amounts of starch. Longitudinal sections of shoot apical meristems of Nittany Lion Red show densely protoplasmic cells with large nuclei present in two to three tunica layers and in the corpus subtending the tunica. Root apical meristems, sectioned longitudinally, are characterized by a small group of initials which collectively give rise to the root cap, epidermis, cortex, and stele. Transverse and longitudinal root sections, taken 5 mm behind the root tip, show a typical young dicotyledonous root. A suberized epidermis and a cortex with an endodermis constitute the outer tissues. Xylem and phloem are in a diarch configuation.

Tomato ringspot virus-infected tissue, the same age as the control material, showed no anatomical changes in leaves, stems, roots, and shoot and root meristems of plants when examined under a light microscope.

A histochemical test for starch (I-KI) in stems and leaves was positive for control and inoculated plants in both studies. Starch detection using polarized light on plant samples was positive in stems, leaves, and root tips from infected and control plants but it was negative in roots and shoot meristems of infected and control plants. Cellulose detection was positive for both control and infected plants.

Reproductive tissues.—The floral meristem in geranium is initiated beside the vegetative meristem. It

then differentiates into several individual floret primordia and forms the characteristic umbel inflorescence of *P.* × *hortorum*. The cells at the apex of each floret primordium contain large nuclei and are densely protoplasmic. Each

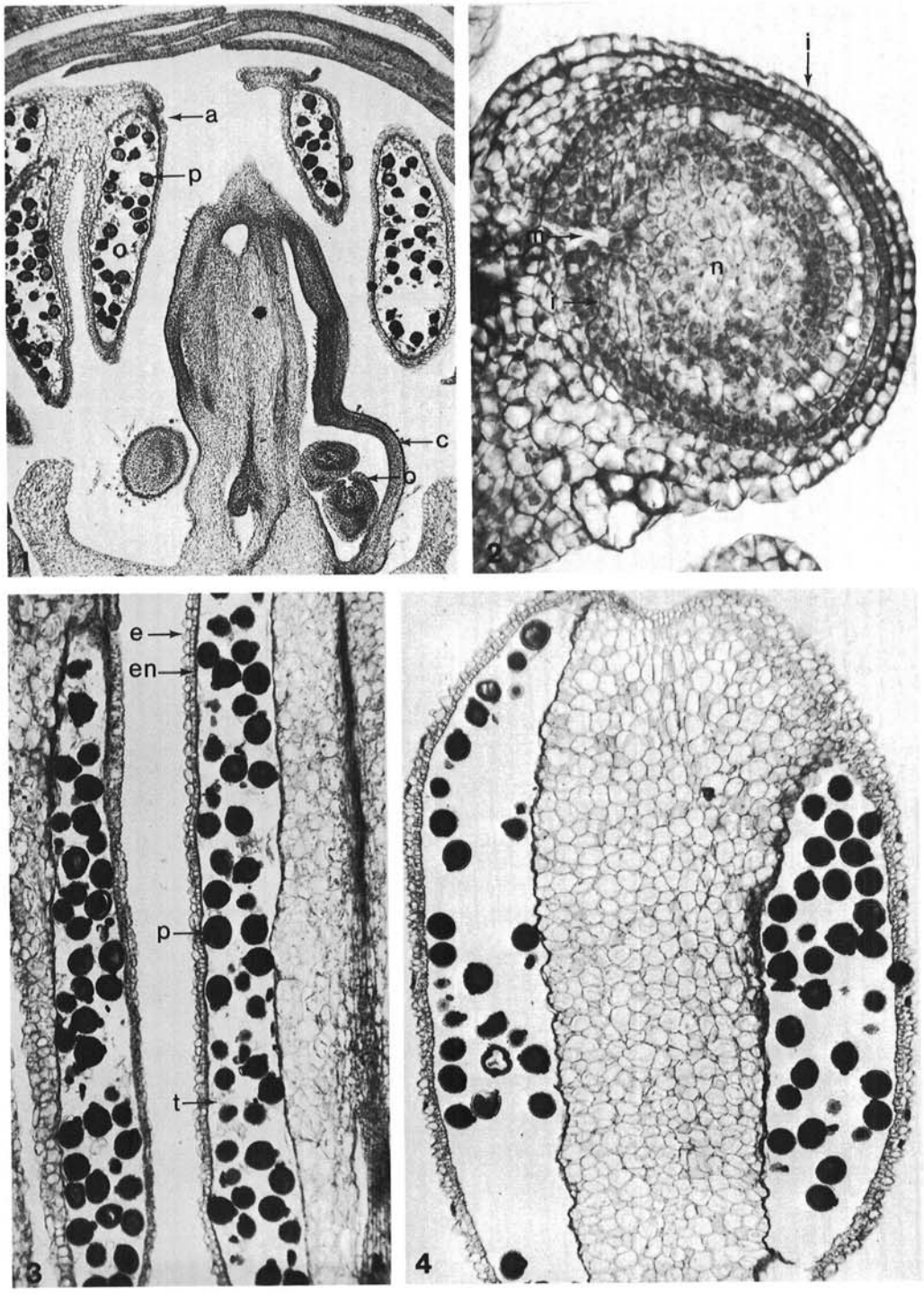

Fig. 1-4. Healthy tissues of *Pelargonium* × *hortorum* 'Nittany Lion Red'. **1)** Portion of a longitudinal section of a healthy floret showing anther (a), pollen (p), carpel (c), and ovule oriented hemianatropously (o) (× 46). **2)** Portion of a longitudinal section of an ovule showing integuments (i), nucellus (n), and micropyle (m) (× 356). **3)** Portion of a longitudinal section of an anther showing epidermis (e), endothecium (en), pollen with spiny exine (p), and disintegrated tapetum (t) (× 85). **4)** Portion of a longitudinal section of a healthy anther taken from a floret at anthesis (× 85).

floret primordium gives rise to sepals, petals, stamens, and carpels in succession and then becomes inactive.

The comparison of floral meristems between control and TomRSV-infected plants was restricted to that stage of development where the floret primordia were visible but had not yet initiated individual floret structures such

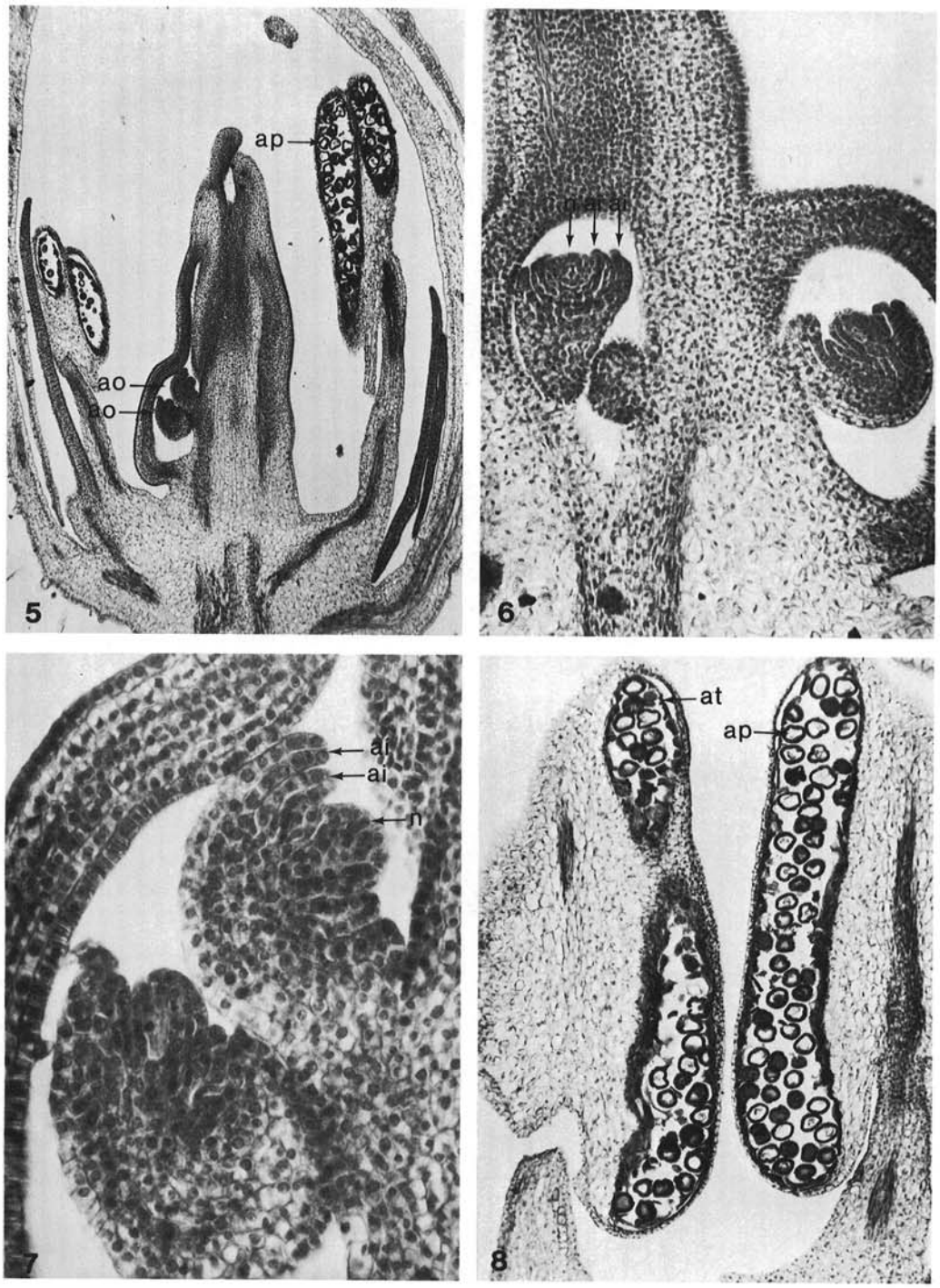

Fig. 5-8. Stage I tomato ringspot virus-infected floret of *Pelargonium* × *hortorum* 'Nittany Lion Red' which macroscopically appeared green and healthy but microscopically showed aborted pollen grains and abnormal ovules. **5)** Portion of a longitudinal section of a floret showing aborted pollen (ap), and abnormal ovules (ao) (× 46). **6, 7)** Portions of a longitudinal section of an ovule showing abnormal integuments (ai) which do not enclose completely the nucellus (n) (Fig. 6, × 178), (Fig. 7, × 356). **8)** Portion of a longitudinal section of an anther showing aborted pollen (ap) and an abnormal tapetum (at) (× 85).

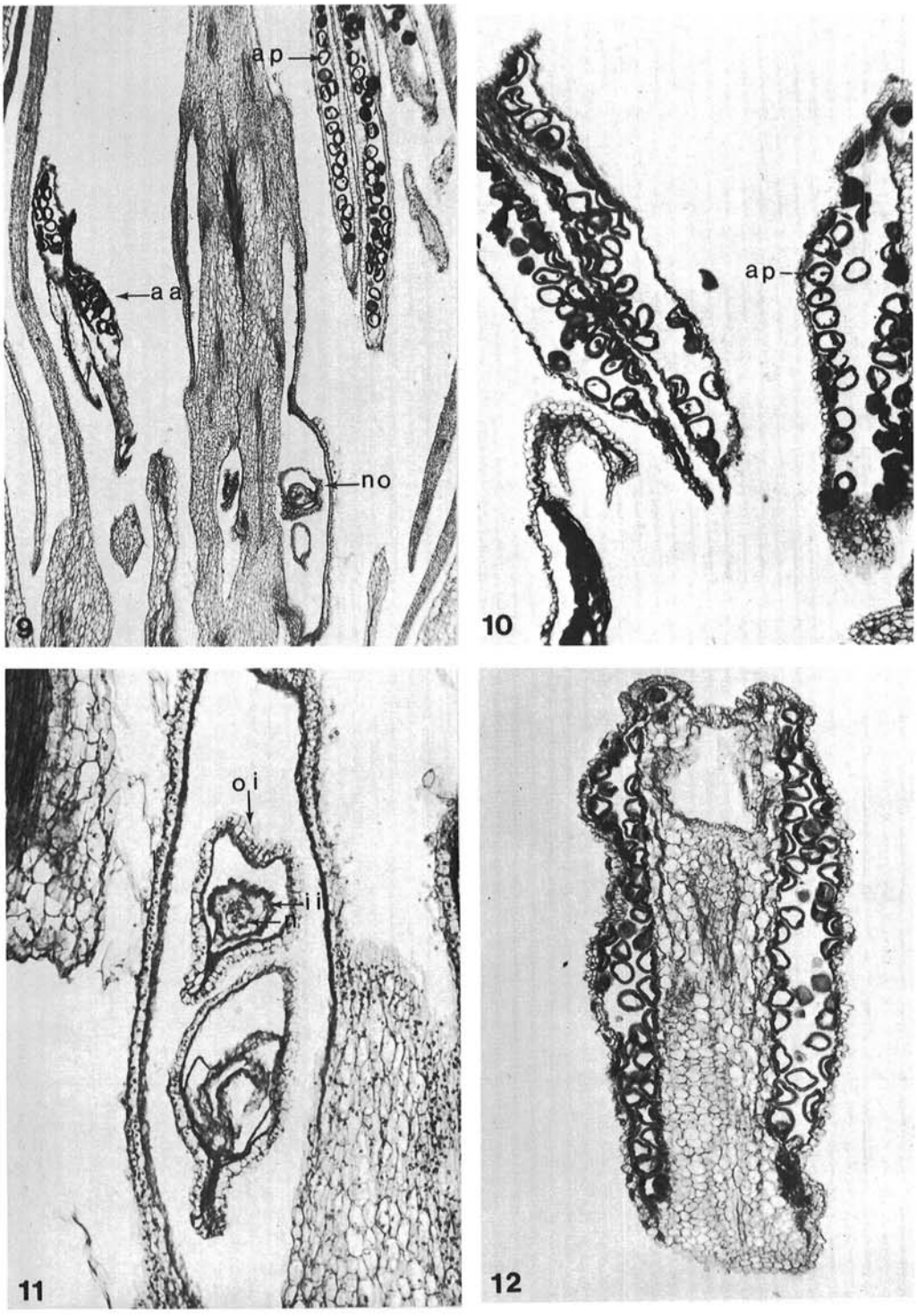

Fig. 9-12. Stage II tomato ringspot virus-infected floret of *Pelargonium* × *hortorum* 'Nittany Lion Red' which macroscopically appeared chlorotic to necrotic and microscopically showed complete degeneration of reproductive tissues. **9)** Portion of a longitudinal section of a floret showing aborted pollen (ap), aborted anther (aa), and a necrotic ovule (no) (× 46). **10)** Portion of a longitudinal section of an aborted anther showing aborted pollen (ap) (× 85). **11)** Portion of a longitudinal section of ovules showing nucellus (n) and inner integument (ii) which have shrunk from the outer integument (oi) (× 178). **12)** Portion of a longitudinal section of an aborted anther taken from a floret at anthesis (× 85).

as petals and stamens. No difference could be detected by the light microscope between control and infected plants.

The florets of cultivar Nittany Lion Red are bisexual structures. Control florets 5-7 mm long were examined macroscopically and microscopically using longitudinal sections (Fig. 1-3). The flower consists of an outer whorl of five sepals surrounding an inner whorl of five petals. The androecium consists of five to seven stamens. Each anther is two-lobed and four-loculed, and near maturity, the only tissues visible are the epidermis, endothecium, a well-disintegrated tapetum, and parenchyma between the lobes and locules. The granular pollen is densely protoplasmic, trinucleate, and has a spiny exine (Fig. 3). The gynoecium is a compound pistil which has a superior ovary and consists of five connate carpels, five styles, and five stigmas. The two ovules in each carpel exhibit axile placentation and are borne vertically with the uppermost ovule in an anatropous orientation and the basal ovule in a hemianatropous orientation (Fig. 1, 2). Two integuments completely surround the nucellus, except for the micropyle (Fig. 2).

Macroscopic symptoms of degeneration of the reproductive tissues of infected florets included chlorosis, which progressed to necrosis of the floret buds. Buds which were chlorotic or necrotic were termed aborted. The number of aborted buds ranged from one to several per inflorescence. By the time the sepals and petals of a floret were chlorotic, the tissues interior to the petals were usually necrotic. Therefore, to obtain samples at an intermediate stage of disease development, green or healthy appearing floret buds also were sampled.

A microscopic examination revealed two general stages of disease development in floret buds:

Stage I.—Stage I was characterized by floret buds that were green and appeared to be normal macroscopically. Microscopically, the most conspicuous symptom was the degeneration of the ovules and pollen. The ovules were oriented correctly within the ovary and the development of the nucellus appeared normal. However, the two integument layers failed to surround the nucellus completely in many virus-infected plants (Fig. 5-7).

Cytoplasm within the pollen grains either was absent or had shriveled to a small mass in the center of the pollen grain. In addition, walls of many pollen grains partially were collapsed, which caused the pollen grains to appear distorted or crescent-shaped (Fig. 8). Occasionally, even though the cytoplasm was still present the pollen grain was distorted. A generalized initiation of cellular disintegration appeared in a few anthers.

The symptoms described for the ovules and pollen grains were usually uniformly present throughout a floret or not present at all. In addition, the symptoms in the ovule and pollen grains occurred independently of each other. Although these symptoms also were detected occasionally in control florets which indexed negative (i.e., were free of TomRSV), they were seen with much greater frequency in TomRSV-infected plants.

The tapetum in anthers of control plants usually was degenerate and well dispersed throughout the anther at this stage of maturity (Fig. 3). Many virus-infected and some healthy florets exhibited a tapetum that was not well degenerated and remained attached or close to the endothecium (Fig. 8). It could not be determined whether this was more prominent in virus-infected plants. The sepals and petals remained unaffected at this stage of disease development.

Stage II.—Stage II florets were characterized macroscopically by chlorotic or necrotic sepals. As mentioned before, by the time the buds were chlorotic, degeneration of the inner tissues was well established (Fig. 9). Cytoplasm in pollen grains had degenerated to a small mass in the center of the pollen or was absent. Wall collapse of pollen grains became more pronounced as overall disintegration of the floret progressed.

Anthers were shrunken and a generalized cellular disintegration was evident in anthers and filaments. Anthers which were most severely affected were shriveled and severely distorted but still retained the pollen grains (Fig. 10). The tapetum of anthers in Stage II was totally disintegrated and not visible.

The gynoecium also exhibited shriveling and generalized cellular breakdown (Fig. 9). The nucellus and inner integument shrank away from the outer integument and were the first tissues to disintegrate (Fig. 11). The funiculus and outer integument were more persistent as were the ovary and style.

Tissues that were not involved in reproduction, the petals and sepals, were the last to be affected by the disease progression. Cells around the vascular system collapsed, after which the remaining cells in those tissues also disintegrated.

Comparisons also were made between healthy and aborted anthers from flowering florets. Macroscopically, healthy anthers were plump and a bright pink color. Aborted anthers were smaller, shriveled, and ranged in color from tan to brown. In addition, aborted anthers failed to release the pollen. Microscopically, symptoms exhibited by aborted anthers and pollen grains were the same as those in a Stage II floret (Fig. 4, 12).

Histochemical tests for cellulose, pectin, callose, gum, and suberin, on control and Stage II florets revealed no major differences between them. Starch tests (polarized light and I-KI) did reveal differences. Control and Stage I florets contained large amounts of starch in the style, ovary, anthers, and filaments whereas Stage II florets contained little or no starch.

DISCUSSION

Disease progression.—Pollen grain abortion and/or abnormal ovules from infected Stage I florets appear to be precursors of the symptoms seen in Stage II florets. In addition, during Stage II, breakdown of reproductive tissues occurs before breakdown of nonreproductive tissues.

Based on the above evidence, the following description of disease progression is proposed: (i) The cytoplasm of the pollen grains disintegrates and is associated with collapse of the pollen grain walls. It is unknown whether abnormal tapetum development contributes to the degeneration of the pollen grains. Abnormal ovules with integuments which do not entirely enclose the nucellus are common at this stage. (ii) Degeneration of the pollen grains and ovules continues and triggers degeneration of all other tissues associated with reproduction. Breakdown products of starch may be transported from the degenerating floret to more viable florets. Petals and sepals still appear normal. (iii) Extreme cellular

breakdown of the reproductive tissues is evident. The nucellus and inner integument disintegrates completely in some florets. Cellular disintegration also is present in the petals and sepals and cellular collapse around the vascular system is evident in all tissues.

Male sterility.—Male sterility has been attributed to recessive genes (4). However, test-cross progeny of several of the lines did not fit the expected test ratios. Cohan's (2) description of genetic male sterility in *P.* × *hortorum* is very similar to the description of male sterility induced by TomRSV. In both studies, an abnormal tapetum was observed which failed to separate from the anther wall and exhibited delayed degeneration. Aborted anthers appeared shrunken and collapsed and pollen grains within such anthers became devoid of cytoplasm, degenerated, and exhibited collapsed walls. Further, in Cohan's study, male-sterile lines were unstable and fertility or sterility often was environment-dependent. Walsh et al. (15) found that symptom expression in tobacco ringspot virus-infected geraniums was influenced by temperature and light. We found that production of aborted florets and aborted anthers declined as the study continued throughout the summer and into early fall. Environmental factors, such as light and temperature or plant age, were possible influences.

Cohan (2) also ran an analysis of free amino acids in male-fertile and male-sterile lines using thin-layer chromatography. In floret buds which were 1.0-1.5 cm long (near anthesis), proline was detected in all samples of male-fertile anthers, but it was consistently absent in male-sterile lines. A preliminary experiment (W. Oglevee and D. J. Murdock, *unpublished*) using the techniques employed by Cohan, was undertaken to analyze the free amino acids of healthy anthers from control plants and aborted anthers from TomRSV-infected plants. The results were very similar to Cohan's results with proline consistently present in healthy anthers and absent in aborted anthers.

Male sterility in geraniums partially is under genetic influence. However, several factors point to the importance of natural infections of TomRSV in male-sterile geranium lines. (i) Plant breeders have not been able to elucidate completely the inheritance of male sterility in geraniums (4). (ii) Symptoms of virus infections are known to be environment-dependent (10, 15). (iii) Scarborough (13) has shown that aborted anthers and nonviable pollen grains occur more frequently in TomRSV-infected geraniums. (iv) Macroscopic and microscopic symptoms of male sterility in geraniums are similar to sterility induced by TomRSV (2). (v) Preliminary analyses of free amino acids from male-fertile and -sterile anthers and healthy and TomRSV-infected anthers are similar (2).

Female sterility.—Female sterility in geranium has not been widely studied by plant breeders. There is evidence that female sterility is influenced by TomRSV infection. Scarborough (13) found that crosses between TomRSV-infected seed parents and healthy pollen grains resulted in a significant reduction in number of fruit produced per pollination, a reduced seed yield per pollination, and a reduced seed content per fruit compared to self-pollinated healthy controls. It is possible that TomRSV infection of the seed parent results in such reductions because of abnormalities in ovule development. Ovules with defective integuments are seen more frequently in TomRSV-infected plants. In such ovules, the development of the integuments does not keep pace with the development of the nucellus and, at maturity, the nucellus is not completely enclosed by the integuments. Following fertilization in normal plants, the integuments develop into the seed coat (5). Therefore, if an ovule with defective integuments does not abort before fertilization, abortion of the seed would be probable.

From the results of our study and those of Scarborough (13), we conclude that vegetative tissues are largely unaffected by TomRSV, but that the morphology of reproductive structures is altered significantly. Both studies also indicate that male sterility owing either to genes or TomRSV infection, cannot be differentiated by macroscopic or microscopic symptoms. A major impediment in elucidating genes for male sterility may be naturally occurring infections of TomRSV and similar viruses. Methods are available for indexing and maintaining healthy breeding stock. Until plant breeders avail themselves of such stock, the true role of genes in male sterility in geranium cannot be determined.

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