# Histopathology of the Chrysanthemum Cultivar Bonnie Jean Infected with Chrysanthemum Stunt Viroid

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## ABSTRACT

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Plants of *Chrysanthemum morifolium* 'Bonnie Jean' were inoculated with chrysanthemum stunt viroid. At 7-day intervals portions of stems and leaves and entire shoot meristems were fixed, dehydrated, embedded, sectioned, and stained to examine changes in the anatomy of infected plants. Apical leaves were checked for the presence of the viroid by polyacrylamide gel electrophoresis and ethidium bromide staining. Infected plants were stunted and developed a pronounced curvature of the stem. There was disruption and dysfunction of the actively dividing cambial initials resulting in the replacement of portions of the xylem and phloem with hypertrophied and hyperplastic tissue. In most stem sections, the vascular cambium was distorted and necrotic. Other anatomical changes included areas of cell hypertrophy and dark staining intercellular material in the cortex. In some cases, the unicellular epidermal layer was replaced by a multicellular layer or by cavities containing wound gum and pectin. In leaf tissue, the palisade cells and upper and lower epidermal cells were necrotic. In the apical meristems, there was a breakdown of individual cells in the rib meristem or groups of cells in vertical files extending through the tunica-corpus region into the rib meristem.

Chrysanthemum stunt was first described on the florists' chrysanthemum, *Chrysanthemum morifolium* (Ramat.) Hemsl. in 1947 (7). The disease rapidly reached epidemic proportions and severe losses were incurred by the industry. The causal organism was thought to be a virus until Diener and Lawson (6) reported that the chrysanthemum stunt pathogen had biochemical properties similar to those described for the potato spindle tuber viroid (PSTV) (5).

Symptom expression on plants infected with chrysanthemum stunt viroid (CSV) varies with the chrysanthemum cultivar and environmental conditions (13). Symptoms on *C. morifolium* 'Bonnie Jean' were described as abnormal upright growth and chlorotic flecking and spotting on the upper and lower leaves (2). Stunting is particularly evident in mature plants, which may be half the height of uninfected plants. Bud formation is generally premature with inferior blooms opening 7–10 days earlier than those on uninfected plants.

The cytopathological effect of CSV on chrysanthemum has not been studied. This study was initiated to determine the histology of stems, leaves, and shoot meristems at different times after infection of the chrysanthemum cultivar Bonnie Jean infected with CSV.

## MATERIALS AND METHODS

Culture-indexed, rooted cuttings of *C. morifolium* 'Bonnie Jean' (supplied by California-Florida Plant Corp., Fremont, CA) were planted in a 1:1:1 soil mixture consisting of peat:perlite:soil. Potted plants were sunk in perlite-containing benches over heating cables to maintain a constant soil temperature of 29 C, the optimum temperature for symptom expression (13). The plants were maintained under continuous light to prevent floral initiation. A Chapin watering system (23) was used for watering each plant, and plants were fertilized at the time of planting with the slow-release fertilizer Osmocote, 14-14-14. Each bench was enclosed with hardware cloth to minimize cross contamination among plants by mechanical means.

Known healthy and CSV-infected indicator plants of the cultivar Bonnie Jean were used for implant inoculations using two surgical cannulas (8). A plug from the succulent tissue of the upper two nodes of the accepter plant was removed, discarded, and replaced with a plug taken from the donor plant or host indicator plant.

Plant stems were sampled before implant inoculations to compare the stem anatomy with stem tissue from plants inoculated with uninfected tissue to confirm that inoculation techniques had not caused significant changes in the host plant anatomy. Sampling of CSV inoculated plants began 1 wk after inoculation and continued weekly for 10 wk. Plants were cut off at the soil line and leaves and branches removed with a razor blade. Before sampling, apical leaves were removed, ground in liquid $N_2$, and assayed for the presence of CSV on cylindrical polyacrylamide gels (18). Stem pieces were fixed in Rawlins formalin-aceto-alcohol fixative (19). Upper and lower leaves with the petioles attached were removed, and 5-mm sections along the midrib were fixed in Craf's solution (3). Vegetative and reproductive shoot meristems and several leaf primordia were removed with a razor blade with the aid of a dissecting microscope. Shoot meristems were fixed using the same procedures described for stem material. Fixed specimens were dehydrated in a tertiary butyl alcohol series (15) and infiltrated and embedded in Paraplast (Sherwood Medical, St. Louis, MO). The stem material was softened for 18–24 hr in a softening solution consisting of 1% sodium lauryl sulfate and 10 ml of glycerol (1) before sectioning. Stem and leaf tissues were sectioned on a rotary microtome at 10–13 $\mu$m. Serial sections of shoot meristems were cut at 10 $\mu$m on a rotary microtome. Haupt's adhesive and a 4% formalin solution were used to mount the paraffin ribbons on chemically cleaned slides. Sections were stained with Johansen's Quadruple Stain (15). Histochemical tests for pectin (iron-absorption), suberin (Sudan IV), gums (orcinol), cellulose (I-KI), callose (lacmoid), and lignin (Maule) as well as the use of polarized light for cellulose detection, were used on selected tissues to determine the chemical constituents of the tissues (14,19). All sections were examined using a Leitz Ortholux research

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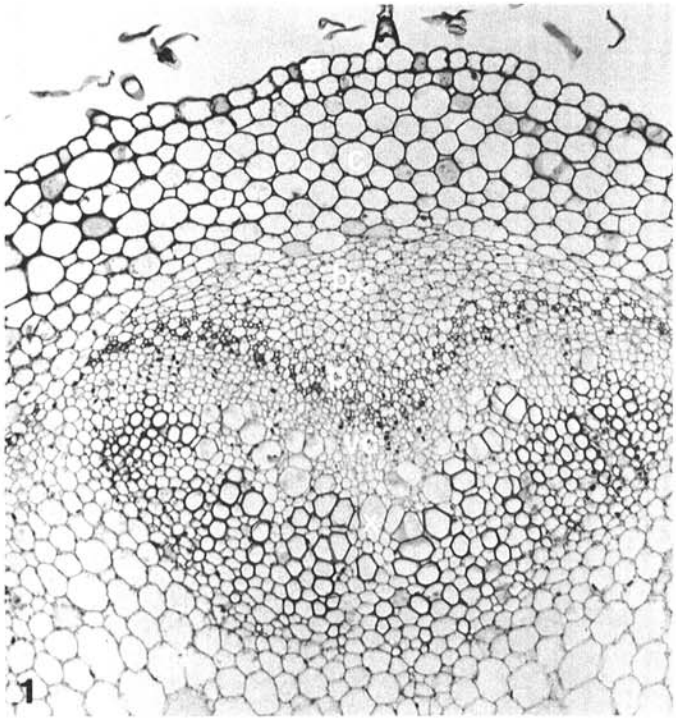
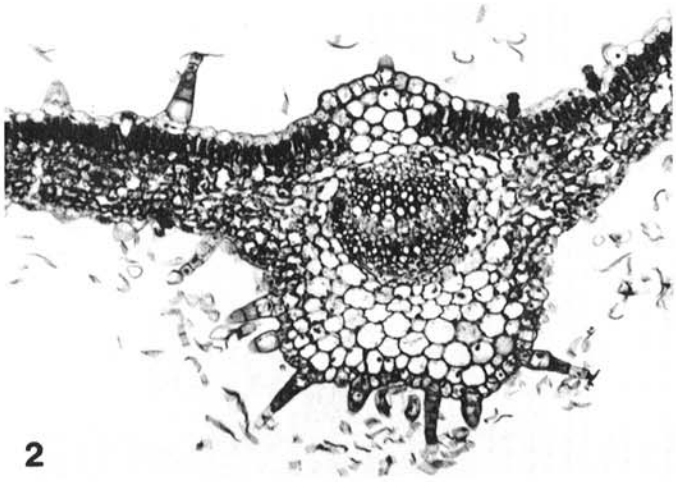
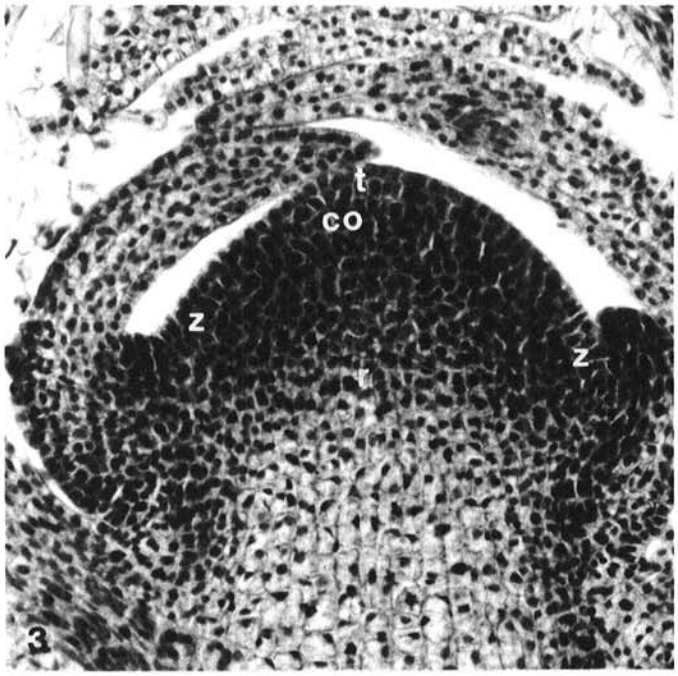







microscope and photographed on Kodak Plus-X-Pan film using a Leitz Aristophot camera with a 10.16 × 12.70 cm Graflex back.

## RESULTS

**Symptomatology.** Symptom expression occurred 4 wk after inoculation when CSV-infected plants began to display a severe curvature of the stem. During the sampling period, 4–10 wk after inoculation, plants often were stunted and showed spotting and flecking on the upper leaves first and eventually on the lower leaves. No consistent pattern of sequential symptoms was observed. Apical meristems removed from CSV-inoculated plants were shaped abnormally compared with those of uninoculated plants. In severely infected plants, the apical meristems were twisted, stunted, and sometimes deformed.

**Pathogen recovery.** CSV was detected by gel electrophoresis at 3 wk after inoculation from all inoculated plants. Nucleic acid bands characteristic of CSV evident in gels layered with tissue extracts from inoculated plants were not evident in preparations from uninoculated plants.

**Histology of uninoculated plants.** Transverse (Fig. 1) and longitudinal stem sections were examined to determine the anatomy of uninoculated Bonnie Jean stems. The stem anatomy was similar to that previously described for the cultivars Yellow Delaware (9) and Mandalay (22). Transverse sections of leaf midrib tissue from apical and basal regions showed an upper and lower epidermis each consisting of a single layer of cells (Fig. 2). Palisade parenchyma cells were generally oblong to angular and compact in arrangement. Spongy mesophyll was loosely spaced and consisted of irregularly shaped cells. The vascular bundles consisted of a bundle sheath, phloem, vascular cambium, and xylem.

Serial longitudinal sections of vegetative and reproductive meristems were examined. Median sections of vegetative meristems consisted of a one to two layered tunica, a corpus and a distinct file of cells comprising the rib meristem, and a peripheral zone on either side of the central tissue region (Fig. 3). The anatomy of reproductive meristems differed from that of vegetative meristems in the flatter and wider apex, which is the region where floral primordia originate.

**Histology of plants inoculated with CSV. Stems.** Anatomical changes in plants infected with CSV were evident 4 wk after inoculation but were most evident in samples taken 7 wk after inoculation. The most extensive changes occurred in the upper portion of the plant. The vascular cambium was the first and most severely affected tissue, and changes in the cambium appeared to be responsible for the subsequent changes in other tissues. Histological changes in the xylem, phloem, and cortex were not as extensive as those observed in the vascular cambium. In the lower portion of the plant, hypertrophied cells were observed occasionally between the thick-walled fiber cells of the bundle cap (Fig. 4). These hypertrophied cells often had cell walls that appeared indistinct, and nuclei were not always visible.

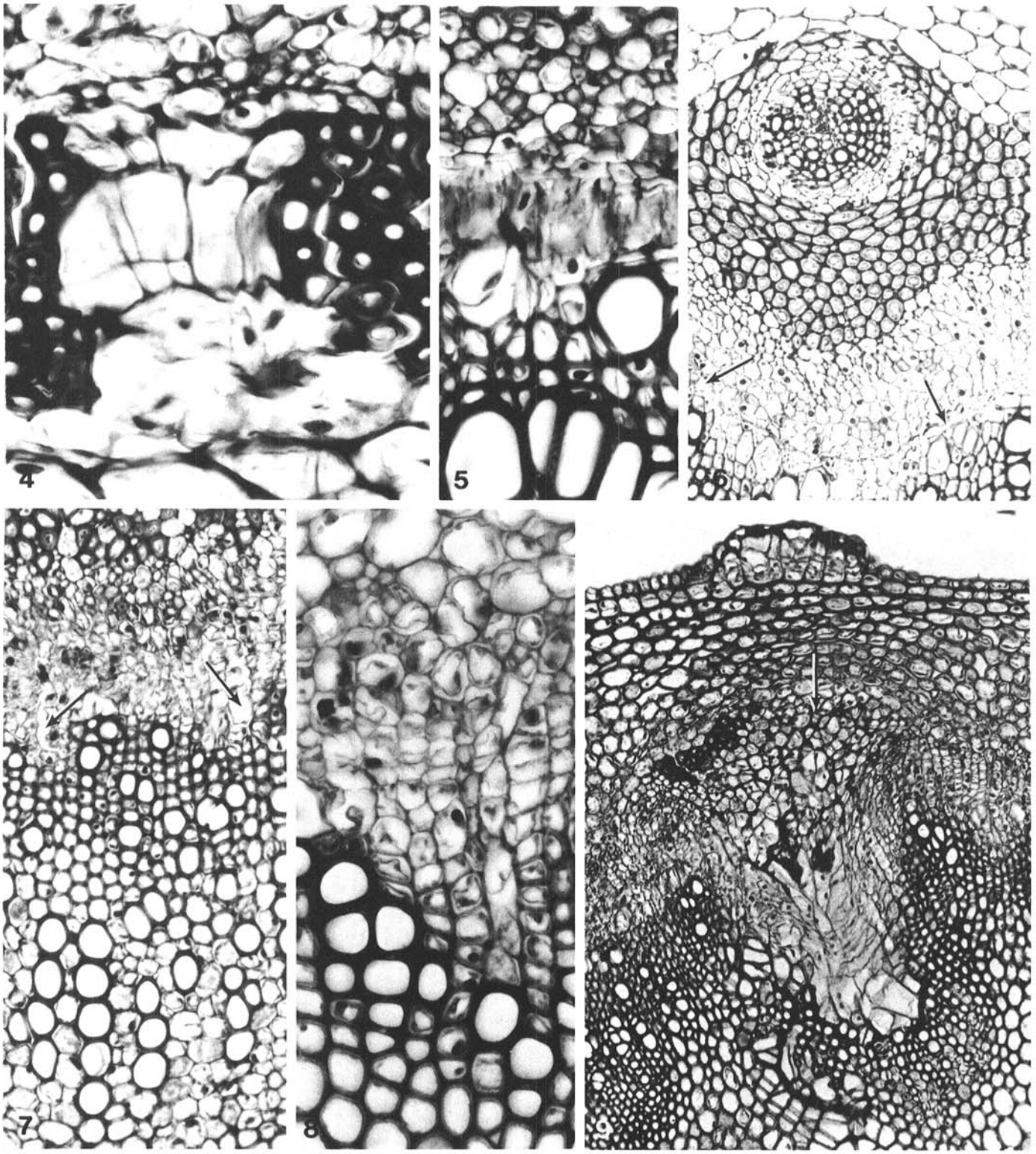
In the upper portion of the plant, cambial cells in some vascular bundles appeared distorted and were difficult to distinguish from the adjacent phloem and xylem cells (Fig. 5). In other areas, the cells of the vascular cambium appeared to be crushed (Fig. 6). Dysfunction of the vascular cambium was evident in vascular bundles in infected tissue and the cambial initials often were not evident and lacked distinct cell walls (Fig. 7). Breakdown of cambial initials occurred as well as incomplete differentiation of the cambial initials, resulting in tissue consisting of thin-walled,

**Figs. 1–3.** Sections of tissue of uninoculated plants of *Chrysanthemum morifolium* 'Bonnie Jean' stained with Johansen's Quadruple Stain. **1,** Transverse section of a portion of a stem showing the epidermis (e), cortex (c), bundle cap (bc), phloem (p), vascular cambium (vc), xylem (x), and pith (pi) (×115). **2,** Transverse section of an apical leaf blade showing the overall arrangement of tissues (×14). **3,** Median longitudinal section of a vegetative shoot meristem showing the one to two layered tunica (t), corpus (co), peripheral zone (z), and the rib meristem (r) (×230).

often rectangular cells arranged in files (Fig. 8). The xylem parenchyma cells were often chromophilic and hypertrophied.

Infected plants showing extensive external symptoms on one side of the plant also showed extensive anatomical changes on the same side of the plant. In these areas, the vascular tissue was replaced by hypertrophied cells (Fig. 9), and nuclei in these cells were elongate and chromophilic. Pectin accumulated in pockets in the center of the hypertrophied and hyperplastic tissue found in apical portions of the stem, and remnants of vascular tissue were observed in the center of these pockets (Fig. 10).

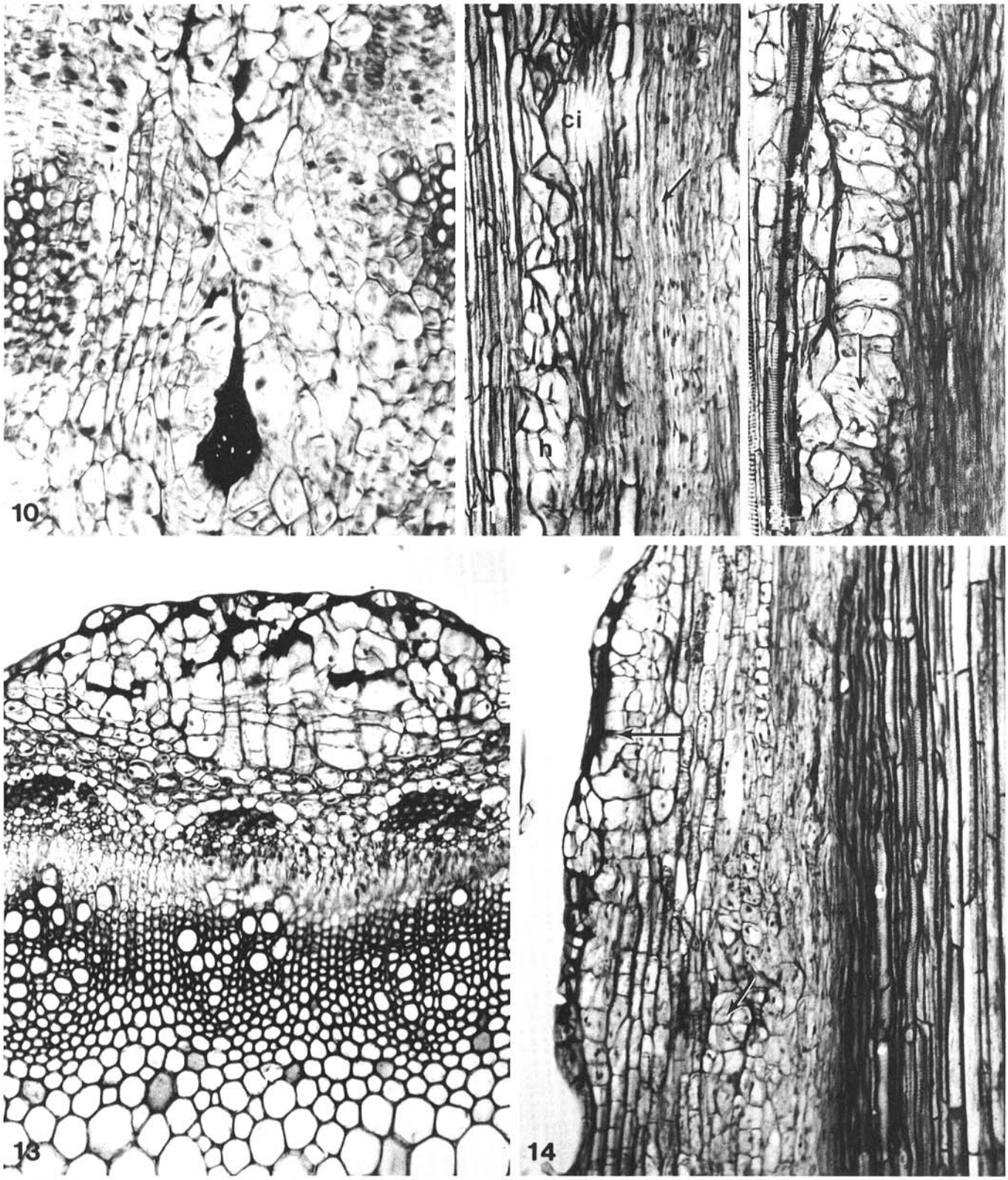
In longitudinal section, the vascular cambium usually consisted of atypically differentiated initials but also was characterized by hyperplastic cell development (Fig. 11). In many sections, the phloem tissue was chromophilic and histochemical tests for callose




**Figs. 4–9.** Transverse sections of stems from plants of *Chrysanthemum morifolium* 'Bonnie Jean' infected with chrysanthemum stunt viroid and stained with Johansen's Quadruple Stain. **4,** Section showing thin-walled hypertrophied cells among the fibers in the bundle cap (×570). **5,** Section showing distortion of cambial initials in the vascular cambium. Note that the cell walls and nuclei are not distinct (×490). **6,** Section showing incomplete differentiation of cambial initials and the breakdown and crushing of cells in the vascular cambium (arrows) (×265). **7,** Section showing gaps in the vascular cambium (arrows) (×280). **8,** Section showing the partial differentiation of the vascular cambial initials (×545). **9,** Transverse section of hypertrophied tissue that has displaced the usual components of the vascular bundle. Note the remnants of the fiber bundle cap (arrow) (×12).

were positive. Longitudinal sections of tissues showing less severe anatomical changes contained pockets of darkly stained material, which gave a positive test for pectin. Hypertrophy of xylem parenchyma cells was also evident in longitudinal sections (Fig. 12).

Other anatomical changes included areas of cell hypertrophy and dark staining intercellular material in the cortex (Fig. 13). The dark staining material gave a positive test for wound gum and in some cases a positive test for pectin using the orcinol and iron-absorption tests (19). The unicellular epidermal layer was replaced



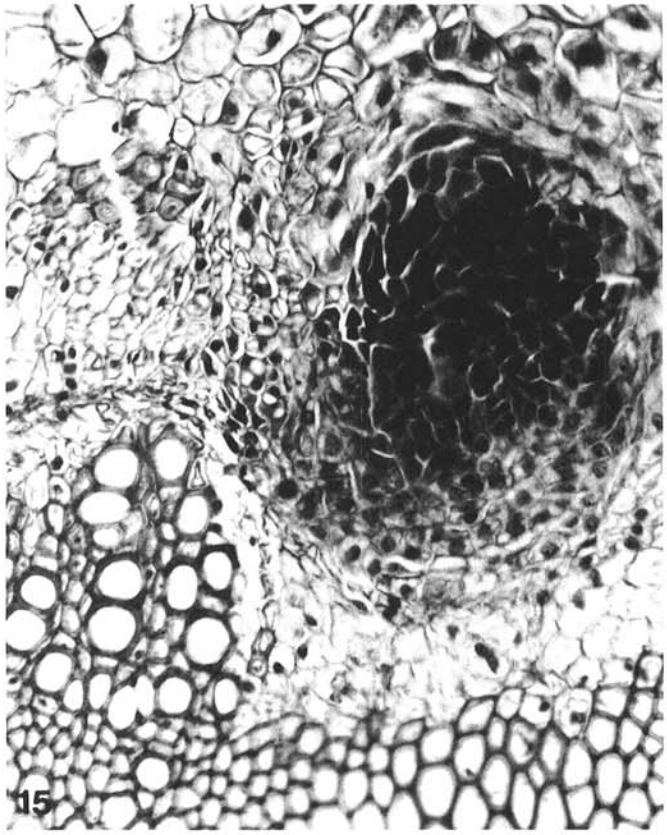

**Figs. 10–14.** Portions of stem sections from plants of *Chrysanthemum morifolium* 'Bonnie Jean' infected with the chrysanthemum stunt viroid and stained with Johansen's Quadruple Stain. **10,** Transverse section showing hypertrophy and hyperplasia of cells in the vascular tissue. Note the dark staining material in the center, which gave a positive test for pectin (×260). **11,** Longitudinal section showing hyperplasia (arrow) of the cambial initials and hypertrophied cells (h) and thin-walled cambial initials (ci) (×12). **12,** Longitudinal section showing hypertrophied cells in the xylem parenchyma (arrow) (×12). **13,** Transverse section showing hyperplasia and hypertrophy in the cortex and dark staining intercellular material (×16). **14,** Longitudinal section showing hyperplasia and hypertrophy in the cortex (arrow) and dark staining intercellular material (arrow) (×11).

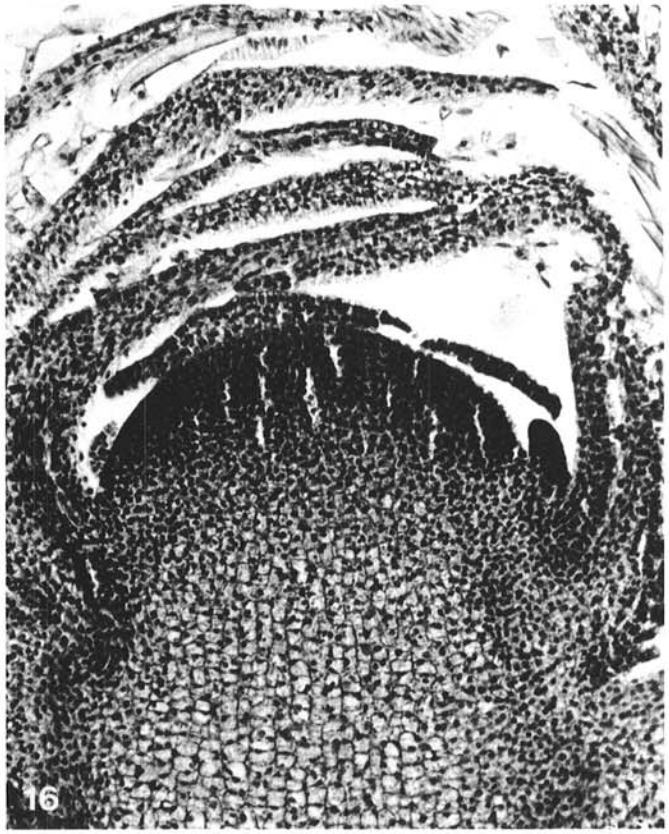
in some instances by a multicellular layer or, often, by cavities containing wound gum and pectin. Cell proliferation of this type was evident in longitudinal sections (Fig. 14).

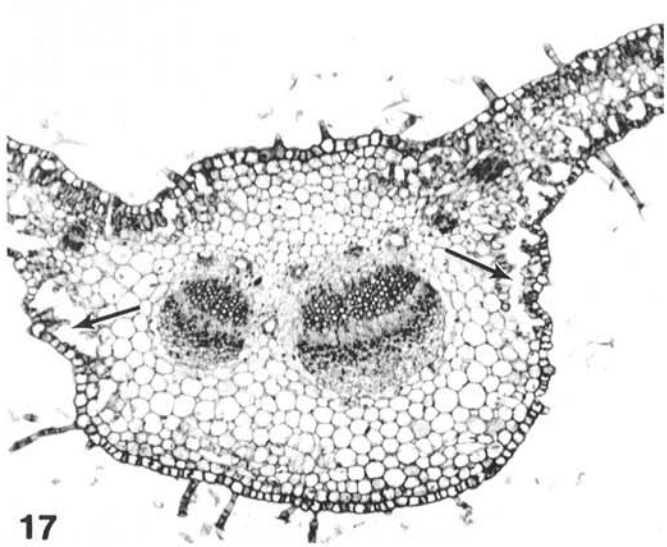
In some sections, a mass of cells resembling in appearance that in apical meristematic tissue replaced the components of the vascular tissue (Fig. 15). These areas often were characterized by darkly stained cells, contained large conspicuous nuclei, and extended through the vascular tissue into the cortex.

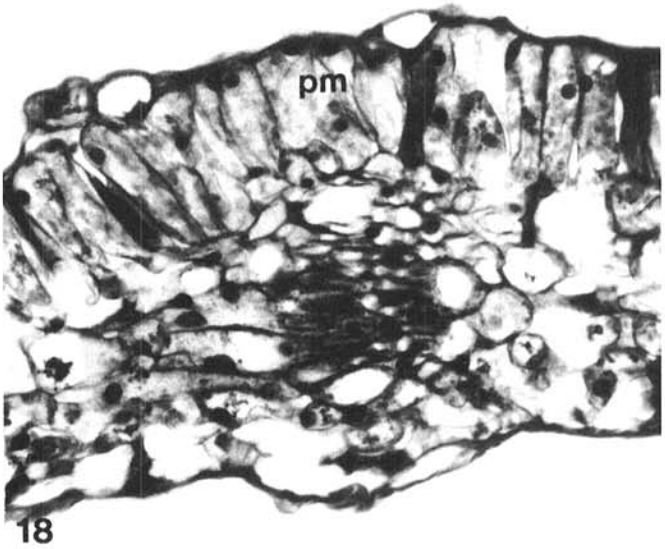
**Apical meristems.** The most common host response to infection observed in vegetative meristems was vertical gaps extending throughout the rib meristem and apex (Fig. 16). The number and size of these gaps varied from meristem to meristem. In reproductive meristems, the response to infection was similar to that observed in vegetative meristems and gaps were observed commonly. Cells in the tissue of reproductive meristems from infected plants were often less compact in arrangement than those observed from uninoculated plants.

**Leaves.** Anatomical changes in young leaves with small chlorotic flecks included a breakdown of mesophyll cells (Fig. 17). The resulting cavities generally were restricted to the abaxial side immediately interior to the lower epidermis. In some areas of the lamina, the lower epidermis was detached from the rest of the tissue by these cavities, whereas in other regions, elongated mesophyll cells bridged these gaps from the spongy mesophyll to the lower




**Figs. 15–18.** Portions of stem section and a vegetative meristem section from plants of *Chrysanthemum morifolium* 'Bonnie Jean' infected with the chrysanthemum stunt viroid and stained with Johansen's Quadruple Stain. **15,** Transverse section showing tissue resembling meristematic tissue occurring in the vascular tissue (×310). **16,** Median longitudinal section of a vegetative shoot meristem showing vertical cavities extending from the tunica-corpus through the rib meristem (×12). **17–18,** Transverse sections of leaf blades. **17,** Section showing cell breakdown in the mesophyll tissues on the abaxial side of the lamina (arrows) (×11). **18,** Section showing the elongated palisade mesophyll cells (pm) and the disintegrated epidermal layers (×57).

epidermis. In some sections, palisade mesophyll cells were less compact in arrangement than in leaves from uninoculated plants.

In leaves with coalescing chlorotic lesions, the palisade mesophyll cells were irregular in size and shape and, in some, the cells were elongate, thin individual cells (Fig. 18). Epidermal layers were not always evident in leaves showing severe symptoms (Fig. 18) and, in some sections, individual mesophyll cells disintegrated. Chloroplasts and nuclei were less visible in these tissues than in mesophyll cells in leaf tissue from uninoculated plants. Chloroplasts and nuclei were indistinct in mesophyll cells in infected plants.

## DISCUSSION

The cultivar Bonnie Jean is used as an indicator host plant for CSV because infection is accompanied by the development of conspicuous symptoms. Symptoms in plants of Bonnie Jean inoculated with CSV were correlated with the severity of anatomical changes observed in the infected tissues. The histological changes observed in this cultivar were restricted to one side of the stem, perhaps indicating a relationship to the curved stem habit of growth. Plants exhibiting extensive symptom expression showed the most extensive anatomical changes.

Because the morphological and physiological changes induced by viruses and viroids are similar, the anatomical changes reported to occur in virus infected host plants were examined. It has been suggested that symptoms resulting from virus infections may be the result of changes in host metabolism (16). Thus, plants infected with a viroid may exhibit an altered metabolic state, which may account for the resulting dysfunction of the cambial initials. Changes observed in other tissues such as the xylem, phloem, and the cortex may be secondary, resulting from the dysfunction of cambial initials. This may explain the appearance of hypertrophied and hyperplastic tissue in the vascular cylinder and what appeared to be a displacement of vascular tissue with meristematic tissue. It was not possible to distinguish between primary and secondary symptoms in this study because of a lack of evidence that individual cells were invaded by the viroid.

Anatomical changes were also evident in shoot meristems of plants inoculated with CSV. Extensive symptom development in this cultivar could be related to the extensive anatomical changes observed in the shoot meristems. Esau (11), studying virus diseases, concluded that the activity of the shoot meristem is not controlled by the subjacent tissues but has an important function in controlling or determining the growth and activity of subjacent tissues (11). The stunting and distortion of stem growth observed in cultivar Bonnie Jean may have resulted from injury to the shoot meristem caused by abnormal metabolism resulting from viroid infection.

Another interesting host response in cultivar Bonnie Jean infected with CSV involved the formation of pockets or cavities of gum-like material in the cortex and xylem. Schneider (20), working with the buckskin-disease of peach and cherry, reported cavities filled with gum in the cortex, phloem, and nonlignified cells of the xylem. Viruses have been reported to alter host metabolism through activation of existing enzymes within host cells or formation of new enzymes (4,17). Alteration of a host enzyme system may explain the breakdown of cells to form pockets containing pectin and wound gum.

In leaves of plants infected with CSV, symptom development resulted in the production of chlorotic lesions that resembled the chlorosis found in mosaic diseases caused by viruses (10,12). The most dramatic response in leaf tissue of Bonnie Jean plants inoculated with CSV was the apparent breakdown of palisade and spongy mesophyll cells, which were replaced with a granular red staining material. Smith and McWhorter (21) detected necrosis of palisade cells in *Vicia faba* infected with tomato ringspot virus (TRSV). Necrosis was initiated in the palisade cells in which swelling and partial breakdown of cell walls occurred in association with dense granulation of the cytoplasm. The epidermis in the infected leaves was necrotic, as were the palisade and spongy mesophyll cells, and these changes resemble those occurring in the early stages of TRSV infection.

Cultivar growth abnormalities observed in this study may be a result of the disruptive effect within the meristematic tissues. Observations of anatomic changes indicated that there may be a distinction between primary and secondary anatomical changes. Changes occurring in the meristematic tissues, the vascular cambium, and shoot meristems may be primary changes and changes evident in the other tissues may be secondary changes. The appearance of hypertrophied nuclei and the disruption of the meristematic tissue suggest a direct activity such as viroid RNA synthesis or replication in the host cell nuclei. These changes may also result from other factors such as hormone imbalance and be secondary effects. Finally, the results indicated that alterations induced in viroid infected chrysanthemum plants were similar to those caused by various viruses in host plants.

## LITERATURE CITED


1. Alcorn, S. M., and Ark, P. A. 1953. Softening paraffin-embedded plant tissues. Stain Technol. 28:55-56.
2. Bachelier, J. C., Monsion, M., and Dunez, J. 1976. Possibilities of improving detection of chrysanthemum stunt and obtaining of viroid-free plants by meristem-culture. Acta Hortic. 59:63-69.
3. Berlyn, G. P., and Miksche, J. P. 1976. Botanical Microtechnique and Cytochemistry. Iowa State University Press, Ames. 326 pp.
4. Cohen, S. S. 1961. Virus-induced acquisition of metabolic function. Fed. Am. Soc. Exp. Biol. 20:641-649.
5. Diener, T. O. 1979. Viroids and Viroid Diseases. John Wiley & Sons, New York. 252 pp.
6. Diener, T. O., and Lawson, R. H. 1973. Chrysanthemum stunt: A viroid disease. Virology 51:94-101.
7. Dimock, A. W. 1947. Chrysanthemum stunt. N.Y. State Flower Grow. Bull. 26:2.
8. Dimock, A. W., Geissinger, C. M., and Horst, R. K. 1971. A new adaptation of tissue implantation for the study of virus and mycoplasma diseases. Phytopathology 61:429-430.
9. Emberger, G., and Nelson, P. E. 1981. Histopathology of a susceptible chrysanthemum cultivar infected with *Fusarium oxysporum* f. sp. *chrysanthemi*. Phytopathology 71:1043-1050.
10. Esau, K. 1944. Anatomical and cytological studies on beet mosaic. J. Agric. Res. (Washington, DC) 69:95-117.
11. Esau, K. 1948. Some anatomical aspects of plant virus disease problems. II. Bot. Rev. 14:413-449.
12. Esau, K., and Cronshaw, J. 1967. Relation of tobacco mosaic virus to the host cells. J. Cell Biol. 33:665-678.
13. Handley, M. K. 1980. The effect of temperature and light on chrysanthemum stunt viroid. M.S. thesis. Cornell University, Ithaca, NY. 53 pp.
14. Jensen, W. A. 1962. Botanical Histochemistry. W. H. Freeman Co., San Francisco. 408 pp.
15. Johansen, D. A. 1940. Plant Microtechnique. McGraw-Hill Book Co., New York. 523 pp.
16. McKinney, H. H., and Clayton, E. E. 1943. Acute and chronic symptoms in tobacco mosaic. Phytopathology 33:1045-1054.
17. McKinney, H. H., and Hills, C. H. 1941. Mosaic, chlorosis and necrosis in virus infected perennial pepper caused directly by products of a deranged metabolism. Science 94:372-373.
18. Pfannenstiel, M. A., Slack, S. A., and Lane, L. C. 1980. Detection of potato spindle tuber viroid in field-grown potatoes by an improved electrophoretic assay. Phytopathology 70:1015-1018.
19. Rawlins, T. E., and Takahashi, W. N. 1952. Technics of Plant Histochemistry and Virology. National Press, Millbrae, CA. 125 pp.
20. Schneider, H. 1945. Anatomy of buckskin-diseased peach and cherry. Phytopathology 35:610-635.
21. Smith, F. H., and McWhorter, F. P. 1957. Anatomical effects of tomato ringspot virus in *Vicia faba*. Am. J. Bot. 44:470-477.
22. Stuehling, B. A., and Nelson, P. E. 1981. Anatomy of a tolerant chrysanthemum cultivar infected with *Fusarium oxysporum* f. sp. *chrysanthemi*. Phytopathology 71:1162-1168.
23. White, J. W. 1971. Irrigation. Pages 94-104 in: Geraniums: A Manual on the Culture, Diseases, Insects, Economics, Taxonomy, and Breeding of Geraniums. J. W. Mastalerz, ed. Pennsylvania Flower Growers Association, Chalfont. 350 pp.