Initiation and development of systemic necrosis in relation to virus concentration in tobacco ringspot virus-infected cowpea plants.

INITIATION AND DEVELOPMENT OF SYSTEMIC NECROSIS IN RELATION TO VIRUS CONCENTRATION IN TOBACCO RINGSPOT VIRUS-INFECTED COWPEA PLANTS

A Thesis Presented

By

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Plant Pathology

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ABSTRACT

Cowpea plants (*Vigna sinensis* var. California Blackeye) mechanically inoculated with tobacco ringspot virus produce local lesions 3-4 days after inoculation. Systemic necrosis of stems first appears 5-7 or more days after inoculation, depending on the concentration of virus in the inoculum. Generally, all plants with a total of 3 or more local lesions develop systemic necrosis, while only about 95% of plants with 2 local lesions and 60% of plants with one local lesion develop systemic necrosis. Systemic necrosis often starts on, and is sometimes limited to, one side of the stem. Histopathological studies show that browning and necrosis of the stem begin in the primary phloem, then spread to the xylem and xylem parenchyma, cambium, epidermis, and subepidermal tissues. Virus is first detectable in the terminal millimeter of roots 3 days after inoculation, but becomes detectable in the upper portion of the stem in 5 days. Virus concentration in stem segments of leaf-inoculated plants increases through the 9th day. Systemic necrosis appears on stems on or soon after the 5th day from inoculation, its extent and severity closely following or coinciding with the rise in concentration of virus in the stem.

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INTRODUCTION

The histopathology of viral infection of plants has been studied extensively. However, most of the research concerning virus-induced necrosis has dealt with the development of local lesions. Very little work has been done on the histopathology of virus-induced systemic necrosis.

Similarly, little is known concerning virus concentration and distribution in plants suffering systemic necrosis and the association, if any, of tissue necrosis to the concentration of virus in these tissues. More research is needed to determine the relation of virus concentration and distribution to the progression of systemic necrosis.

Cowpea plants infected with tobacco ringspot virus (TRSV) provide a good system for studying the development of systemic necrosis and its relation to virus concentration. Mechanically inoculated primary leaves develop local lesions 3 to 4 days after inoculation; systemic necrosis is manifested 5 to 6 days after inoculation. Systemic necrosis usually begins on one side of the stem, then spreads laterally and vertically until the entire stem is involved. Occasionally, the necrosis is asymmetric, being limited to one side of the stem even though both primary leaves may be inoculated. The time required for symptom development varies with inoculum concentration and with the number of local lesions on the primary leaves. Occasionally, however, some plants with well-developed local lesions do not develop systemic symptoms.

The objective of this study was to determine the sequence of events in virus induced systemic stem necrosis. More specifically, research was conducted to determine the initial point along the stem at which necrosis begins, and to follow the types and sequence of cells and tissues killed by the virus. Furthermore, studies were also initiated to determine the minimum virus concentration required to induce systemic necrosis and to correlate virus concentration and distribution in plant tissues with stages of necrosis of those tissues.

LITERATURE REVIEW

Tobacco ringspot virus (TRSV) is an isometric RNA virus approximately 28 nm in diameter. The geographical distribution of TRSV is North America, Europe, and Australia, and this virus causes ringspot diseases on tobacco, cucumber, Easter lily, hydrangea, iris, Pelargonium and blueberry, and bud blight on soybean (Stace-Smith, 1970). It has a wide host range, including 246 species in 54 families (DeZeeuw, 1965).

An important assay species for TRSV is Vigna sinensis (cowpea), on which it produces local lesions followed by a systemic necrosis. As pointed out by Bawden (1964), in virus infections, localized infections can occur without necrosis, and systemic infections can occur despite necrosis. Interestingly, infection of cowpea by TRSV usually results in a systemic necrosis. This TRSV-induced systemic necrosis is by no means unique; other viruses also cause systemic necrosis. However, this subject is not treated very extensively in the literature (Porter, 1954; Smith and McWhorter, 1957; Worley, 1965; White and Horn, 1975).

Tomato ringspot virus (TomRSV) is often considered similar to TRSV although the two are not serologically related (Smith, 1972). The type strain of TomRSV is usually symptomless in broad bean (Vicia faba), but infection by the cucumber strain results in formation of local lesions and a progressive necrosis of the stem and roots. Typically, macroscopic symptoms begin with the formation of local lesions 4 to 6 days after inoculation. This is followed by withering of the

inoculated leaves and development of brown streaks extending down the petioles and into the stems. The upper shoot darkens, collapses, and the root tips become necrotic. Finally, the entire stem blackens and the plant dies (Smith and McWhorter, 1957). Necrosis originates in the epidermis of inoculated leaves, and spreads down into the palisade and spongy parenchyma until it reaches the veins. A brown streak, indicating necrosis, then spreads down the petiole. The necrosis actually develops in parenchyma tissue near the primary phloem fibers. Stem necrosis, however, begins in the primary phloem fibers and is preceded by inhibition of cambial activity. Occasionally, even complete suppression of the interfascicular cambium occurs. Necrosis then progresses laterally throughout the pith and cortex, eventually affecting the vascular bundles (Smith and McWhorter, 1957).

Worley (1965) studied the translocation of southern bean mosaic virus (SBMV) and the histopathology of necrotic stems in SBMV-infected Pinto beans. Necrosis first became visible 2 to 4 days after superficial inoculation of the stem. Cross-sections made at or near the point of inoculation showed the inward spread of necrosis extending all the way to, and including part of, the pith. Epidermis, cortex, endodermis, part or all of the phloem, and the xylem parenchyma were all necrotic. Necrosis then spread both up and down the stem. However, necrotic streaks were usually limited to endodermal and phloem fiber cells. Similar histological data concerning the same virus and host was reported by Mitchell et al. (1956).

Tobacco etch virus (TEV) causes a severe wilt and death in tabasco peppers. Stem and petiole cortex, and leaf mesophyll cells become

plasmolyzed. Necrosis actually occurs first in the phloem and cambium of the roots, and is followed by degeneration of the cortex and plastids. No blockage of xylem occurs, the xylem being left apparently unaffected (White and Horn, 1975).

Cucumber mosaic virus (CMV) also is capable of inducing necrotic reactions in host plants such as Easter lily, tulip, and broad bean. In each case, necrosis originates in the leaf mesophyll, and, in Easter lily and tulip, it is restricted primarily to the mesophyll, spreading to the vascular tissues only during the late stages of mesophyll degeneration. Degeneration of the vascular tissues appears to be an indirect effect due to association with the surrounding dead tissues rather than a direct result of virus infection (Porter, 1954).

In CMV-infected broad bean, however, vascular degeneration occurs first, and general necrosis of leaves and stems spreads rapidly throughout all parts of the plant. In leaves, necrosis spreads rapidly from the mesophyll to the epidermal and vascular tissues. In stems, necrosis begins in the phloem of a single vascular bundle, then spreads through the cortex, phloem, cambium, xylem, and pith (Porter, 1954).

Several of the potato viruses induce systemic necrosis. The leaf roll, leaf-drop streak, and top necrosis diseases of potato all involve systemic necrosis. In leaf roll (caused by potato leaf roll virus) and top necrosis (caused by several different viruses in different varieties), necrosis first develops in the phloem. Both diseases involve the deposition of a yellow or yellowish-brown gum-like substance

in collapsing cells of the primary phloem. Necrosis in plants suffering from top necrosis spreads into surrounding tissues in all directions, whereas necrosis in plants afflicted with potato leaf roll is limited to the phloem strands. Also, phellogens are formed around the necrotic tissues in top necrosis. Systemic necrosis in leaf-drop streak, caused by potato virus Y (PVY), is quite different, affecting chiefly the collenchyma. Although necrosis may spread to other tissues of the cortex, it does not spread to the vascular bundles (Quanjer, 1931; Bawden, 1932, 1964; Esau, 1938, 1948).

Barley yellow dwarf virus (BYDV) and beet yellows virus (BVY) cause phloem degeneration and necrosis similar to that in potato leaf roll. In several grasses, BYDV induces phloem degeneration resulting in necrosis of sieve elements, companion cells, and neighboring parenchyma. Degeneration resembles normal obliteration, but is more extensive, involving necrosis and affecting cells that in normal phloem (at a similar age) would be intact (Esau, 1957). Phloem of sugar beet infected with BYV undergoes similar degeneration and necrosis (Esau, 1948, 1960).

No abnormal cell growth was observed in any of the above diseases, with the exception of phellogen formation in top necrosis of potatoes. In curly top of sugar beets, on the other hand, hypertrophy and hyperplasia within the phloem are the first symptoms in the phloem degeneration syndrome caused by the virus (Esau, 1938; Rasa and Esau, 1961). These tissues then become necrotic and collapse, while further hypertrophy and hyperplasia occur around the necrotic areas. Much of the more recent research deals with electron microscope studies of hyper-

sensitive local lesion reactions rather than systemic necrosis. Abnormal chloroplasts, ruptured chloroplasts, and general cell disorganization have been observed in cells undergoing necrosis in local lesions (Hayashi and Matsui, 1965; Chalcroft and Matthews, 1966; Israel and Ross, 1967; Allison and Shalla, 1974; DaGraca and Martin, 1975).

The relation between virus concentration and symptom development varies with the host-virus combination in question. Usually, the rate of symptom development and the severity of symptoms in systemic infections is directly related to the concentration of the inoculum. This appears quite logical when one considers that a higher inoculum concentration provides a greater number of virus particles spreading from an increased number of infection sites. The uninoculated areas of the plant can then be more quickly invaded by the virus (Schneider, 1965).

Hooker and Benson (1960) found that the time required for symptom response in Datura tatula to potato virus X was a function of virus concentration. When inoculum concentration was decreased, the time required for symptom development increased. Similar results occurred when the size of the area inoculated was restricted.

Schneider (1965) showed that inoculation of primary leaves of Pinto bean with relatively high concentrations of TRSV resulted in all plants becoming systemically infected. Plants inoculated with various dilutions of the same inoculum contained no detectable virus in the uninoculated leaves.

Bennett (1960) observed a distinct correlation between the number

of local lesions induced by the sugar beet yellows virus on Chenopodium capitatum and the occurrence of systemic infection. Only 10 percent of plants with 25 lesions or less developed systemic symptoms. In order to obtain systemic infection in all plants, at least 200 local lesions per plant were required.

Apparently direct relationships between virus concentration and symptom development suggest that virus must be present in a plant tissue for disease symptoms to occur. Indeed, Milne (1966) found necrosis in TMV-infected leaves of Chenopodium amaranticolor only after virus had been synthesized.

Hoefert et al. (1970), however, found this not to be the case. In beet leaves infected with beet yellow stunt virus, there was no correlation between virus distribution and internal symptoms. Degenerative changes were observed in chloroplasts of mesophyll and phloem parenchyma, and in plastids of sieve elements. Yet virus was absent from these cells. It was speculated that the presence of virus in one cell could have been disturbing the metabolism in neighboring cells, or that the apparently non-infected cells did indeed contain virus, but in incomplete form so that it was not detectable.

Obviously then, distribution of virus in systemically infected plants is not necessarily uniform. Schneider (1964) found TRSV could not move into the uninoculated primary leaf opposite the inoculated leaf in Black Valentine bean, while SBMV could. Both TRSV and SBMV move rapidly from the inoculated leaves to other parts of the plants. When SBMV and TRSV were inoculated onto the same primary leaf, only SBMV could be detected in the noninoculated opposite leaf. Schneider

proposed that both viruses moved through the phloem to uninoculated parts of the plant, but that TRSV was restricted to slow cell-to-cell movement in the petiole of the uninoculated leaf.

Karle and Shalla (1966), studying the movement of peach yellow bud mosaic virus (YBMV) in cowpea, could not detect YBMV in the opposite noninoculated primary leaf. Tracer studies using $C^{14}O_2$ indicated that the photosynthate flow through the phloem is directed away from primary leaves. They therefore speculated that the failure of YBMV to invade noninoculated primary leaves was due to its inability to move against the carbohydrate flow.

Resconich (1963) studied the movement of tobacco necrosis virus (TNV) in systemically infected soybeans. When plants were inoculated on only one primary leaf, systemic infection was asymmetric throughout the entire plant. Stem necrosis occurred only on the same side of the plant as the inoculated leaves. Asymmetry of necrosis on the trifoliate leaves was striking. Only the half of the center leaflets and the lateral leaflets from the same side of the plant as the inoculated leaves usually became necrotic. Roots appeared normal, but local lesion assays indicated they were also asymmetrically infected. No local lesion assays were completed to study the actual virus distribution in other portions of the plant.

More specific data dealing with asymmetric virus infection is needed. A great deal of useful information can be gained by studying the relationship of virus presence and concentration in various plant tissues to the initiation and development of systemic necrosis in these tissues. Much of the literature concerning the histology of systemic necrosis deals

with phloem-limited necrosis. When generalized necrosis has been studied, the sequence of tissue death in most cases has not. Developmental studies are required in order to obtain a clear and complete understanding of the infection process. Therefore, a histopathological study of TRSV-infected cowpeas may help in understanding the nature of plant viruses and their relationship with various plant tissues.

MATERIALS AND METHODS

The Plants

Cowpea plants (*Vigna sinensis* var. California Blackeye) were grown in a 1:1:1 soil-sand-peat moss mixture in the greenhouse, or under artificial light in growth chambers at a daytime temperature of 85 F and a nighttime temperature of 65 F. Day length was 16 hours. Plants were fertilized with a water-soluble fertilizer (16-32-16) every two weeks.

Two weeks after planting, the primary leaves of the plants were inoculated with a 1:50 dilution of tobacco ringspot virus. The plants were then allowed to grow under observation and were used for various experiments.

The Virus

The grapevine strain of TRSV was used throughout these studies. The virus was propagated in tobacco (*Nicotiana tabacum* var. Turkish) or in cowpeas. For cowpea inoculations, inoculum was prepared by grinding systemically-infected tobacco leaves and straining the extract through cheesecloth. This extract was then diluted with 0.01 M phosphate buffer, pH 7.2, and used to inoculate primary leaves of cowpeas. Virus concentration was assayed by inoculating celite-dusted cowpea primary leaves using glass rods as outlined below.

Histopathological Studies

If fresh sections were to be made, plants in various stages of necrosis were harvested, sectioned free-hand and observed immediately

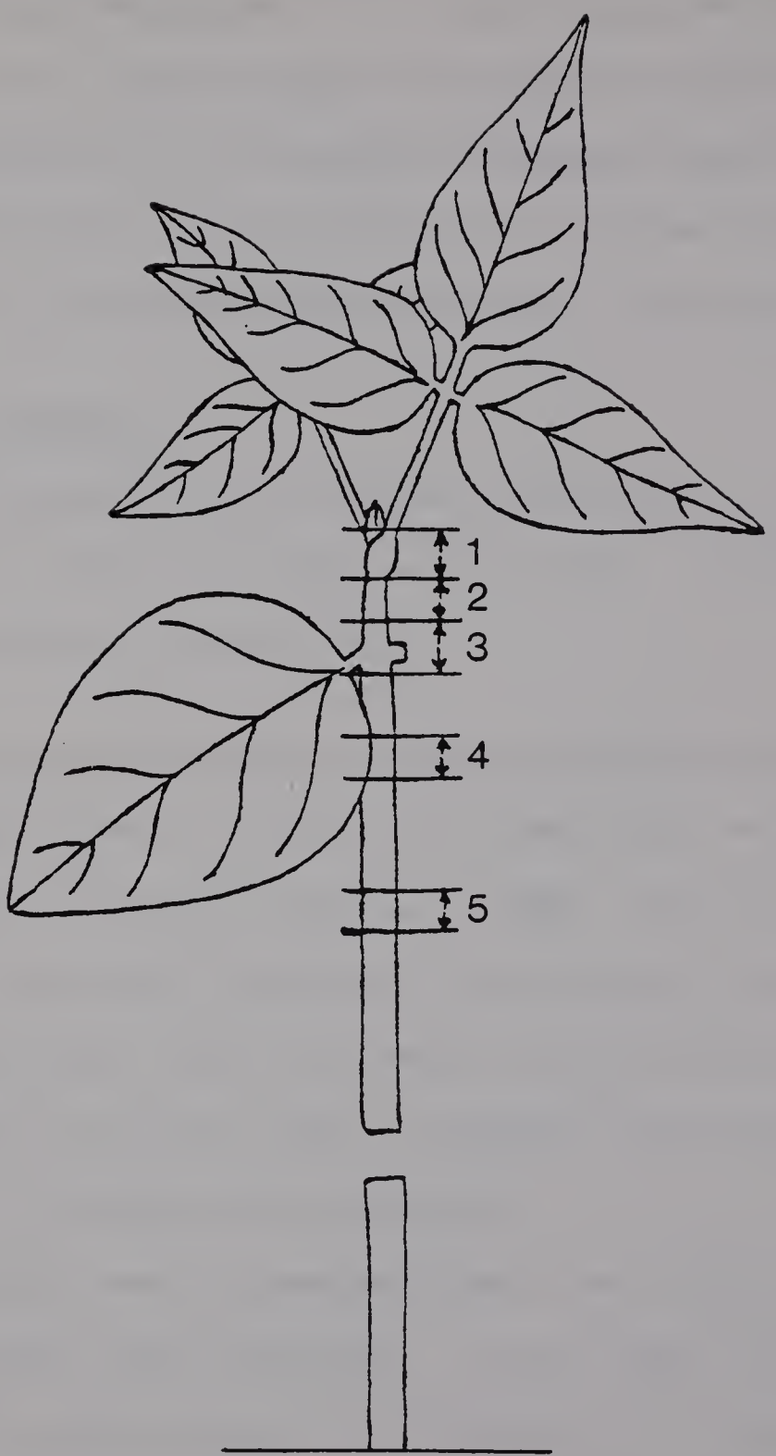
under the microscope.

Plants to be used for paraffin sectioning were harvested daily, beginning on the first day after inoculation and continuing for eight days. Both healthy (controls) and diseased plants were collected. Five sections of approximately five millimeters in length were excised from each plant harvested and immediately placed in the fixative solutions. Each section was taken from a different portion of the plant and included: (1) apex and node, (2) stem just below the apex, (3) primary leaf node, (4) one centimeter below the primary leaf node, (5) two centimeters below the previous section (Fig. 1).

Tissue fixation, dehydration, and embedding procedures were modifications of those used by Johansen (1940), Jensen (1962), Sass (1968), and Feder and O'Brien (1968). Three different fixatives (FAA, CRAF III, 2.5% glutaraldehyde) were tested to determine which was most suitable for this work. Glutaraldehyde provided the best fixation and was used in all subsequent work.

The tissue samples were immersed in glutaraldehyde and aspirated immediately in order to speed infiltration of the fixative and to remove any trapped air. Samples were fixed for 20-24 hours and then dehydrated in an ethyl alcohol and tertiary butyl alcohol (TBA) series, beginning at 10 percent alcohol. Lower alcohol solutions contained ethyl alcohol and water; TBA was added when the total alcohol content of the solutions reached 50 percent. Alcohols were changed in steps of 10 percent, after periods of two hours each, until 70 percent was reached. Samples were left in the 70 percent solution overnight, and then placed in 85,95, and 100 percent alcohol for 10-12 hours each.

Fig. 1. Stem section of cowpea plants collected for paraffin embedding and sectioning were taken from various portions of the plant: (1) apex and node, (2) stem just below the apex, (3) primary leaf node, (4) 1 cm below the primary leaf node, (5) 2 cm below the previous section.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This was followed by three changes (10-12 hours each) of pure TBA. Samples were placed in a mixture of equal parts paraffin oil and TBA for 1 day, then placed in the paraffin oven overnight. Finally, the samples were taken through four changes of paraffin (Tissue Prep, 56.6 C melting point) and embedded. Embedded tissues were sectioned on a Lipshaw Model 50 AB microtome at a thickness of 12-15 microns. Sections were affixed to slides with Haupt's adhesive.

Staining Procedures

The safranin/fast green staining combination was used because of safranin's ability to stain necrotic tissue. Staining procedures were as outlined by Jensen (1962).

Number of Local Lesions and Initiation of Systemic Necrosis

Primary leaves of two-week-old cowpea plants were inoculated with 1:1000 and 1:10,000 dilutions of TRSV. Local lesions were counted 4-5 days after inoculation, and the plants were divided into groups according to the total number of local lesions on each plant. Plants were observed for signs of systemic necrosis and necrosis of the apex for 60 days after inoculation.

All plants used in these experiments were grown in growth chambers up to two weeks after inoculation. At that time, the plants were transferred to the greenhouse for the remainder of the experiment.

Determination of Vertical Distribution and Concentration of TRSV

Two-week-old cowpea plants grown in the growth chamber were inoculated with a 1:50 (v/v) dilution of TRSV. Each day following inoculation

five plants were harvested and four one centimeter long sections were excised from each plant. Each of the sections was ground in 1.0 ml of 0.01 M phosphate buffer, pH 7.2, and used as inoculum on eight primary half-leaves of cowpea plants kept in the greenhouse. Inoculations were carried out using glass rods of equal length so that equal width strips were inoculated on each half-leaf (DeZeeuw and Timmer, 1964). Such inoculations were carried out for eight days in one experiment and ten days in two experiments. The sections were as follows (Fig. 2): (1) apex to primary leaf node (including base of petioles of trifoliate leaves), (2) primary leaf node, (3) 1-cm section taken 1 cm below the primary leaf node, and (4) 1-cm section taken 1 cm above the soil line. Local lesions were counted 4-5 days after inoculation.

Local Lesion Assays of Asymmetrically Necrotic Stem Areas

Two-week-old cowpea plants in the growth chamber were inoculated on only 1 primary leaf with a 1:10 (w/v) dilution of TRSV-infected cowpea tissue. Beginning on the fourth day after inoculation, five plants were harvested daily. Two sections of the stem were removed from each plant (Fig. 3). The first section extended from the base of the petioles of the trifoliate leaves to the primary leaf node (no longer than 1.5 cm). The second section (1.5 cm long) was taken one centimeter below the primary leaf node. Each section was then split longitudinally; one side being from the inoculated side of the plant and generally representing the more necrotic area, and the other side being from the noninoculated side of the plant and generally representing

Fig. 2. Stem sections removed from cowpea plants and assayed to determine the vertical distribution and concentration of TRSV in relation to symptom development: (1) apex to primary leaf node, (2) primary leaf node, (3) 1-cm section taken 1 cm below the primary leaf node, (4) 1-cm section taken 1 cm above the soil line.

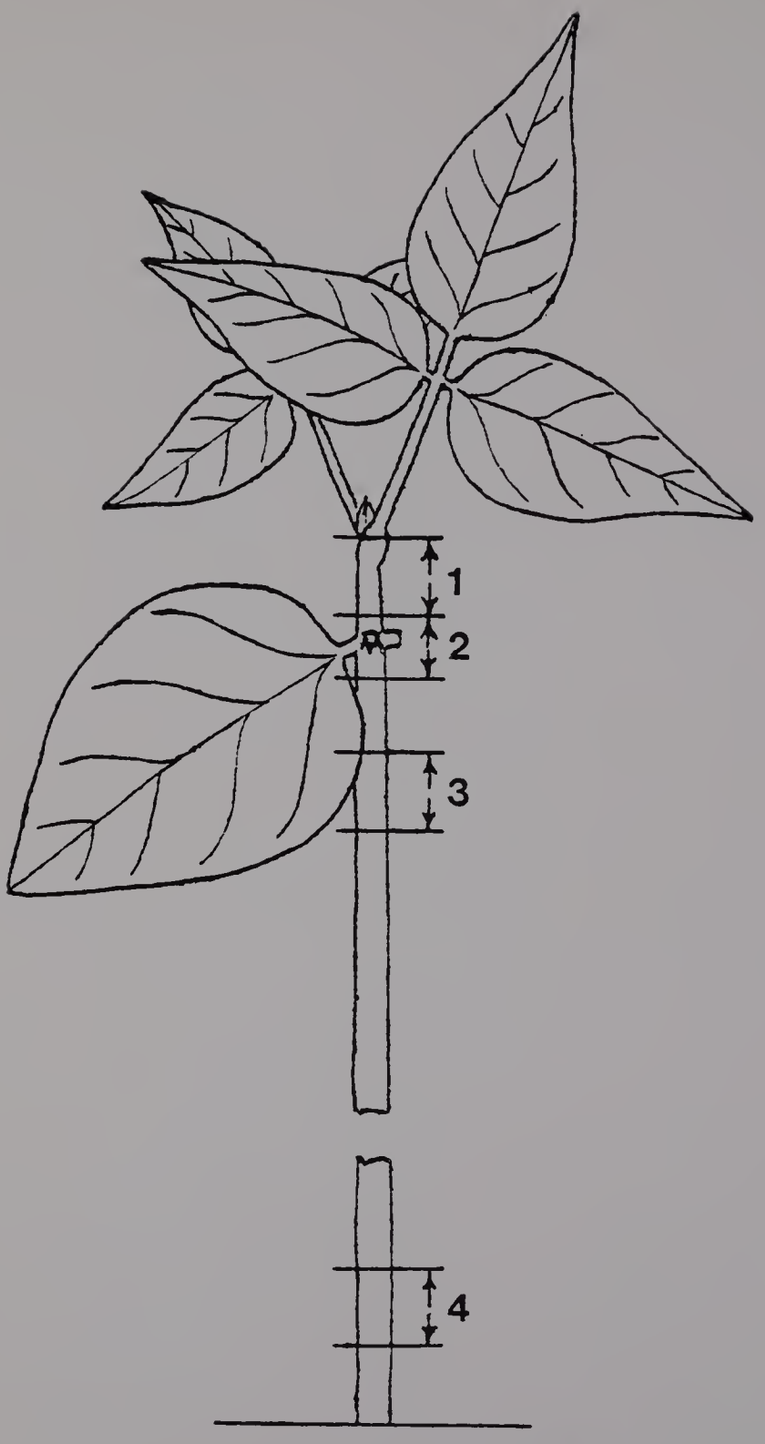
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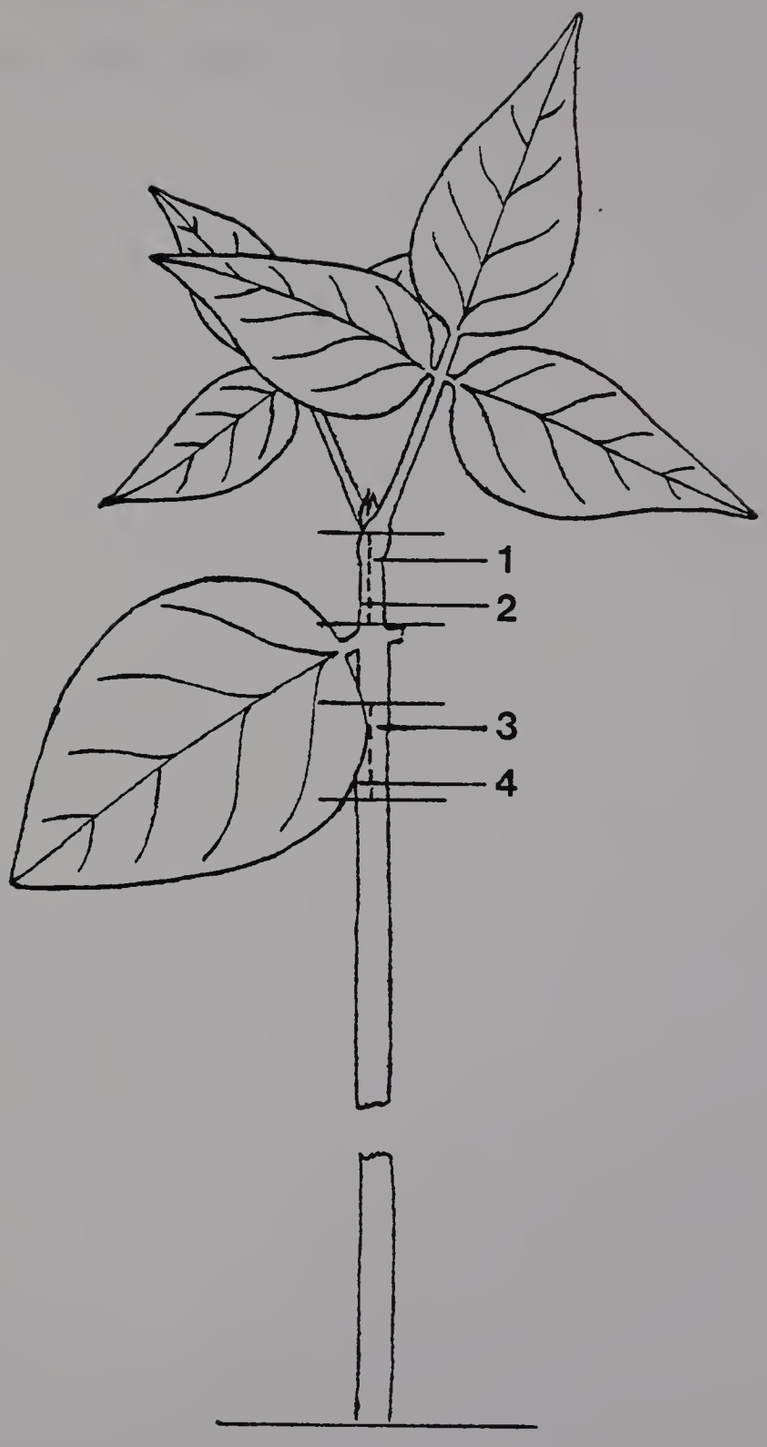
Fig. 3. Stem sections removed from cowpea plants and assayed to study TRSV distribution and concentration in asymmetrically necrotic stem areas: (1) apex to primary leaf node ("inoculated side"), (2) apex to primary leaf node ("noninoculated side"), (3) 1 cm below primary leaf node ("inoculated side"), (4) 1 cm below primary leaf node("noninoculated side").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the healthy or less necrotic area. All four sections were then ground in 1.0 ml of 0.01 M phosphate buffer, pH 7.2, and inoculated onto eight half-leaves of cowpea plants grown in the greenhouse. Local lesions were counted 4-5 days after inoculation.

RESULTS

External Development of TRSV-Induced Necrosis in Cowpeas

Distinct, reddish-brown local lesions appear on mechanically inoculated primary leaves 3-4 days after inoculation (Fig. 4). Systemic symptoms appear 5-7 days after inoculation, depending on the concentration of virus in the inoculum. Cowpea plants inoculated with a 1:50 (v/v) dilution of sap from TRSV-infected tobacco leaves show the first signs of systemic necrosis on the fifth day after inoculation. Rapidly growing trifoliate leaves develop necrotic spots (secondary local lesions) in the lamina, and necrotic areas along the veins of individual leaflets. Six days after inoculation, necrosis of the trifoliate leaves is more severe and some petiole necrosis may be evident. Stem necrosis first appears as a slight reddish-brown coloration of the top internode, between the apex and the primary leaf node (Fig. 5). By the seventh day, necrosis of the top internode is much more severe, and is spreading down the stem past the primary leaf node. Internode necrosis is so severe by the eighth day that the stem in this region has a collapsed and constricted appearance (Fig. 6). Trifoliate leaves show necrotic veins and appear withered.

Occasionally, necrosis is restricted to one side of the stem, even though both primary leaves were inoculated (Fig. 7). Necrosis starting in one trifoliate leaf tends to spread down the same side of the stem as the trifoliate leaf. However, if only one primary leaf is inoculated, necrosis is limited to the side of the stem directly

Fig. 4. Cowpea primary leaf showing reddish-brown local lesions about four days after mechanical inoculation with tobacco ringspot virus.

Fig. 5. Cowpea plants showing various stages of systemic stem necrosis following inoculation of the primary leaves with tobacco ringspot virus.

Fig. 6. Cowpea stems showing severe necrosis eight days after inoculation of the primary leaves with tobacco ringspot virus. Stem area just below apex is collapsing. Necrosis extends down the stem, past the primary leaf node.

Fig. 7. Cowpea stems showing one-sided necrosis following inoculation of one or both primary leaves with tobacco ringspot virus. (A) Necrosis proceeding down the stem on the same side as the inoculated leaf (only one leaf inoculated). (B) Necrosis spreading down the stem on the same side as the necrotic trifoliate leaf (both leaves inoculated).


a
b

below that primary leaf. Age of the inoculated plant is a factor in determining whether the necrosis remains limited or spreads laterally around the stem. Generally, the older the plants, the greater the frequency of one-sided necrosis. In cowpea plants inoculated when two weeks old (as in these experiments), necrosis begins on one side but eventually spreads laterally around the stem.

Histology of Healthy Stems

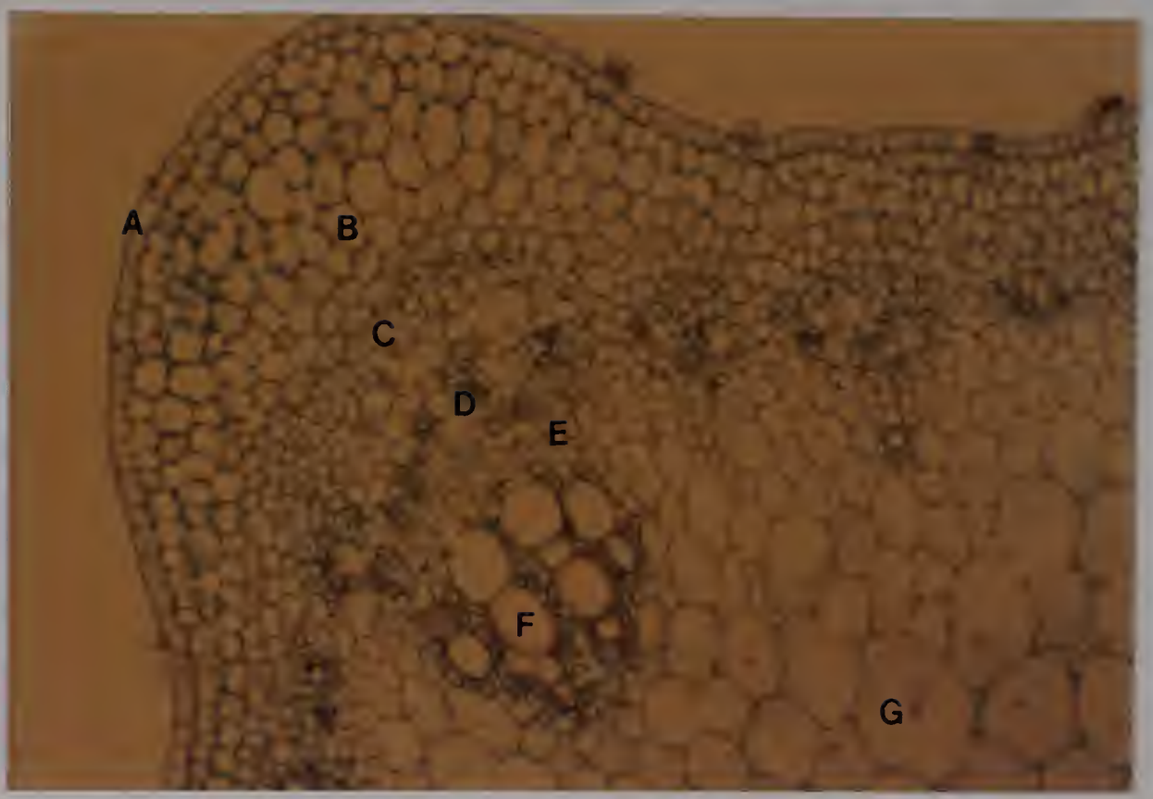
The various stem tissues and cell types of healthy cowpea plants are easily distinguished in transverse sections of stems as in Figure 8. Just beneath the epidermis there are several layers of collenchyma cells. A band of chlorenchyma appears beneath the collenchyma. Major vascular bundles are located in the ridges of the stem,with other vascular tissues differentiating in the interfascicular regions. Protophloem which is present in the upper portion of the stem is composed largely of immature primary phloem fibers (Fig. 9). Metaphloem consists of sieve tubes, companion cells, phloem parenchyma, and secretory elements in the forms of enlarged parenchyma cells. Pith in the upper stem consists of continuous parenchyma, but in lower stem (older portions of the stem) disintegrates and is hollow. Lower regions of the stem develop a more woody appearance with much more thick-walled primary phloem fibers, well-developed interfascicular cambium, and xylem vessels eventually forming a continuous band around the stem.

Histopathology of TRSV-infected Stems

Internal stem necrosis originates in the immature primary phloem

Fig. 8. Transverse section of a healthy cowpea stem showing the type and arrangement of the various cells and tissues (100X): (A) epidermis, (B) collenchyma, (C) protophloem (primary phloem fibers), (D) metaphloem, (E) cambium, (F) xylem, (G) pith.

Fig. 9. Enlargement of part of the previous section showing healthy, immature primary phloem fibers arising in the protophloem (arrow) (400X).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E
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G

fibers in the protophloem of a main vascular bundle in the upper stem (Figs. 10,11,12,13). At first, there is only a slight brown discoloration of the walls and lumen of some cells due to an apparent accumulation of melanoid pigments (Fig. 10). Accumulation of dark-colored substances continues until dense dark masses are formed within or between primary phloem fiber cells (Fig. 11). Eventually, these immature fiber cells collapse, forming a necrotic line just outside the metaphloem (Figs. 11,12,13). If enough of the cells collapse, the bundle may have a flattened appearance (Figs. 12,13).

Initially, only one or two main bundles on one side of the stem are involved. Necrosis then spreads laterally as well as vertically through the primary phloem fibers of the smaller vascular bundles. The primary phloem cells in each vascular bundle appear as a line of collapsed, necrotic cells. Eventually, cambium cells in interfascicular areas also become necrotic, and the line of collapsed, necrotic cells extends around various portions of the stem (Figs. 14,16,18), until, finally, the entire stem is affected.

Necrosis also spreads away from the primary phloem, and into the epidermis and the xylem. Although necrosis of epidermal cells usually follows that of primary phloem fibers, there is often no necrosis in the intervening tissues of chlorenchyma and collenchyma (Figs. 14,15). In the meantime, xylem vessels also become discolored and xylem parenchyma becomes necrotic as the disease progresses. Vessels occasionally appear clogged with brown-colored materials of undetermined composition (Figs. 16,17,21). Although both the proto-

Fig. 10. Fresh section of tobacco ringspot virus-infected cowpea stem showing early stages of discoloration and necrosis in the immature primary phloem fibers (400X).

Fig. 11. Fresh section of tobacco ringspot virus-infected cowpea stem showing slightly more severe necrosis than is evident in Fig. 10. Note the accumulation of dark melanoid pigment and the obliteration of some affected cells (400X).

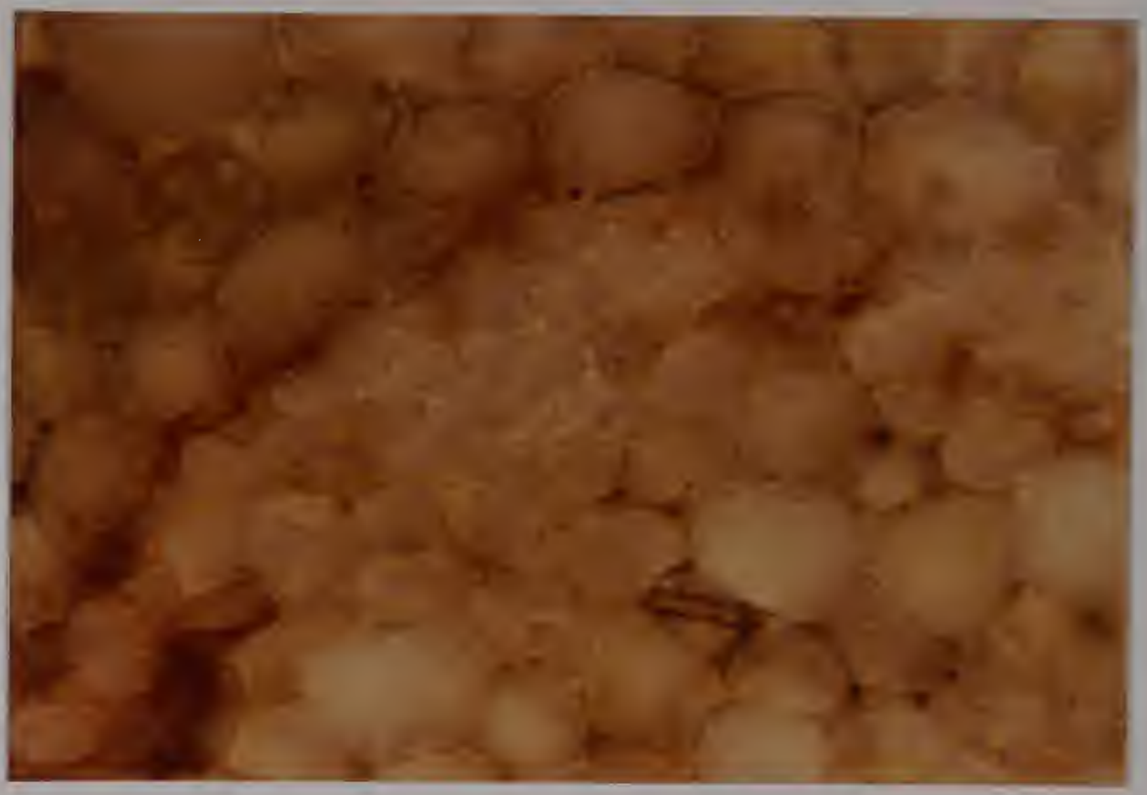
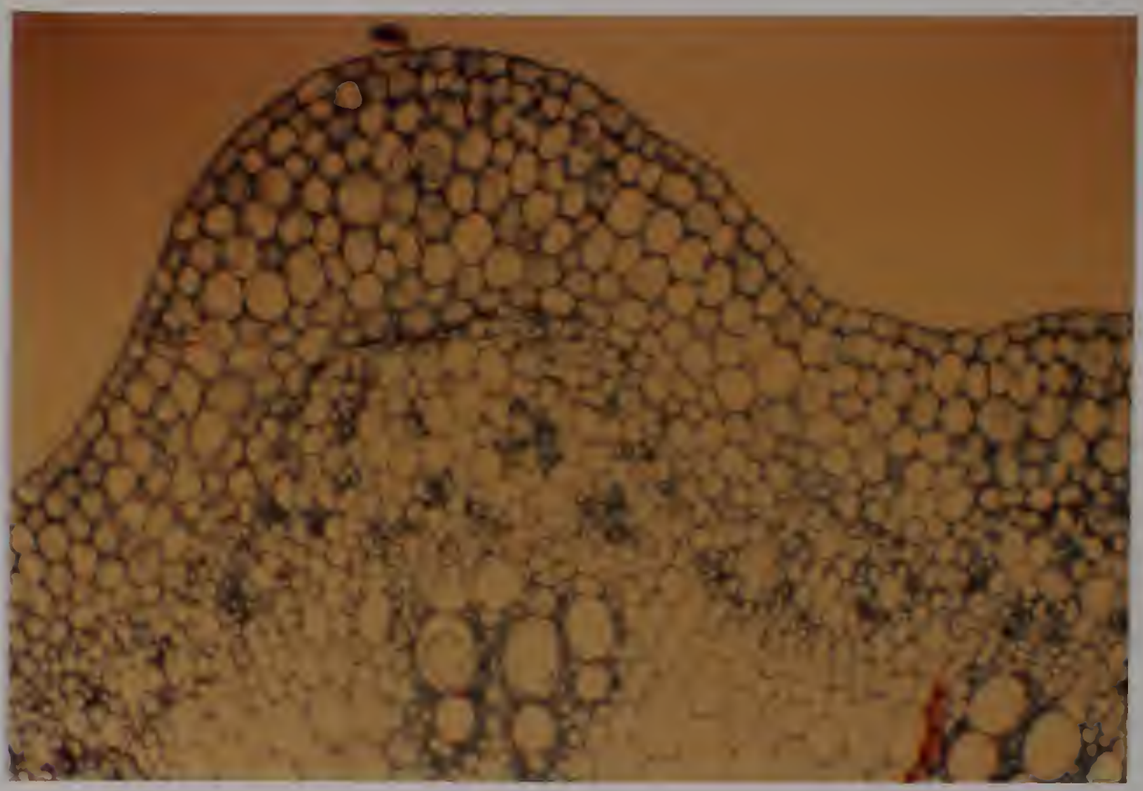


Fig. 12. Paraffin section of tobacco ringspot virus-infected cowpea stem showing necrosis of fibers in the proto-phloem of a main vascular bundle. Cells are collapsed, forming a necrotic line capping the vascular bundle (100X).

Fig. 13. Enlarged section of Fig. 12 showing collapsed and necrotic primary phloem fibers (400X).



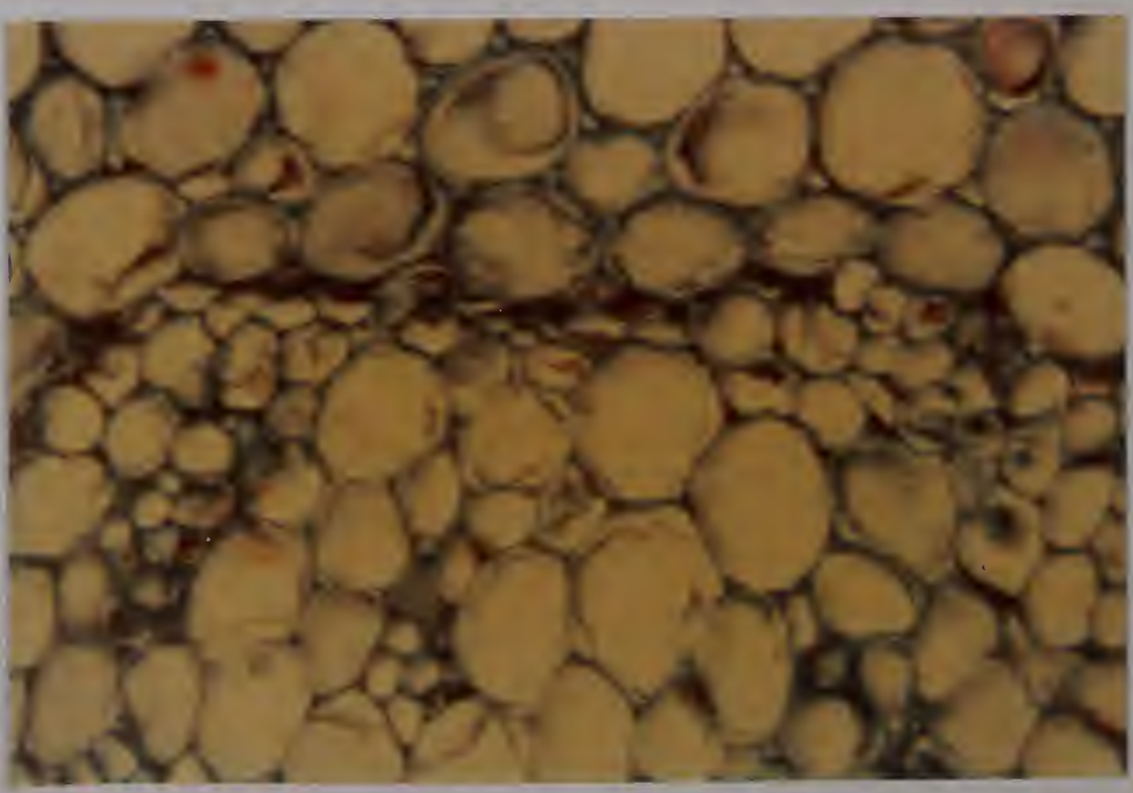
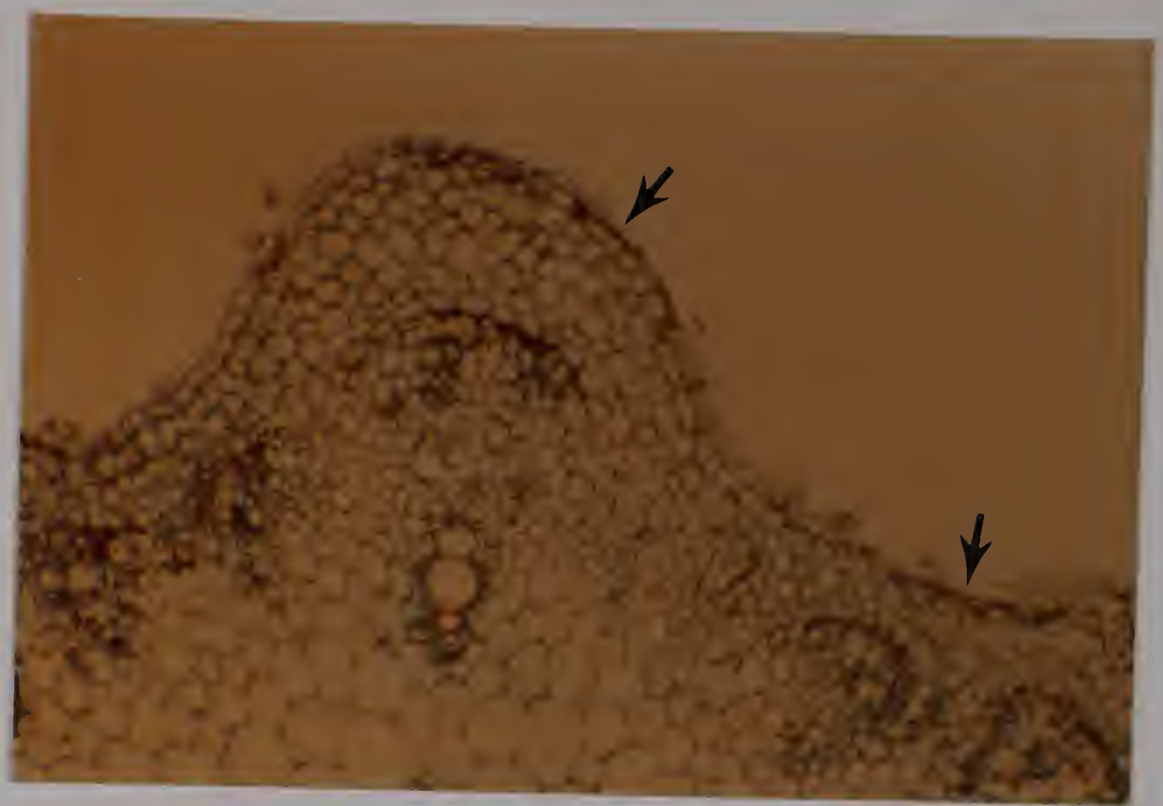


Fig. 14. Paraffin section of tobacco ringspot virus-infected cowpea stem showing early stages in the lateral spread of necrosis. Line of necrosis advancing laterally from the main vascular bundles. Note the epidermal necrosis at arrows (100X).

Fig. 15. Enlarged segment of Fig. 14 showing necrosis of epidermal and subepidermal cells and of primary phloem fibers. Note that the collenchyma between them is only mildly affected (400X).



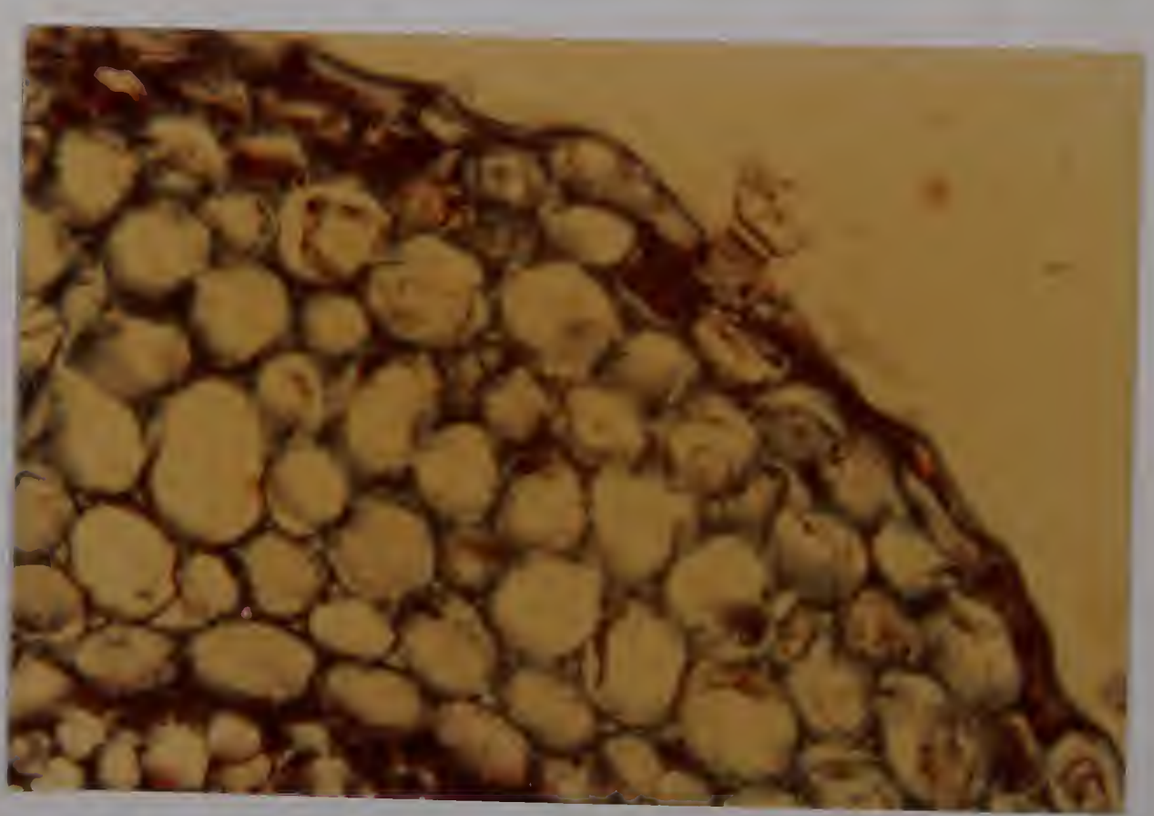


Fig. 16. Paraffin section of tobacco ringspot virus-infected cowpea stem showing discoloration and necrosis in the protophloem and in xylem and xylem parenchyma of a main vascular bundle. Note the apparently occluded xylem vessel (arrow). Some necrosis is evident in the cambium and primary phloem fibers of the interfascicular region at right as well as in the main bundle (400X).

Fig. 17. Enlarged segment of Fig. 16 showing the necrosis of xylem parenchyma and the occlusion of some xylem vessels (400X).

phloem and the xylem are necrotic at this point, the metaphloem appears healthy. Soon cells of the cambium, especially in the interfascicular areas, become necrotic, while the metaphloem is still healthy in appearance (Fig. 18). The necrosis of protophloem and cambium, but not metaphloem, results in a double line of necrosis extending around much of this stem(Fig. 22). Eventually, necrosis also develops in the metaphloem, but at first it involves only individual cells or small groups of cells (Figs. 19,20,21). Collenchyma and pith parenchyma become discolored and necrotic in later stages of necrosis (Figs. 22,23). Occasionally, necrosis is visible in these tissues at a much earlier stage, but usually it occurs only when these cells are located immediately adjacent to other necrotic cells. In the final stages, a generalized necrosis is apparent. Massive vascular necrosis occurs, as well as necrosis of the collenchyma, epidermis and pith (Figs. 22,23,24)

In the older tissues of the lower stem, necrosis does not usually originate in the primary phloem fibers. Rather, necrosis occurs first in the metaphloem followed by discoloration and necrosis of xylem, xylem parenchyma, cambium, and pith (Fig. 25).

Some variability exists in the developmental stages and distribution of necrosis. Collenchyma necrosis may precede that of the epidermis. Some randomly distributed necrotic cells are often observed in the pith, cambium, collenchyma, or epidermis.

Number of Local Lesions in Relation to Initiation of Systemic Necrosis

A total of 336 plants were tested to determine the relationship

Fig. 18. Fresh section of tobacco ringspot virus-infected cowpea stem showing well-developed line of necrosis, extending through the protophloem of the main vascular bundle, and the interfascicular regions. Necrosis is also evident in the xylem, interfascicular cambium, epidermis, and collenchyma.

Fig. 19. Patchy necrosis developing in the interfascicular region, in this case extending through the entire phloem, including the metaphloem. Some discoloration of vessels, and some necrosis of primary phloem fibers, cambium, and metaphloem can be seen in main vascular bundle (100X).

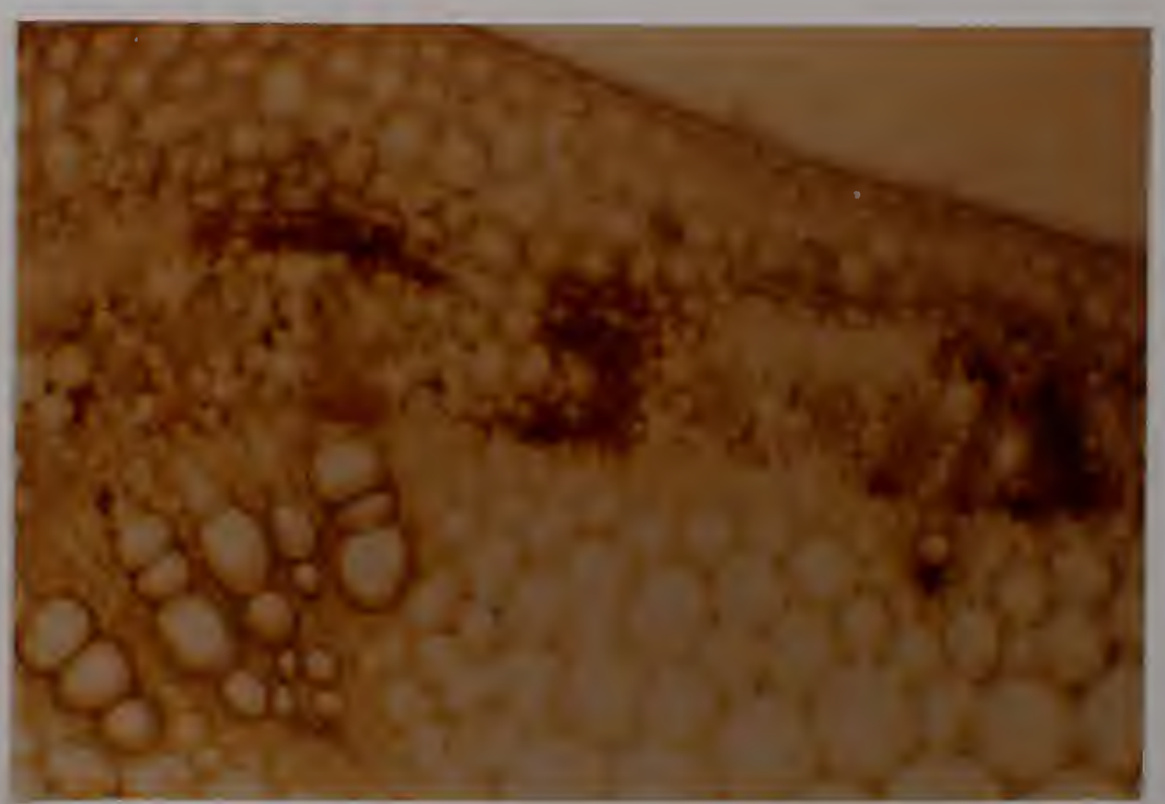


Fig. 20. Fresh section of tobacco ringspot virus-infected cowpea stem showing severe necrosis of xylem, xylem parenchyma, cambium, and metaphloem cells in a main vascular bundle (100X).

Fig. 21. Fresh section of tobacco ringspot virus-infected cowpea stem showing more severe necrosis. The entire phloem, cambium, and xylem of area at left is necrotic. Primary phloem fibers, xylem, and xylem parenchyma of main vascular bundle are discolored and necrotic, while necrosis is just beginning to develop in the metaphloem. Some necrosis of neighboring parenchyma is evident (100X).

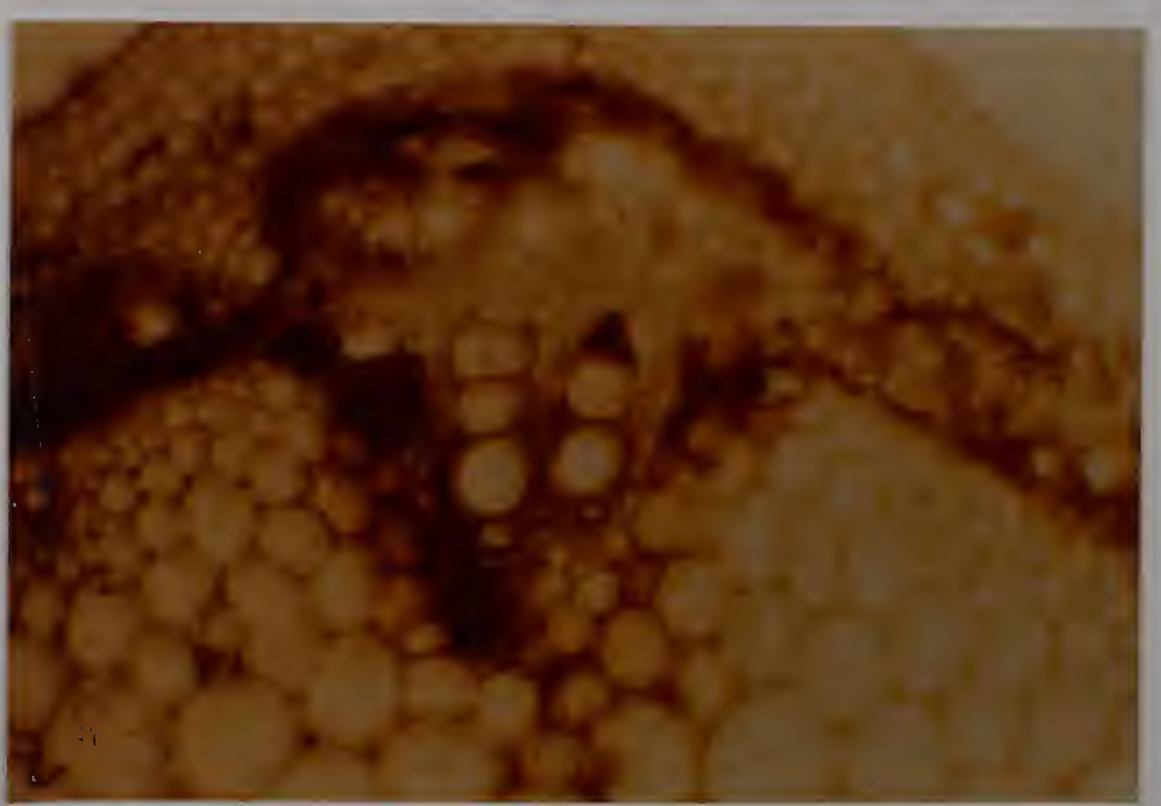


Fig. 22. Fresh section of tobacco ringspot virus-infected cowpea stem showing necrosis advancing through interfascicular cambium and primary phloem fibers in a double line, apparently originating from the main vascular bundle at the right and spreading towards the left of the photograph. Note the discoloration and necrosis of collenchyma (100X).

Fig. 23. Fresh section of tobacco ringspot virus-infected cowpea stem showing the final stages of stem necrosis in various tissues: epidermis, collenchyma, phloem (in its entirety), xylem, and pith. Massive necrosis of the vascular tissues is evident. Xylem vessels are occluded (100X).

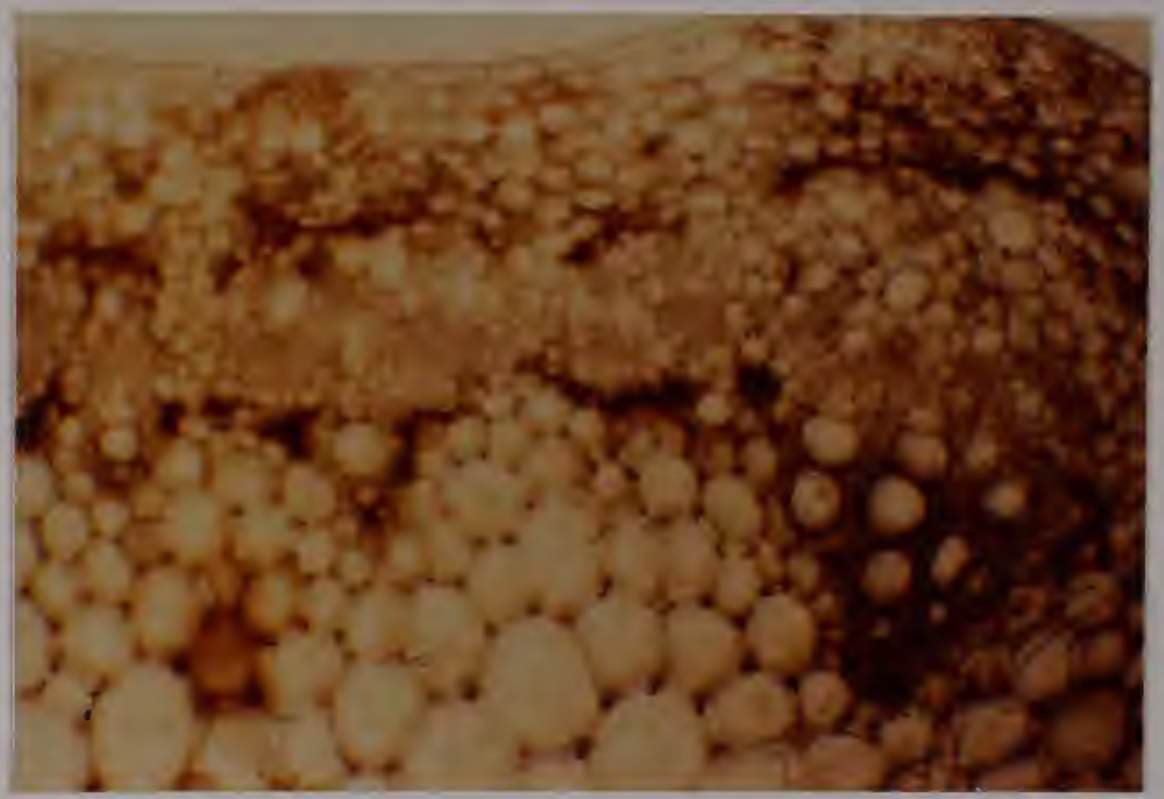
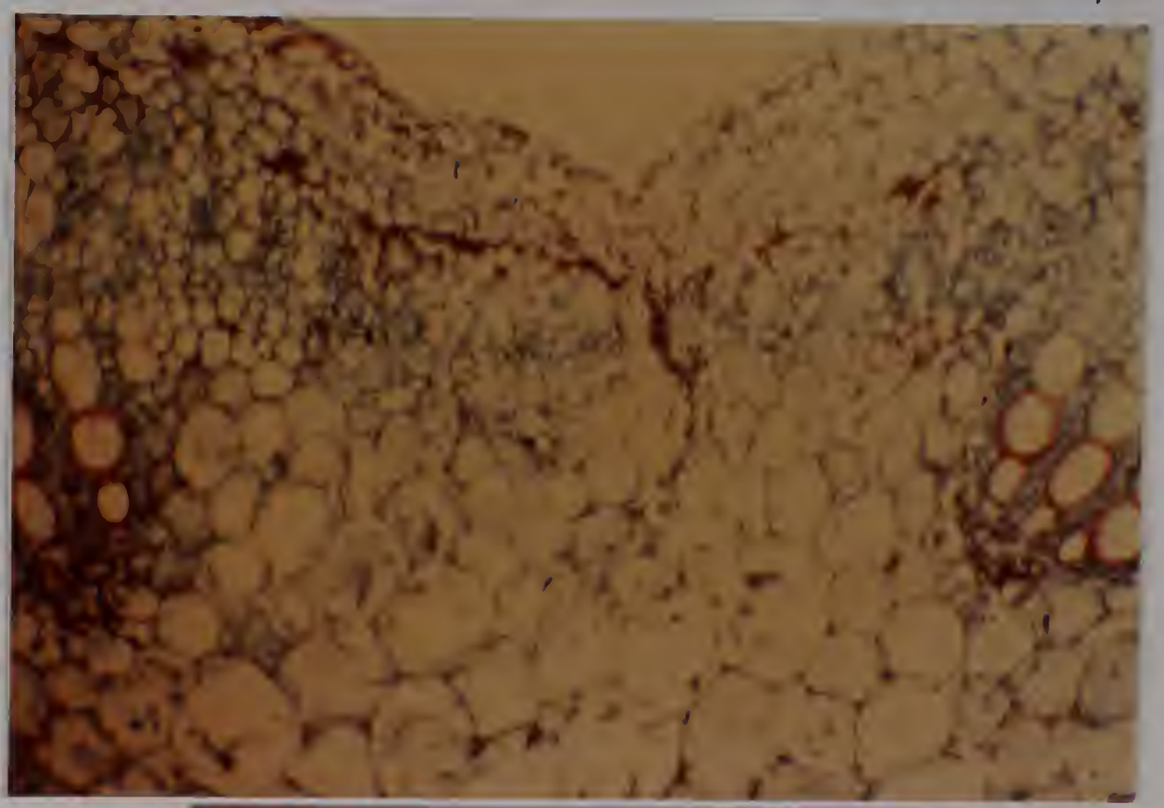
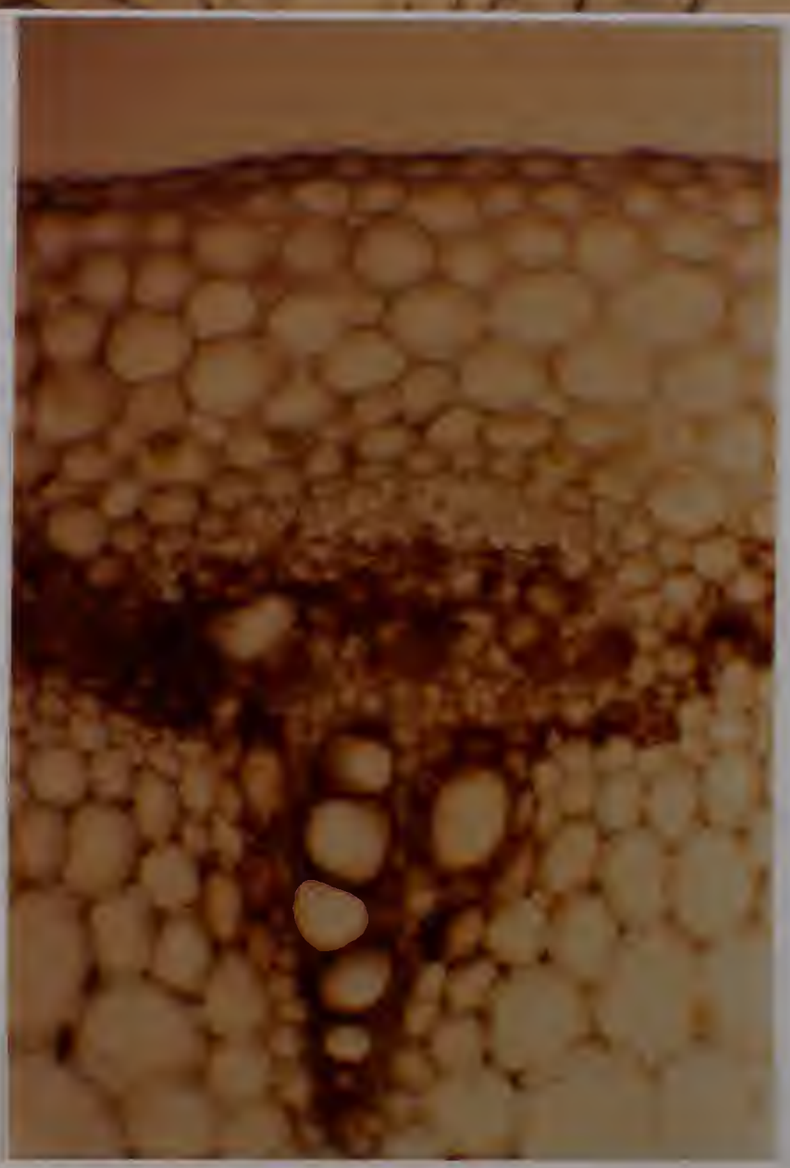


Fig. 24. Paraffin section of cowpea stem infected with tobacco ringspot virus showing extensive necrosis. Epidermis, collenchyma, primary phloem fibers, xylem and some cambium affected (100X).

Fig. 25. Fresh section of lower stem of cowpea plant infected with tobacco ringspot virus showing discoloration and necrosis in the metaphloem, xylem, xylem parenchyma, and cambium. Only slight discoloration of primary phloem fibers has occurred (100X).





between the number of local lesions per plant and the initiation and development of systemic necrosis.

Of the 100 plants with one local lesion, 61 developed systemic necrosis. Plants with two or more local lesions, however, developed systemic necrosis in approximately 90% of the cases (Table 1).

Most of the plants that developed systemic necrosis died, but some of them survived. Of the 61 plants with one local lesion that developed systemic symptoms, only 46 developed sufficient necrosis to kill at least the apex of the plants. In contrast, approximately 85% of plants with 2 or more local lesions developed severe enough necrosis to kill at least their apices (Table 1).

An inverse relationship was observed between the number of local lesions present per plant and the length of time required for the initiation and development of systemic necrosis. Generally, plants with few local lesions (1-2) required much more time to develop systemic symptoms than those with many local lesions (11-50).

Virus Concentration and Distribution in Relation to Development of Necrosis

No virus was detectable in any portion of the stem tested until the fifth day after inoculation (Table 2). By the fifth day, local lesions were well developed on the inoculated primary leaves. The only systemic symptoms visible were small necrotic areas in the tri-

Table 1. Relationship between number of local lesions and initiation and development of systemic necrosis in tobacco ringspot virus-infected cowpea plants

	Local Lesions Per Plant										
	1	2	3	4	5	6	7	8	9	10	11-50
No. of plants tested	100*	55	36	27	14	22	9	5	7	6	55
No. of plants with systemic necrosis	61	52	33	25	12	21	8	5	6	5	50
% of plants with systemic necrosis	61	95	92	93	86	96	89	100	86	83	91
No. of tested plants in which at least the apex died	46	47	31	22	12	21	8	5	6	5	49
% of tested plants in which at least the apex died	46	85	86	82	86	96	89	100	86	83	89

*Each number represents the combined results of six replications.

Table 2. Vertical distribution and concentration of tobacco ringspot virus in cowpea plants in relation to development of systemic necrosis (LL/half leaf, 8 half leaves, 3 replications).

Section of inoculated plant used for sub-inoculation	Days after original inoculation						
	1-4[a]	5[b]	6[c]	7[d]	8[e]	9[f]	10[g]
Apex to primary leaf node (PLN)	0	11	43	82	92	115	94
PLN	0	2	29	62	73	103	126
1 cm below PLN	0	1	23	48	51	112	114
1 cm above soil line	0	0	19	24	45	65	49

Symptoms on originally inoculated plants at time of subinoculation:

[a]Local lesions appear 3-4 days
[b]Necrosis first appears in trifoliates
[c]Necrosis first appears in upper stem just below apex
[d]Necrosis more severe, spreading below PLN
[e]Stem between apex and PLN collapsing
[f]Collapsing of stem more severe
[g]Stem between apex and PLN shriveled and drying out

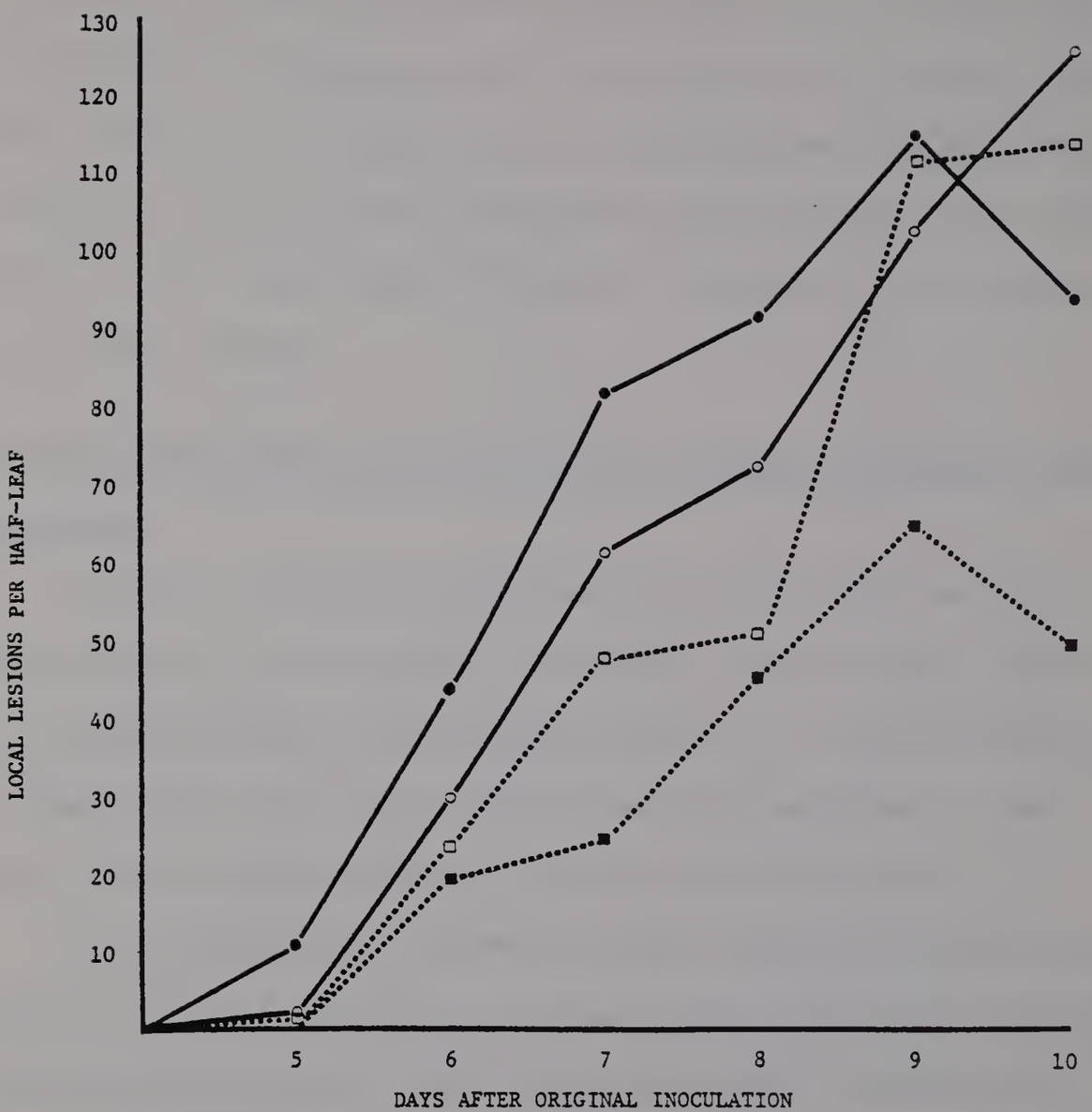
foliate leaves. The highest concentration of virus along the stem on the fifth day was detected in the uppermost portion of the stem, between the apex and primary leaf node (Table 2).

Virus concentration continued to increase in most sections tested until the ninth day after inoculation, but in the section including the primary leaf node, virus concentration continued to increase through the tenth day (Table 2, Fig. 26).

Distribution of virus in the different stem areas varied somewhat. Largest concentrations of virus were present in the upper stem, i.e., in the internode between the apex and primary leaf node, up until the ninth day. At that point, distribution of virus in the three upper stem areas was quite uniform (Fig. 26). Final virus concentration in the lowermost section tested (1 cm above the soil) remained consistently lower than the virus levels detected in the other stem sections (Table 2, Fig. 26).

Development of systemic necrosis corresponded with the rise in virus concentration. Six days after inoculation, stem necrosis was first visible in the upper stem, just below the apex. At that point, a rapid increase in virus concentration was observed. This rate of increase was continued until the seventh day in all but the lowest portion of the stem. Necrosis in the internode between the apex and primary leaf node became severe and spread down the stem past the primary leaf node. During the seventh and eighth days there seemed to be a degree of leveling off of virus production in the top of the plant. A similar rate reduction appeared to occur in the lower stem between the sixth and seventh days. By the eighth day

Fig. 26. Graphic representation of the data in Table 2 showing vertical distribution and concentration of tobacco ringspot in cowpea plants in relation to development of systemic necrosis. Section of plant assayed: ●——● apex to primary leaf node: o——o primary leaf node; □·····□ 1 cm below primary leaf node; ■·····■ 1 cm above soil line.


130
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LOCAL LESIONS PER HALF-LEAF
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10
DAYS AFTER ORIGINAL INOCULATION

necrosis became severe, and the stem between the apex and primary leaf node was collapsing. Collapse and constriction of the top internode was even more severe on the ninth day, when the virus concentration throughout most of the plant was at its peak. By the tenth day, the stem at the top internode was shriveled and drying out, and the virus concentration was leveling off or dropping. Virus concentration continued to increase in the primary leaf node, however.

Virus Concentration and Distribution in Plants Showing One-sided Necrosis.

The first detectable amount of virus in the stem was obtained again on the fifth day after inoculation, when the first signs of trifoliate necrosis were appearing (Table 3). Local lesions on primary leaves were quite distinct and well-developed by then, since they first appeared three or four days after inoculation.

Virus concentration increased fastest and was greatest on the side of the stem between the apex and primary leaf node just above the inoculated primary leaf ("inoculated side"). Maximum virus concentration in sections of the "inoculated side" of the plant was reached on the seventh day after inoculation; maximum virus concentration in sections of the "noninoculated side" of the plant was reached on the eighth or ninth day after inoculation (Table 3, Fig. 27). Virus concentration in opposites sides of the upper stem was approximately equal by the eighth day, and higher in the noninoculated side by the ninth day. The lowest concentrations of virus occurred

Table 3. Distribution and concentration of tobacco ringspot virus in cowpea plants inoculated on one primary leaf and showing primarily one-sided necrosis (LL/half leaf, 8 half-leaves, 4 replications)

Sections of inoculated plant used for sub-inoculation		Days after original inoculation						
		4[a]	5[b]	6[c]	7[d]	8[e]	9[f]	10[g]
Apex to primary leaf node	Inoculated (necrotic) side	0	37	82	116	110	48	20
	Noninoculated side	0	15	48	94	106	70	26
1 cm below primary leaf node	Inoculated (necrotic) side	0	10	34	74	68	51	30
	Noninoculated side	0	1	5	8	18	28	23

Symptoms on originally inoculated plants at time of subinoculation:

[a]Local lesions appear in 3-4 days

[b]Necrosis first appears in trifoliates

[c]Necrosis first appears in upper stem just below apex on the inoculated side

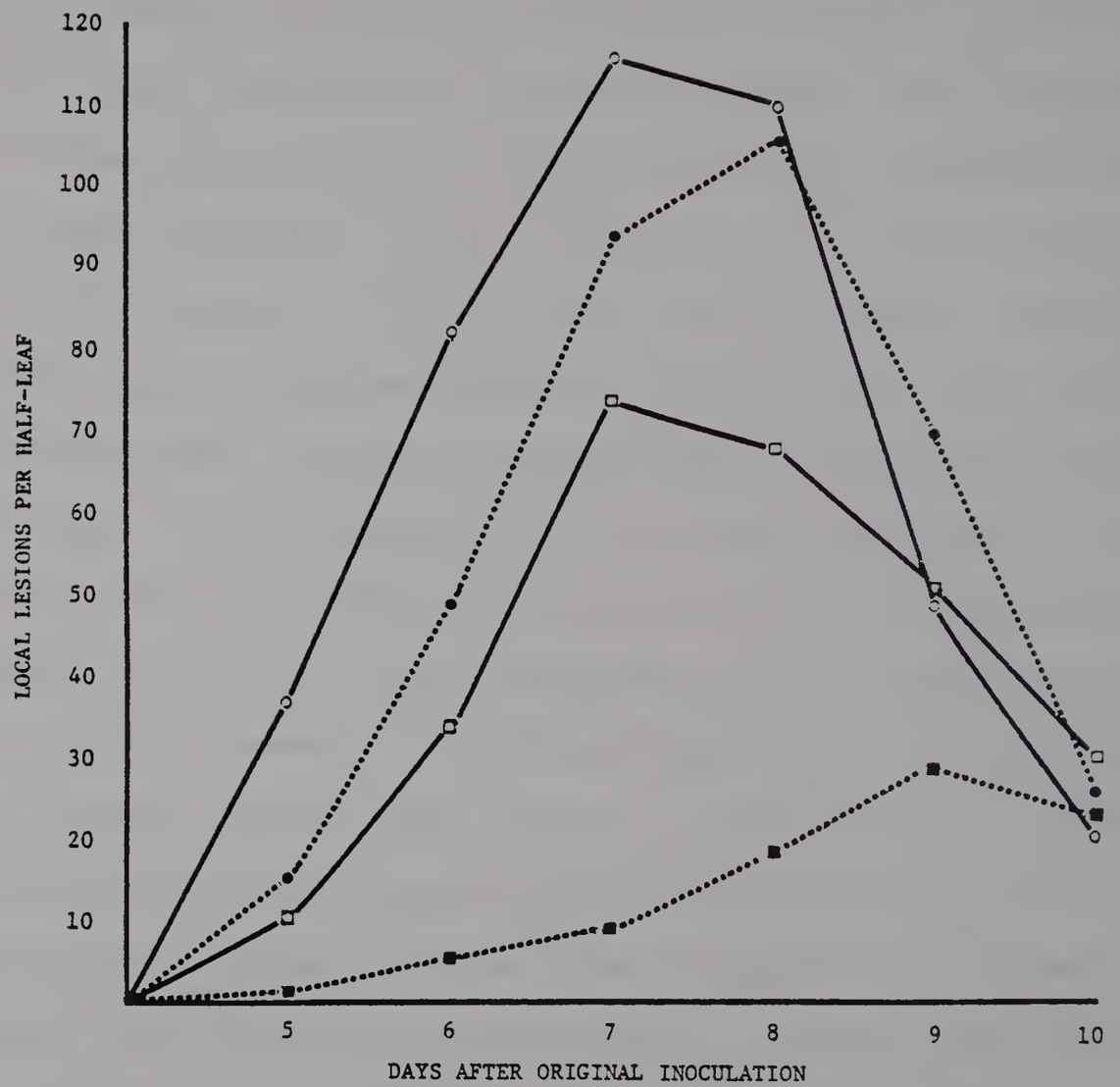
[d]Necrosis more severe, spreading below primary leaf node (PLN) and around stem to noninoculated side

[e]Collapse of inoculated side of stem just below apex, noninoculated side becoming more necrotic

[f]Necrosis and collapse of both sides of upper stem more severe

[g]Stem between apex and PLN shriveled and drying out

Fig. 27. Graphic representation of the data in Table 3 showing distribution and concentration of tobacco ringspot virus in cowpea plants inoculated on one primary leaf and showing primarily one-sided necrosis. Section of plant assayed: ○——○ apex to primary leaf node (PLN)/inoculated side; ●·····● apex to PLN/noninoculated side; □——□ 1 cm below PLN/inoculated side; ■·····■ 1 cm below PLN/noninoculated side.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LOCAL LESIONS PER HALF-LEAF
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DAYS AFTER ORIGINAL INOCULATION

in the "noninoculated side" of the stem, in the section one centimeter below the primary leaf node (Fig. 27).

Virus distribution was asymmetric, as was symptom development. In the section of the stem above the primary leaf node, the concentration of the virus was consistently higher on the "inoculated side" than the corresponding "noninoculated side" through the seventh day after inoculation. Up until the sixth day, necrosis was very slight and limited to the "inoculated side" of the stem. By the seventh day, necrosis was spreading around the stem to the "noninoculated side", as well as extending down the stem past the primary leaf node. On the eighth day after inoculation, the amount of virus in each side of the stem above the node was virtually identical. However, by that day, virus concentration in the "inoculated side" was declining, whereas in the "noninoculated side" it was just reaching its peak. By day eight, necrosis was quite severe on both sides of this portion of the stem, and the "inoculated side" of the stem had begun to collapse. Ten days after inoculation, virus concentration in both sides had declined greatly, while the stem itself was shriveled and drying out.

Symptom development and virus distribution were more asymmetric in the stem section below the primary leaf node. Virus concentration in the "noninoculated side" remained at a level much below that in the "inoculated side", although by the tenth day virus concentration in the "inoculated side" had decreased to the level of that in the "noninoculated side". Necrosis was first visible on the "inoculated side" of the stem on the seventh day after inoculation, when virus

concentration in this section of the stem reached its peak. Some slight necrosis of the "noninoculated side" of the stem of some plants was first observed on the eighth day. On the ninth day, all plants exhibited some degree of necrosis on the "noninoculated side" of the stem below the primary leaf node. By this time, necrosis of the "inoculated side" of the stem below the primary leaf node was quite severe.

DISCUSSION

TRSV-induced necrosis of stems of cowpea plants occurs in addition to the formation of local lesions on inoculated primary leaves. The external symptoms of such necrosis are similar to those produced in certain other systemic necroses, such as Nicotiana glutinosa infected with glutinosa necrosis virus (Miyamoto and Miyamoto, 1971), tabasco pepper infected with tobacco etch virus (White and Horn, 1975), Pinto bean infected with southern bean mosaic virus (Worley, 1965), broad bean infected with cucumber mosaic virus (Porter, 1954), and broad bean infected with the cucumber strain of tomato ringspot virus (Smith and McWhorter, 1957).

Both fresh and paraffin sections were used in studying the histopathology of TRSV-induced necrosis of cowpea stems. Paraffin sections were stained with safranin and fast green due to the superior ability of this stain combination to reveal necrotic areas. Diseased tissue can be distinguished from healthy tissue in sections stained with safranin and fast green by the amount of safranin taken up during staining. Much more safranin is taken up by diseased tissue than healthy tissue, even though both may look identical. However, because processing and staining can obscure changes in the tissue, if those changes only involve discoloration, fresh sections of necrotic stems were also examined.

In both paraffin and fresh sections, the origin of stem necrosis appears to be in the primary phloem fibers of one main vascular bundle (Figs. 10,11,12,13). This corresponds closely with the observations

of Porter (1954), Smith and McWhorter (1957), and Worley (1965). Porter found that stem necrosis began in a single vascular bundle in CMV-infected broad beans. Worley, working with SBMV-infected Pinto beans, and Smith and McWhorter, working with TomRSV-infected broad beans, found that initial necrosis of stems involved the primary phloem fibers. They speculated that cyclosis in the fiber cells caused rapid virus transport down the stem. It would seem reasonable then, to assume that TRSV also moves through the phloem fibers of systemically infected cowpeas.

Phloem fibers of older tissues, such as those of the lower stem, usually do not develop necrosis in the same manner as those in the upper stem (Fig. 25). This could be due to the age of the fibers, since in the lower stem the fibers are less active than in the upper stem.

Other data suggests that TRSV moves in the phloem with the general carbohydrate flow. Kuriger and Agrios (1977) were able to detect TRSV in the terminal millimeter of cowpea roots 3 days after inoculation of the primary leaves, which indicates that the virus moves rapidly from the primary leaves to the roots. The virus presumably moves through the phloem, without infecting and replicating in many cells along the way, since in our experiments TRSV could not be detected in the stem until 5 days after inoculation of the primary leaves. By the fifth day, virus concentration was highest in the uppermost portion of the plant (Tables 2,3; Figs. 26,27). This indicates that this virus, too, moves first down to the roots through the stem via the phloem, and then back up to the young, rapidly growing tissues of the upper stem in

a way similar to that of TMV in tomato, as established by the classic work of Samuel (1934). Although Samuel did not prove it then, it has since been well established that viruses move over long distances via the phloem (Esau et al., 1967).

Phloem degeneration and necrosis in TRSV-infected cowpea plants does not involve hypertrophy, hyperplasia, or phellogen formation as is commonly observed in top necrosis of potatoes, potato leaf roll, and curly top of sugar beet (Quanjer, 1931; Bawden, 1932, 1964; Esau, 1938, 1948; Rasa and Esau, 1961). Cambial activity appears normal, unlike the suppression of the interfascicular cambium observed by Smith and McWhorter (1957) just prior to stem necrosis in TomRSV-infected broad beans. In fact, no abnormalities of any kind were observed in cell growth or differentiation; nor is the necrosis limited to the phloem, as it is in potato leaf roll, curly top of sugar beet, barley yellow dwarf, and beet yellows (Quanjer, 1931; Bawden, 1932, 1964; Esau, 1938, 1948, 1957, 1960; Rasa and Esau, 1961).

In TRSV-infected plants, necrosis spreads laterally from the main bundles into the interfascicular regions (Figs. 14,18). Following necrosis of the protophloem, xylem and cambium become necrotic next (Figs. 16,17,18,21,22), and these are followed by necrosis of the metaphloem (Figs. 19,20,21). Epidermis, collenchyma, and pith are generally affected in later stages of necrosis, although certain cells in these tissues may become necrotic even before the metaphloem (Figs. 22,23,24). Similar complete necrosis was observed in SBMV-infected Pinto bean (Worley, 1965), TomRSV-infected broad bean (Smith and Mc-

Whorter, 1957), and CMV-infected broad bean (Porter, 1954). However, the sequence of appearance of necrosis in the stem tissues was not determined in the above studies. Worley, who inoculated bean stems with SBMV, inferred the sequence of tissue necrosis to begin with the epidermis, and subsequent tissue death was apparently caused by cell to cell movement(and replication) of the virus until the phloem was reached. Therefore, the cortex, endodermis, phloem, xylem parenchyma, and pith probably underwent necrosis in that order.

In cowpea plants infected with TRSV, lateral symptom development in the nonvascular tissues can be explained as the result of cell to cell movement of the virus. Even apparently isolated necrotic cells (in the pith, for example) may not be really isolated, but instead they may be adjacent to other necrotic cells and only appear to be isolated due to the orientation of the cut when sectioning. However, it should be noted that in contrast to the results of White and Horn (1975), no root necrosis was observed until after stem necrosis was quite severe, despite the fact that virus was shown to be present in roots since the third day after inoculation (Kuriger and Agrios, 1977). Virus would, presumably, also be present in sieve elements of the metaphloem through which the virus moved from the primary leaves to the roots, and yet these elements were not necrotic. Obviously, then, presence of virus in individual cells does not necessarily lead to necrosis. Instead, there seems to be a differential sensitivity in the various types of cells to the presence of virus. Another possibility, of course, is that the seemingly isolated necrotic

cells are indeed isolated in terms of virus invasion and reproduction. In this case, it would be interesting to know whether or not virus is actually present in the necrotic cells, or whether it is present in them earlier or in greater concentration than in adjacent necrotic cells.

Studies of beet leaves infected with beet yellow stunt virus suggest that virus may well be absent from isolated, necrotic cells (Hoefert et al., 1970). Although degenerative changes occurred in mesophyll parenchyma, phloem parenchyma, and sieve elements of beet leaves, no virus was present within the affected cells. It was speculated that the presence of virus in one cell could have been disturbing the metabolism in adjacent cells, or that the virus was present in incomplete (and therefore undetectable) form.

The data obtained in our experiments support the contention that virus is present in all symptom-showing tissues. There was a direct correlation between a rise in virus concentration and macroscopic symptom development. Appearance, extent, and severity of systemic necrosis closely followed or coincided with the rise in concentration of virus in the stem (Tables 2,3; Figs. 26,27). The rate of virus increase was affected to some extent by the necrosis it caused. When virus concentration was determined in relation to symptom development, the rate of virus increase was slightly lower between the seventh and eighth days after inoculation, possibly because of the massive necrosis induced in the stem (Table 2; Fig. 26). This was followed by an unexplained greater rate of virus increase. It is possible that the renewed increase was due to the invasion of previously

uninfected pith parenchyma cells. By the tenth day, the total virus concentration was decreasing, presumably due to the shriveled volume and dried state of the stem, combined with inactivation of virus by high concentrations of phenolic compounds in the necrotic tissues. The relatively low levels of virus detected in the stem just above the soil line are probably due to the fact that the stem in that region is hollow and consists of older, less active cells, since there is very little active growth there in comparison with the upper stem.

In the experiment designed to study virus distribution in asymmetrically necrotic stems, the same correlation between virus concentration and symptom development was observed (Table 3; Fig. 27). Asymmetry in symptom development was shown to be directly related to asymmetry in virus distribution. As virus distribution became more uniform, so did symptom development. This confirms similar results obtained by Resconich (1963), who found that in soybean plants inoculated with tobacco necrosis virus on only one primary leaf, the appearance of systemic symptoms was also strongly asymmetric. However, Resconich did not determine the actual virus distribution in the plant, but only assumed that the virus was also asymmetrically distributed.

Not all cowpea plants inoculated with TRSV develop systemic necrosis. Some variability exists, and is reflected in the different percentages of plants developing systemic symptoms (Table 1). Schneider (1965) obtained similar results with TRSV in Pinto bean. When he attempted to detect virus in uninoculated portions of plants, he found that unless high concentrations of TRSV were used, he could not detect any systemic

spread of the virus. Apparently, TRSV moves through cowpea plants much more easily than it does through Pinto beans, since 61% of cowpea plants with only one local lesion developed systemic necrosis. In contrast, sugar beet yellows virus (BYV) moves with difficulty through Chenopodium capitatum, since systemic infection of Chenopodium with BYV cannot be assured unless there are at least 200 local lesions per plant (Bennett, 1960). Only 10% of Chenopodium plants with 25 local lesions or less developed systemic symptoms. According to Schneider (1965), this occurs because the uninoculated areas of a plant can be more quickly and efficiently invaded by a virus if a higher inoculum concentration is used, since this results in a greater number of virus particles spreading from a greater number of infection sites. Apparently, in TRSV-infected cowpeas, the amount of increase in inoculum concentration required for a significant increase in efficiency of systemic spread of virus is fairly low, since the greatest difference in percentage of plants systemically infected was between plants with 1 and 2 local lesions. On the other hand, in BYV-infected Chenopodium, the amount of increase in inoculum concentration required for a significant increase in efficiency of systemic spread of the virus is considerably higher.

Although Table 1 does not show the length of time required for the initiation of systemic necrosis in relation to the number of local lesions, that time was generally inversely proportional to the number of local lesions present. Usually, plants with fewer local lesions required more time to develop systemic symptoms, if they developed systemic symptoms at all. The same relationship between symptom de-

velopment and time was observed in potato virus X-infected Datura tatula by Hooker and Benson (1960), who found that when inoculum concentration was decreased, the time required for symptom development increased.

SUMMARY

In tobacco ringspot virus-infected cowpea plants, external symptoms first become evident with the development of local lesions 3-4 days after inoculation of the primary leaves. Systemic symptoms appear 5 days after inoculation in plants inoculated with a 1:50 dilution of sap from TRSV-infected tobacco leaves. Necrotic spots or secondary lesions, appear in the lamina of trifoliate leaves, and necrotic areas develop along the veins of individual leaflets as well. Stem necrosis first appears on the sixth day in the top internode, between the apex and the primary leaf node. By the seventh day,necrosis is spreading down the stem past the primary leaf node. Internode necrosis is so severe by the eighth day that the stem in this region appears collapsed and constricted.

Internal stem necrosis originates in the immature primary phloem fibers in the protophloem of a main vascular bundle in the upper stem. Necrosis spreads laterally as well as vertically through the primary phloem fibers of the interfascicular regions. This is followed by discoloration and necrosis of the xylem and xylem parenchyma, cambium, metaphloem, epidermis, collenchyma, and pith. Necrosis of the xylem and cambium occurs much earlier than that of the metaphloem. Necrosis of protophloem on one side, and xylem and cambium on the other, but not of metaphloem, results in a double line of necrosis extending around much of the stem. Epidermis, collenchyma, and pith are affected in later stages of necrosis, although some necrosis of these tissues may be evident before that of the metaphloem. Seemingly

discontinuous effects of the virus on different cells indicates a differential sensitivity in the various types of cells to the virus.

In attempts to determine the minimum virus concentration required to induce systemic necrosis, local lesion assays were carried out that produced a range of local lesion numbers per plant. It was thus shown that only one local lesion is required to induce stem necrosis. However, efficiency of systemic infection increases with inoculum concentration. Of the plants with one local lesion, only 61% developed systemic necrosis. On the other hand, approximately 90% of plants with two or more local lesions developed systemic necrosis.

In attempts to correlate virus concentration and distribution in plant tissues with stages of necrosis of those tissues, cowpea plants were inoculated on the primary leaves and in subsequent days, sections of stems of such plants were used as inoculum to produce local lesions on other cowpea primary leaves. The number of local lesions produced, corresponding to the concentration of virus in the stem section, showed that there is a direct correlation between a rise in virus concentration and macroscopic symptom development. Appearance, extent, and severity of systemic necrosis closely follows or coincides with the rise in concentration of virus in the stem. Local lesion assays using split stem sections of cowpea plants showing asymmetric symptom development showed that virus distribution in these plants is also asymmetric,with earlier and greater virus production on the side of the stem showing symptoms first.

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