Initiation and Development of Systemic Necrosis in Relation to Virus Concentration in Tobacco Ringspot Virus-Infected Cowpea

M. C. Edwards and G. N. Agrios

Graduate research assistant and professor of plant pathology, respectively, Department of Plant Pathology, University of Massachusetts, Amherst 01003. Present address of senior author: Department of Plant Pathology, Cornell University, Ithaca, NY 14853.
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ABSTRACT

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Tobacco ringspot virus-infected cowpea plants developed local lesions 3–4 days after inoculation of the primary leaves and systemic stem necrosis, often on one side of the upper stem, 5 days after inoculation. Internally, stem necrosis originated in the immature primary phloem fibers in the protophloem of a main vascular bundle and then spread laterally and vertically to the primary phloem fibers of the interfascicular regions. Subsequently, discoloration and necrosis appeared in the xylem, xylem parenchyma, and cambium and (in later stages) in the metaphloem, epidermis, collenchyma, and pith. The frequency and severity of systemic infection increased with inoculum concentration. Appearance, extent, and severity of systemic necrosis closely followed or coincided with the rise in concentration of virus in the stem. In plants exhibiting asymmetric symptom development virus distribution also was asymmetric.

Sap inoculation of tobacco ringspsot virus (TRSV) on cowpea (*Vigna unguiculata* [L.] Walp.) results in formation of local leaf lesions followed by a systemic stem necrosis. Systemic necrosis of various types occurs, of course, in several other host-virus combinations. However, little work has been done on the initiation and development of systemic necrosis and most of this deals with phloem-limited necrosis (2,8,9,19,23) while much more research deals with electron microscope studies of hypersensitive local lesion reactions (1,4,5,10,13, and others).

The relation between virus concentration and distribution on one hand, and symptom development on the other appears to vary with the particular host-virus combination. Usually, the rate of symptom development and severity of symptoms in systemic infections is directly related to concentration of inoculum (3,12,18,22). The distribution of virus in systemically infected plants, however, is not necessarily uniform (11,16,20,21).

Cowpea infected with TRSV provide a good system for studying the relationship of virus presence and concentration in various plant parts to the initiation and development of systemic necrosis in those parts. So far, even in the few cases in which generalized necrosis has been studied, the sequence in which the various types of plant cells and tissues die has seldom been studied. This paper describes the sequence of events in TRSV-induced systemic stem necrosis in cowpea and the relation of virus concentration to the initiation and development of systemic necrosis.

MATERIALS AND METHODS

Cowpea plants (*Vigna unguiculata* [L.] Walp. 'California Blackeye') were grown in a soil-sand-peat moss mixture (1:1:1, v/v) in the greenhouse, or in growth chambers at day and night temperatures of 30 and 18 C respectively, with a daylength of 16 hr and a light intensity of about 7,000 lux. Two weeks after planting, the primary leaves of the plants were inoculated with a 1:50 dilution (v/v) of sap from TRSV-infected *Nicotiana tabacum* (L.) 'Turkish' in 0.01 M phosphate buffer pH 7.2. The New York grapevine strain of TRSV was used throughout these studies.

The histopathology of development of systemic necrosis was followed by examining both fresh and paraffin sections. For fresh sections, plants in various stages of necrosis were harvested, sectioned freehand, and observed immediately with a microscope. For paraffin sections, healthy (controls) and diseased plants were harvested daily, from day 1 to day 8 after inoculation. Five 5-mm-long sections were excised from each harvested plant and immediately placed in the fixative solutions. Each section represented a different portion of the plant: apex and node, stem just below the apex, primary leaf node 1 cm below the primary leaf node, and 2 cm below the previous section.

Standard histological procedures were used for tissue processing. Fixation was carried out in 2.5% glutaraldehyde, followed by dehydration in an ethanol/*t*-butanol series. Samples were embedded in Tissue Prep (Fisher Scientific Co., Fair Lawn, N.J. 07410) (56.5 C melting point) and sectioned at a thickness of 12–15 μm. Staining was done with safranin and fast green (14,15).

To determine the relationship between the number of local lesions and initiation of systemic necrosis, primary leaves of 2-wk-old pea plants were inoculated with 1:1,000 and 1:10,000 dilutions of TRSV-infected sap. Local lesions were counted 4–5 days after inoculation, and the plants were grouped according to the total number of local lesions per plant. Plants were observed for signs of systemic necrosis for 60 days after inoculation.

To determine the vertical distribution and relative concentration of TRSV in plants, 2-wk-old growth chamber grown cowpea plants were inoculated with a 1:50 (v/v) dilution of sap from TRSV-infected tobacco leaves. Each day following inoculation, five plants were harvested and four 1-cm-long sections were excised from each plant. The sections were as follows: apex to primary leaf node (including base of petioles of trifoliolate leaves), primary leaf node, a 1-cm section taken 1 cm below the primary leaf node, and a 1-cm section taken 1 cm above the soil line. 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 made with a glass rod so that strips of equal width were inoculated on each half-leaf (6). Inoculations were carried out for 8 days in one experiment and 10 days in two experiments. Local lesions were counted 4–5 days after inoculation. The number of local lesions was taken to be proportional to the amount (concentration) of infective virus present in the tissue used as inoculum.

To assay the distribution and relative concentration of TRSV in asymmetrically necrotic stem areas, 2-wk-old cowpea plants grown in the growth chamber were inoculated on only one primary leaf with a 1:10 (w/v) dilution of TRSV-infected cowpea tissue.

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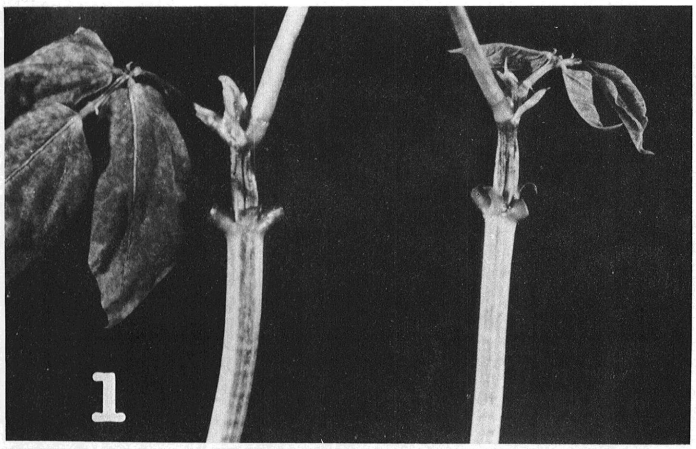
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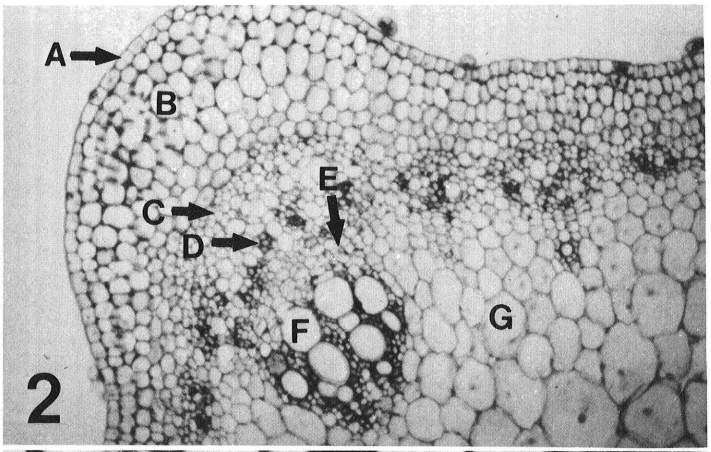
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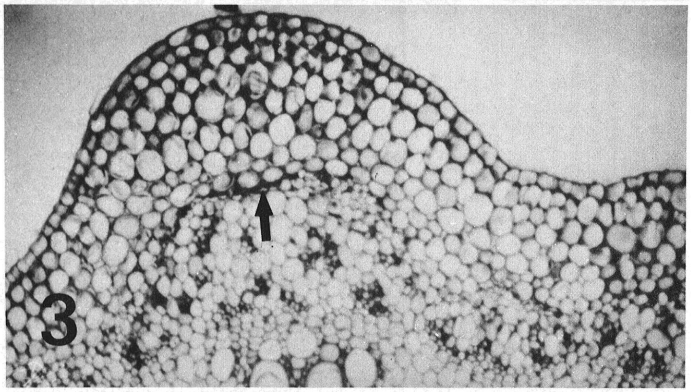
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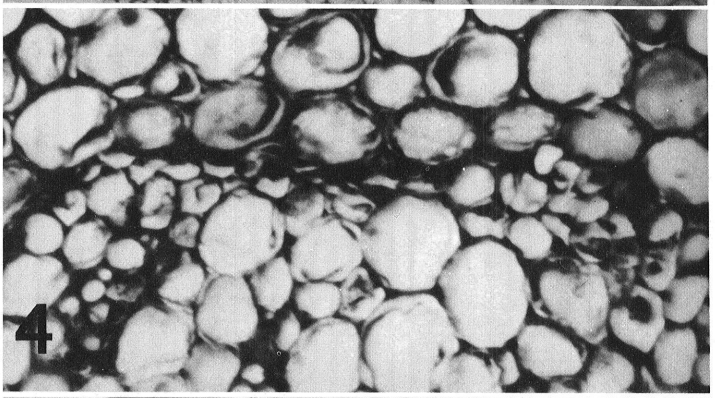
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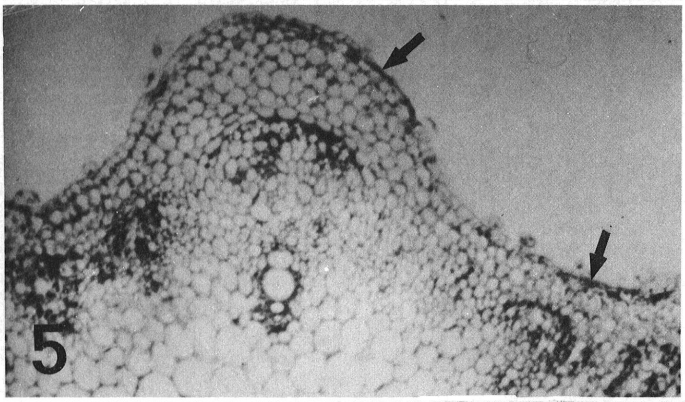
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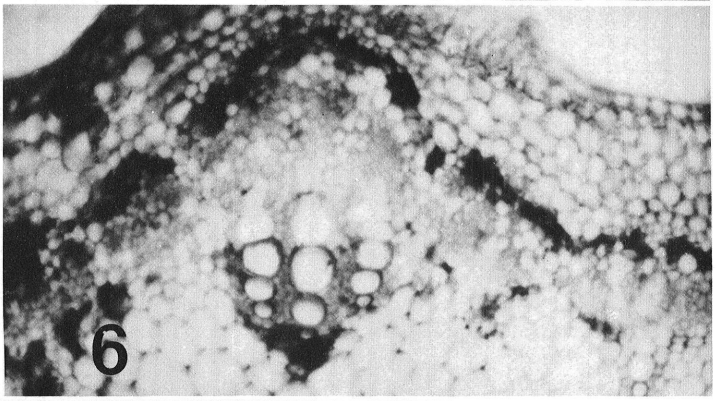
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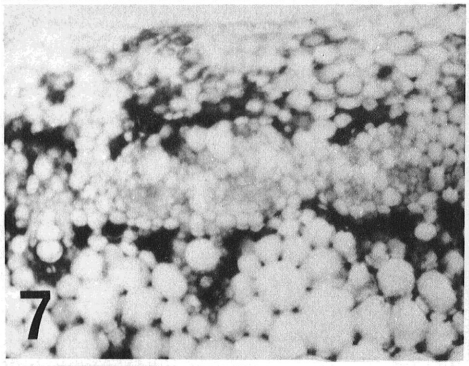
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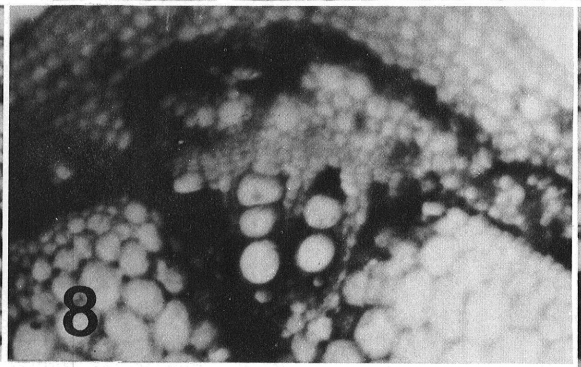
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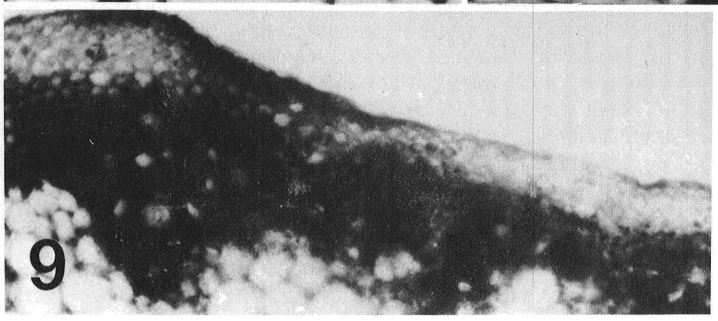
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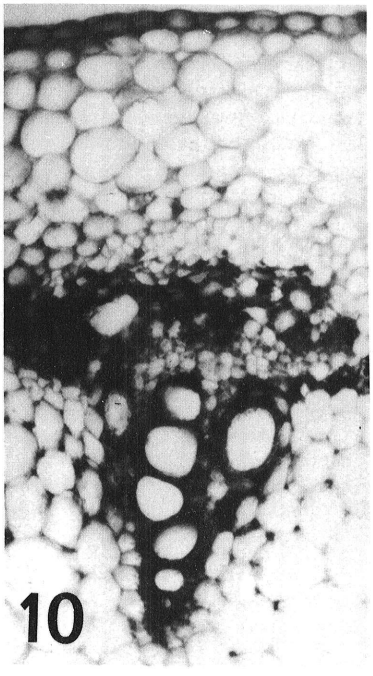
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Beginning on the 4th day after inoculation, five plants were harvested daily. Two stem sections were removed from each plant. One section extended from the base of the petioles of the trifoliolate leaves to the primary leaf node (no longer than 1.5 cm). The other section (1.5 cm long) was taken 1 cm below the primary leaf node. Each section was split longitudinally so that one side would include the inoculated side of the plant and generally represent the more necrotic areas, and the other side would include the noninoculated side of the plant and generally represent the healthy or less necrotic area. All four sections were 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. Again, the infectivity of the virus was used as a measure of virus concentration in the tissues used as inoculum.

RESULTS

External development of TRSV-induced necrosis in cowpeas. Cowpea plants inoculated with a 1:50 dilution (v/v) of homogenate from TRSV-infected tobacco leaves developed local lesions 3–4 days after inoculation and the first signs of systemic necrosis 5 days after inoculation. Rapidly growing trifoliolate leaves developed necrotic areas in the lamina and along the veins of individual leaflets. Stem necrosis first appeared as a slight reddish-brown coloration of the top internode, between the apex and the primary leaf node. While necrosis spread down the stem past the primary leaf node, necrosis of the upper stem (top internode) became so severe by the 8th day that it had a collapsed and constricted appearance (Fig. 1). At this point, trifoliolate leaves were quite wilted and withered.

In plants inoculated on both primary leaves when 2 wk old, stem necrosis began on one side but eventually spread laterally around the entire stem. Occasionally, necrosis was restricted to one side of the stem. There was a marked tendency for necrosis starting in one trifoliolate leaf to spread down the same side of the stem to which the trifoliolate leaf was attached. However, if only one primary leaf was inoculated, necrosis was limited to the side of the stem directly below that primary leaf. Age of the plant at the time of inoculation was a factor in determining whether the necrosis remained limited or spread laterally around the stem. Generally, the older the plants, the greater the frequency of one-sided necrosis.

Histopathology of TRSV-infected stems. Internal stem necrosis originated in the immature primary phloem fibers in the protophloem of a main vascular bundle in the upper stem (Figs. 2 and 3). After turning slightly brown and then being filled with dark brown substances, the immature fiber cells eventually collapsed, forming a necrotic line just outside the metaphloem (Figs. 3 and 4).

Initially, discoloration and necrosis appeared in only one or two main bundles on one side of the stem. Subsequently, however, they spread laterally as well as vertically through the primary phloem fibers of the smaller vascular bundles. The primary phloem fibers in each vascular bundle appeared as a line of collapsed, necrotic cells, eventually extending around the entire stem (Figs. 5 and 6).

Necrosis also spread away from the primary phloem, and into the epidermis and the xylem. Although necrosis of epidermal cells usually followed that of primary phloem fibers, there often was no necrosis in the intervening tissues of chlorenchyma and collenchyma (Fig. 5). In the meantime, xylem vessels and xylem parenchyma cells became discolored. Vessels occasionally appeared clogged with brown-colored materials of undetermined composition. Cambial cells, especially in the interfascicular areas, also became necrotic (Figs. 6 and 7). Although protophloem, xylem, and cambium were necrotic at this point, the metaphloem generally still appeared healthy (Figs. 6 and 7). The necrosis of protophloem and cambium, but not metaphloem, resulted in a double line of necrosis extending around much of the stem (Figs. 7 and 8). Eventually, necrosis also developed in the metaphloem, at first involving only individual cells or small groups of cells (Fig. 8). Collenchyma and pith parenchyma became discolored and necrotic in later stages of necrosis although, occasionally, necrosis was visible in these tissues at a much earlier stage. In the final stages, a generalized necrosis was apparent (Fig. 9). Massive vascular necrosis occurred in the vascular bundles, the cambium, collenchyma, epidermis, and pith.

In the older tissues of the lower stem, necrosis occurred first in the metaphloem rather than in the primary phloem fibers and it was followed by discoloration and necrosis of xylem, xylem parenchyma, cambium, and pith (Fig. 10).

Number of local lesions in relation to initiation of systemic necrosis. A total of 336 plants were tested to determine the relationship 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. On the other hand, approximately 90% of plants with two or more local lesions developed systemic necrosis (*unpublished*). The time required for the initiation of systemic necrosis was generally inversely proportional to the number of local lesions present; ie, plants with fewer local lesions required more time to develop systemic necrosis than did plants with more local lesions.

Virus concentration and distribution in relation to development of necrosis. No virus was detectable in any portion of the stem tested until the 5th day after inoculation, when the only systemic symptoms visible were small necrotic areas in the trifoliolate leaves (Table 1). Virus concentration in the stem continued to increase until the 9th or 10th day after inoculation.

Distribution of virus in the different stem areas varied somewhat (Table 1). Largest concentrations of virus were present in the upper stem (ie, in the internode between the apex and primary leaf node) up until the 9th day. At that point, concentration of virus in the three upper stem areas was similar. Final virus concentration in the lowermost section tested (1 cm above the soil) remained consistently lower than those detected in the other stem sections.

Development of systemic necrosis corresponded with the rise in virus concentration (Table 1). Following appearance of local lesions on the primary leaves (3–4 days after inoculation) and of systemic necrotic areas in trifoliolate leaves (5 days after inoculation), necrosis was first visible in the upper stem, just below the apex, on the sixth day after inoculation, where a rapid increase in virus concentration was observed. This rate of increase was continued until the 7th day in all but the lowest portion of the stem, while necrosis became severe in the internode between the apex and primary leaf node and spread down the stem past the primary leaf node. During the 7th and 8th days there seemed to be a degree of leveling off of virus production in the top of the plant while a

Figs. 1–10. Systemic necrosis caused in cowpea stems by tobacco ringspot virus (TRSV): **1,** Cowpea stems showing severe necrosis 8 days after inoculation of the primary leaves with TRSV. Stem area just below the apex is collapsing and necrosis extends down the stem, past the primary leaf node (left). **2,** Transverse section of a healthy cowpea stem showing the type and arrangement of the various cells and tissues: A, epidermis; B, collenchyma; C, protophloem (primary phloem fibers); D, metaphloem; E, cambium; F, xylem; and G, pith. **3,** Paraffin section of TRSV-infected cowpea stem showing necrosis of fibers in the protophloem of a main vascular bundle. Some cells have collapsed, forming a necrotic line capping the vascular bundle (arrow). **4,** Enlarged section of Fig. 3 showing collapsed and necrotic primary phloem fibers. **5,** Paraffin section of TRSV-infected cowpea stem showing line of necrosis beginning to form in the protophloem. Note epidermal necrosis at arrows. **6,** Fresh section of TRSV-infected cowpea stem showing well-developed line of necrosis, extending through the protophloem of the main vascular bundle as well as through interfascicular regions. Necrosis also is evident in the xylem, interfascicular cambium, epidermis, and collenchyma. **7,** Fresh section of TRSV-infected cowpea stem showing necrosis advancing through interfascicular cambium, xylem, and primary phloem fibers in a double line. Note also the discoloration and necrosis of collenchyma. **8,** More advanced stages of 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. **9,** Fresh section showing final, massive stages of necrosis. Many xylem vessels are occluded. **10,** Fresh section of lower stem of TRSV-infected cowpea plant showing discoloration and necrosis in the metaphloem, xylem, parenchyma, and cambium. Only slight discoloration of primary phloem fibers has occurred. (Magnifications: Fig. 4, × 400; all others, × 100).

similar rate reduction appeared to occur in the lower stem between the 6th and 7th days. By the 8th day, 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 9th day, when the virus concentration throughout most of the plant was at its peak. By the 10th 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 5th day after inoculation, when the first signs of trifoliolate necrosis were appearing (Table 2).

Virus distribution was asymmetric, as was symptom development. In the section of the stem above the primary leaf node, the concentration of virus was consistently higher on the "inoculated side" than on the corresponding "uninoculated side" through the 7th day after inoculation. Up until the 6th day, necrosis was very slight and limited to the "inoculated side" of the stem. By the 7th day necrosis was spreading around the stem to the "uninoculated side," as well as extending down the stem past the primary leaf node. On the 8th 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 "uninoculated side" it was just reaching its peak. By the 8th day, 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 "uninoculated side" remained at a level much below that in the "inoculated side," although by the 10th day virus concentration in the "inoculated side" had decreased to the level of that in the "uninoculated side" (Table 2). 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 "uninoculated side" of the stem of some plants was first observed on the 8th day. On the 9th day, all plants exhibited some degree of necrosis on the "uninoculated side" of the stem below the primary leaf node. By this time, necrosis on the "inoculated side" of the stem below the primary leaf node was quite severe.

TABLE 1. Distribution and relative concentration of tobacco ringspot virus in stems of cowpea in relation to time after inoculation and development of systemic necrosis as determined by infectivity assays on cowpea

Section of inoculated plant used for subinoculation	Local lesions per half leaf[a] at (days after original inoculation[b])						
	1–4	5	6	7	8	9	10
Apex to primary leaf node (PLN)	0	11	43	82	92	115	94
PLN	0	2	29	42	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

[a] Eight half leaves, three replications.

[b] Symptoms on originally inoculated plants at time of subinoculation: 1–4 days—local lesions appear in 3–4 days; 5 days—necrosis first appears in trifoliolate leaves; 6 days—necrosis first appears in upper stem just below apex; 7 days—necrosis more severe, spreading below PLN; 8 days—stem between apex and PLN collapsing; 9 days—collapsing of stem more severe; and 10 days—stem between apex and PLN shriveled and drying out.

TABLE 2. Distribution and relative concentration of tobacco ringspot virus in stems of cowpea inoculated on one primary leaf and showing primarily one-sided stem necrosis as determined by infectivity assays on cowpea

Sections of inoculated plant used for subinoculation		Local lesions per half leaf[a] at (days after original inoculation[b])						
		1–4	5	6	7	8	9	10
Apex to primary leaf node	Inoculated (necrotic) side	0	37	82	116	110	48	20
	Uninoculated side	0	15	48	94	106	70	26
1 cm below primary leaf node	Inoculated (necrotic) side	0	10	34	74	68	51	30
	Uninoculated side	0	1	5	8	18	28	23

[a] Eight half leaves, four replicates.

[b] Symptoms on originally inoculated plants at time of subinoculation: 1–4 days—local lesions appear in 3–4 days; 5 days—necrosis first appears in trifoliolate leaves; 6 days—necrosis first appears in upper stem just below apex on the inoculated side; 7 days—necrosis more severe, spreading below primary leaf node (PLN) and around the stem to the uninoculated side; 8 days—collapse of inoculated side of stem just below apex, uninoculated side becoming more necrotic; 9 days—necrosis and collapse of both sides of upper stem more severe; and 10 days—stem between apex and PLN shriveled and drying out.

DISCUSSION

Stem necrosis appears to originate in the primary phloem fibers of one main vascular bundle, as revealed by both paraffin and fresh sections. This corresponds closely with the observations of Porter (19) who found that stem necrosis began in a single vascular bundle in cucumber mosaic virus (CMV)-infected broad beans, and of Smith and McWhorter (23) and Worley (24) who found that primary phloem fibers are involved in the initial necrosis of stems in tomato ringspot virus (TomRSV)-infected broad beans and in southern bean mosaic virus (SBMV)-infected Pinto beans, respectively.

In older tissues, however, such as those of the lower stem, necrosis developed first in the metaphloem rather than the protophloem. This could be due to the age of the phloem fiber cells, since in the lower stem the primary fiber cells are older and less active than in the upper stem.

Distribution of TRSV depends on local cell-to-cell movement and on long distance transport through the phloem. TRSV has been detected in the terminal millimeter of cowpea roots just 3 days after inoculation of the primary leaves, indicating that the virus moves rapidly from the primary leaves to the roots (17). The virus presumably moves through the phloem, without infecting and replicating in many cells along the way; in our experiments TRSV could not be detected in the stem until 5 days after inoculation of the primary leaves. By the 5th day, virus concentration was highest in the uppermost portion of the plant, while no stem necrosis was yet evident.

Phloem degeneration and necrosis in TRSV-infected cowpea does not involve hypertrophy, hyperplasia, or phellogen formation as seems to occur in top necrosis of potatoes, potato leaf roll, and curly top of sugar beet (2,7). Cambial activity in TRSV-infected cowpea also appears to be normal, unlike the suppression of the interfascicular cambium observed just prior to stem necrosis in TomRSV-infected broad beans (23). In fact, tissue necrosis caused by TRSV infection does not seem to be accompanied by any apparent abnormalities in growth or differentiation of necrotic and adjacent tissues in cowpea plants.

The sequence of the lateral spread of necrosis from the main bundles into the interfascicular regions, xylem, cambium, metaphloem, epidermis, collenchyma, and pith appears similar to those observed in SBMV-infected Pinto bean (24), TomRSV-infected broad bean (24), and CMV-infected broad bean (19).

Although in the stem, tissue necrosis appears to follow or coincide with cell-to-cell movement and replication of virus, virus presence does not necessarily result in necrosis. Thus, despite the fact that virus was present in detectable quantities in roots the 3rd day after inoculation (17), root necrosis was not observed until after stem necrosis was quite severe. Also, the sieve elements of the metaphloem, through which the virus probably moved from the primary leaves to the roots, were not necrotic until late in the

infection.

When whole stem sections were considered, however, there was a direct correlation between virus concentration and macroscopic symptom development. Appearance, extent, and severity of systemic stem necrosis closely followed or coincided with the rise in concentration of virus in the stem. The rate of virus increase (or decrease) was affected to some extent by the amount of necrosis present, the virus concentration decreasing as the stem became shriveled and dry and as virus-inhibiting compounds presumably increased in the necrotic tissues. The relatively low levels of virus detected in the lower stem are probably due to the fact that the stem in that region is hollow and consists of fewer, older, and less active cells compared to those in the upper stem.

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. Appearance of systemic symptoms also was strongly asymmetric in soybean plants inoculated with tobacco necrosis virus on only one primary leaf, although the actual distribution of TNV in the plants was not determined (20).

The fact that not all cowpea plants inoculated with TRSV develop systemic necrosis (only 61% of plants with one local lesion did), is indicative of the variability which can be present in this system at low inoculum levels. Apparently, however, the amount of increase in inoculum concentration required for a significant increase in efficiency of systemic spread of virus is fairly low, since the percentage of plants infected systemically increased to approximately 90% for plants with two or more local lesions. On the other hand, the fact that plants with fewer local lesions required more time to develop systemic symptoms than those with more local lesions, also supports the conclusion that there is a direct correlation between virus concentration and macroscopic systemic necrosis.

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