Irregular Distribution of Tomato Ringspot Virus in Apple Trees

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ABSTRACT

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Leaf, bark, and root samples of Malling-Merton 106 (MM 106) and cultivar/MM 106 trees were assayed for tomato ringspot virus (TmRSV) by enzyme-linked immunosorbent assay (ELISA) at various times during the growing season. TmRSV was not regularly distributed within trees. In MM 106 trees, TmRSV was detected most consistently in leaves, slightly less so in bark, and only erratically in roots. The average absorbance values at 405 nm declined toward the end of the growing season and were always highest in leaves and lowest in roots. TmRSV was also detected in Golden Delicious bark tissue just above the graft union. The location and/or mode of inoculation seemed to influence the virus distribution within the trees.

In 1976, tomato ringspot virus (TmRSV) (22), which causes diseases in fruit and berry crops, eg, in peaches (21,25), grapevines (8,28,29), and red raspberries (12), was found associated with apple union necrosis and decline (AUND) disease (26). Since then, the disease has been reported to occur in the northeastern (5,6,23,24) and western United States (17). Delicious, McIntosh, and Tydeman's Early on Malling-Merton 106 (MM 106) rootstocks are especially affected. Because nearly 40% of all apple trees planted in New York State in the 1965–1975 decade are on MM 106 rootstocks (5), AUND may soon become a serious economic problem. Rosenberger et al (20) reported that AUND is indeed a serious problem in the Hudson Valley of New York.

Detecting TmRSV in woody plants has been a problem. Young, expanding leaves from the tips of shoots or root suckers have been used as tissue sources for detecting TmRSV in apple (4,17,24,26), grapevine (9,10,28), blueberry (11), and strawberry (3). However, TmRSV was not recovered consistently, probably because of irregular distribution within the plants (2,4,7,13–16). Lister et al (13) suggested that bark might be a more suitable tissue for virus detection than leaves. Using a bark sampling method, Rosenberger et al (20) detected TmRSV in 89% of trees that showed AUND symptoms.

The time of year also seems an important factor for virus detection (18,29). L. B. Forer (*personal communication*) consistently detected TmRSV in the leaves of orchard peach trees in May and in the roots and bark in July. Gonsalves (10) studied the distribution of TmRSV in grapevine and found only a few leaves virus-positive during a test period of 6 wk.

The purpose of this study was to determine the distribution of TmRSV in MM 106 and cultivar/MM 106 trees over a summer by repeated assaying of leaf, bark, and root tissues, using the enzyme-linked immunosorbent assay (ELISA) technique, and to determine when during summer an apple tree should be indexed for TmRSV, what type of tissue should be sampled, and from which location in a tree the sample should be taken in order to get a reliable evaluation of TmRSV infection.

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MATERIALS AND METHODS

Trees. Nine trees at the New York State Agricultural Experiment Station in Geneva were investigated in detail (Table 1). TmRSV had been detected in these trees by ELISA either 1 wk before the detailed study began or at least once in previous years.

Tree 1 was a potted MM 106 in the greenhouse that had been bud-inoculated in June 1980 with the Chickadee isolate of TmRSV (4). The eight other trees were orchard trees 6–20 yr old. Trees 2–4 had been bud-inoculated with the Chickadee isolate of TmRSV in either 1976 or 1977 and kept in the greenhouse until planted in the field in 1979. The precise inoculation site was not determinable for each tree in summer 1982. Trees 5 and 6 had a joint root system. Tree 6, a Golden Delicious/MM 106, was planted in 1970 and bud-inoculated in the rootstock with the Amberg isolate of TmRSV (4) in May 1977. Tree 5 originated as a root sucker from tree 6 and had formed its own root system (but had not been detached); it could be regarded as a low branch of its mother tree and hereafter will be presented in the group of bud-inoculated trees.

Three trees had been naturally inoculated, presumably by nematodes (27). The virus isolate was not defined but was serologically similar to the Amberg isolate (hereafter called the Amberg-like isolate). Tree 7 was 20 yr old, consisted of three stems, and had grown from an old stool bed. Trees 8 and 9 were 13-yr-old Macspur/MM 106 and McIntosh/MM 106, respectively, standing in a well-managed orchard.

Tissue sampling. Samples of leaves, bark, and roots were collected between 11 June and 13 August 1982. Trees 1–5 were sampled three times at 3- or 4-wk intervals; tree 6 was sampled three times within 3 wk, tree 7 four times within 5 wk, and trees 8 and 9 once in August.

Leaf samples were chosen from different positions on the branches and from branches on different parts of a tree. Where present, at least one young expanding leaf from the tip of each branch was included at the first sampling (Fig. 1). The samples were put in an ice chest in the field immediately after collection. When leaves were sampled only once (trees 6–9), the whole leaf was collected. For sequential study (trees 1–5), each leaf sample was taken by cutting perpendicular to the main vein about one-third of the way from the apical end of the leaf for the first sampling. About half of the remaining leaf section was cut at the second sampling, and the rest was taken at the

last sampling date. In order to uncover an effect of leaf-cutting on virus detection, a whole leaf that developed immediately above and below the sampled tip leaf of each branch was included in the second and third sampling, respectively.

Bark samples were obtained by cutting with a knife a lanceolate- to oval-shaped slice 3–5 cm long and 1–2 cm wide, removing it, and scraping tissue from the cambial zone of the bark slice as well as from the exposed wood of the tree. The knife was swabbed with chlorine bleach, rinsed with water, and blotted dry between samplings. Samples were taken from the trunk and branches (minimum 2 cm diameter) where leaf samples were also taken (Fig. 1). Samples for the second and third indexings were taken 1–5 cm from the first one.

Root samples were taken by cutting with a knife down to the central cylinder, then proceeding in the manner described for the bark samples. Samples were collected from roots ranging from <1 to about 12 cm in diameter.

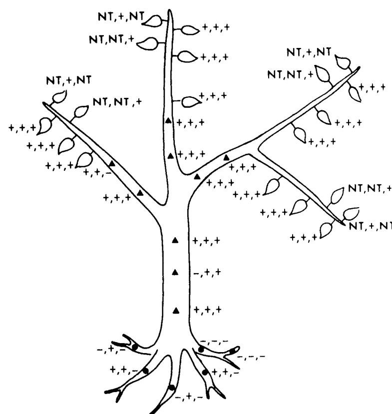

Fig. 1. Distribution of tomato ringspot virus (TmRSV) in bud-inoculated, orchard-grown Malling-Merton 106 (MM 106) tree 5 on three sampling dates (June, July, and August). The tree originated as a rootsucker from tree 6; its root system was still physically connected with the MM 106 rootstock of tree 6. ◯ = Leaf sample, ▲ = bark sample, ● = root sample, + = ELISA-positive, – = ELISA-negative, and NT = not tested.

ELISA procedure. The procedure described by Clark and Adams (1) and by Gonsalves (9) was followed. Leaf samples were weighed and ground in 1:20 ratio (w/v) cold-extraction buffer. If 0.35 g of tissue was available, it was ground with a Tissumizer (Tekmar Co., Cincinnati, OH 45222); otherwise, it was ground in a mortar and pestle. All bark and root samples were ground in cold mortars and pestles in 4 ml cold-extraction buffer (approximately in a 1:20 ratio, w/v) in the field immediately after collection, poured into centrifuge tubes, and kept cold in an ice chest until taken to the laboratory. All samples were processed in ELISA plates the same day they were collected.

Because the Amberg and Chickadee isolates differ serologically (13; D. Gonsalves, *unpublished*), two antisera were used for their detection. Antisera to peach yellow bud mosaic (PYBM) and to grape yellow vein (GYV) strains of TmRSV (27) were used for detecting Amberg and Chickadee isolates, respectively. ELISA plates (Substrate and Immulon 2, Dynatech Co., Alexandria, VA 22314) were coated with gamma-globulin at 5 μg/ml for GYV and at 2 μg/ml for PYBM, with the exception of the first assay of tree 5, where 5 μg/ml was used. Alkaline phosphatase-labeled gamma-globulin was used at 1/400 and 1/500 (v/v) dilutions for GYV and PYBM, respectively. The unknown tissue samples were tested in one or two wells each. At least two wells each with healthy apple leaf tissue, extraction buffer, and infected apple or *Gomphrena globosa* tissue were included as controls in each plate. The absorbance readings from replicate wells were averaged.

Plates were read visually at least twice, and in addition, the absorbance at 405 nm was measured with a Microelisa Autoreader MR 580 (Dynatech) or with a Beckman Model 25 spectrophotometer (Beckman Instruments, Palo Alto, CA 94300). In the latter case, the color reaction was stopped by adding 50 μl of 3 M NaOH per well after about 1 hr. Substrate buffer was used as a reference for the readers. For visual examinations, a distinct yellow color was scored positive and a colorless well negative; faintly yellow samples were regarded as questionable. With the photometric readers, a test was scored positive if the absorbance reading at 405 nm was at least twice that of the healthy control and at least 0.20.

RESULTS

The PYBM antiserum, used for detecting the Amberg and the Amberg-like isolates of TmRSV (Table 1), produced a stronger color reaction in ELISA than the GYV antiserum, which was used for detecting the Chickadee isolate. Yellow color developed faster and was more intense with the former, and no nonspecific background reaction was observed. In all cases, visual and photometric ratings of ELISA plates were in agreement for positive and negative results. However, the photometric readings allowed quantitative classification of 21 samples (of a total of 432) that visually had been rated questionable; 20 of the 21 were rated positive. The variation of absorbance values in replicate wells of the same sample was usually below 1/10 of an absorbance unit.

Generally, leaf samples resulted in higher absorbance values than bark and root samples. Because the trees chosen for this study were not uniform in age, date and mode of inoculation, number of samplings, etc., the results of each tree group are presented separately.

Distribution of TmRSV in a bud-inoculated, greenhouse-grown MM 106 tree. The potted tree 1 (Table 1), bud-inoculated 2 yr earlier with the Chickadee

Table 1. Distribution of tomato ringspot virus (TmRSV) in nine apple trees as determined by ELISA

Tree	Cultivar or rootstock[a]	Environment	Tree age (yr)	Virus isolate[b]	Mode/year of inoculation	No. of indexings	Infected/tested (no.)[c]		
							Leaf	Bark	Root
1	MM 106	Greenhouse	3	C	Bud/1980	3	1/8	1/3	0/2
2	MM 106	Orchard	7	C	Bud/1976	3	12/12	5/5	0/3
3	MM 106	Orchard	7	C	Bud/1976	3	10/12	5/7	0/4
4	MM 106	Orchard	6	C	Bud/1977	3	5/9	3/5	0/4
5	MM 106	Orchard	?[d]	A	Bud/1977	3	12/12	9/9	4/6
6	GD/MM 106	Orchard	12	A	Bud/1977	3	NT	3/8	···
							1/1	5/6	2/2
7	MM 106	Old nursery	20	AL	Natural/?[e]	4	0/6	3/10	3/3
8	MS/MM 106	Orchard	13	AL	Natural/?[e]	1	0/9	0/13	···
							···	1/3	1/4
9	MI/MM 106	Orchard	13	AL	Natural/?[e]	1	0/9	0/12	···
							···	2/3	2/4

[a] MM 106 = Malling-Merton 106, GD = Golden Delicious, MS = Macspur, MI = McIntosh. The first data row of the grafted trees 6, 8, and 9 refers to the scion; the second data row refers to the rootstock portion.
[b] A = Amberg, C = Chickadee, AL = Amberg-like and is serologically similar to Amberg.
[c] Sample was rated infected if TmRSV was detected by ELISA at least once during the whole test period at the corresponding location (numerator is number of TmRSV-infected samples, denominator is total number of samples tested); NT = not tested.
[d] Age not known, but certainly less than 12 yr (see text).
[e] Year of inoculation unknown.

isolate of TmRSV, had grown vigorously with the exception of a 5 cm long shoot growing from the inoculum bud.

TmRSV was detected only from a single, sequentially indexed leaf near the tip of the one branch that had the inoculum shoot at its base (Fig. 2). Leaves that developed immediately above and below the ELISA-positive leaf were included in the second and third indexings and were also infected. The absorbance values of the positive samples ranged from 1.08 to 1.15, the healthy controls from 0.06 to 0.08. TmRSV was detected also in bark between the inoculum and the ELISA-positive leaves but only in the last indexing in August; absorbance at 405 nm was 0.36.

Distribution of TmRSV in bud-inoculated, orchard-grown MM 106 trees. Four trees in this group were similar in that they had been bud-inoculated with TmRSV either 6 yr (trees 2 and 3) or 5 yr (trees 4 and 5) before this study and had grown vigorously in the orchard. Data for all four trees are summarized in Figure 3, and data from tree 5 are presented in Figure 1.

During the indexing period, TmRSV was detected consistently from leaves, somewhat less frequently from bark, and only erratically from roots. All 38 leaves that indexed positive in June were still positive in July and August; one leaf was ELISA-positive only in July. Leaf position on a branch generally did not influence virus detectability; that is, on a given branch, either all leaves or none were ELISA-positive. All sampled leaves from four branches of trees 2 and 5 (Fig. 1) tested positive during the entire indexing period. In tree 4, however, no TmRSV was detected in leaves from two of four branches at any time. In tree 3, all leaves from two branches were always positive; on a third branch, however, only the most apical leaf that was assayed was positive, and this only in July. No significant differences in absorbance values were found between samples taken from ELISA-positive cut leaves and their adjacent whole leaves that were included in the July and August samplings (data not shown). Apparently, leaf cutting did not affect subsequent detection of TmRSV in the leaf.

TmRSV was detected in bark samples from all four trees in this group. From 26 sample locations, TmRSV was detected at least once in 22 during the survey, in 16 of them consistently, and in two locations just once. Bark samples from branches without ELISA-positive leaves were always negative; however, three negative bark samples were from branches without ELISA-negative leaves.

No TmRSV was detected in roots of three trees. TmRSV was detected in tree 5 from two of five roots sampled in June, from four in July, but from none in August (Fig. 1).

The average absorbance values decreased in leaf samples taken between June and August, were highest in bark samples taken in July, and were similar in root samples taken in June and July (Fig. 3). However, at any sampling date, leaf tissue gave the highest average absorbance values, followed by bark and root, respectively.

Distribution of TmRSV in a naturally infected, nursery-grown MM 106 tree. The 20-yr-old, three-stem tree 7 (Fig. 4) was never inoculated artificially and was not indexed for TmRSV until this study began. The date of inoculation, presumably by nematodes, is therefore unknown.

Bark tissue was sampled four times between 8 July and 10 August; leaves and roots were sampled once, on 20 July and 10 August, respectively. TmRSV was detected in all three sampled roots and in bark tissue from the lower parts of two stems (three of 10 samples). TmRSV was not detected from bark samples taken above a stem height of 45 cm or from any leaf.

Distribution of TmRSV in a bud-inoculated, orchard-grown Golden Delicious/MM 106 tree. Tree 6 was inoculated (in the MM 106 rootstock) in May 1977, but the inoculum-shoot died. The tree showed normal growth and had an average crop load in the summer of 1982. No AUND symptoms such as invagination or necrotic plate at the graft union or tree decline were observed.

TmRSV was detected from five of six bark samples taken from around the trunk circumference of the MM 106 rootstock 2–10 cm below the graft union, from two root samples, and from a leaf on the single available root sucker about 60 cm from the trunk. One of the positive samples was taken directly adjacent to the negative one only 3 days later. TmRSV was also detected in three of eight bark samples taken from the Golden Delicious scion 2–15 cm above the graft union. However, all ELISA-positive Golden Delicious samples were located within 5 cm of the union. Two were exactly in line above an ELISA-positive rootstock sample, but one was above a negative MM 106 sample. The 405-nm absorbance values were between 0.74 and 2.30 for the positive samples and 0.00 for the healthy controls.

Distribution of TmRSV in naturally infected, orchard-grown Macspur/MM 106 and McIntosh/MM 106 trees. The inoculation dates of these two 13-yr-old trees (8 and 9, respectively) are not known. They were first indexed by ELISA on 29 June 1982 by taking two bark samples from opposite sides of the MM 106 rootstock just below the graft union. TmRSV was detected in both trees. The trees were retested on 6 August 1982 by taking three bark samples on

Fig. 2. Distribution of tomato ringspot virus (TmRSV) in bud-inoculated, greenhouse-grown Malling-Merton 106 (MM 106) tree 1 on three sampling dates (June, July, and August). ◌ = Leaf sample, ▲ = bark sample, ● = root sample, + = ELISA-positive, − = ELISA-negative, and NT = not tested.

Fig. 3. Detection of tomato ringspot virus (TmRSV) by enzyme-linked immunosorbent assay (ELISA) from different parts of four bud-inoculated Malling-Merton 106 (MM 106) orchard trees on three dates. Percent infection and absorbance (o.d. 405 nm) represent averages from 45, 26, and 17 samples of leaf, bark, and root, respectively.

Fig. 4. Distribution of tomato ringspot virus (TmRSV) in naturally infected Malling-Merton 106 (MM 106) tree 7 in an old nursery. ◌ = Leaf sample, ▲ = bark sample, ● = root sample, a = assayed 8 July, b = assayed 20 July, c = assayed 4 August, d = assayed 10 August, + = ELISA-positive, and − = ELISA-negative.

different sides of the trunk just below the graft union, three just above, and nine or 10 more throughout the scion portion. Nine scion leaf samples and four MM 106 root samples also were included for both trees (Table 1).

TmRSV was detected in one root sample and one bark sample from the MM 106 rootstock just below the graft union in tree 8. These two positive samples were not in a vertical line. In the stock of tree 9, there were two ELISA-positive bark samples above two positive root samples. The absorbance values at 405 nm ranged from 0.43 to 0.62 for the positive samples, and the healthy controls were 0.00. These ELISA-positive samples represented 29 and 57% of the total number of MM 106 samples taken from trees 8 and 9, respectively.

DISCUSSION

Although absorbance values at 405 nm only measured the relative concentration of TmRSV-specific antigen in plant tissues, we believe these measurements also reflected the relative concentrations of TmRSV. Therefore, we will discuss our results in terms of TmRSV detectability or relative TmRSV concentration. Time of the sampling, type of tissue sampled, and location on a tree from which the sample was taken were important factors that affected detection of TmRSV in apple trees during June through August. Although the data indicated that the distribution of TmRSV might be unique in each individual tree, the following trends were revealed.

In MM 106 trees, leaves were the most reliable source for TmRSV detection from June through August, although the detectability of the virus in leaves by ELISA declined toward the end of the growing season (Fig. 3). Leaf position on a given branch was not important for detection, ie, the virus concentration in young leaves from the tip of a branch was about the same as in older leaves from the base of a branch. However, TmRSV was not evenly distributed within trees; some branches appeared to be entirely TmRSV-free, or the virus concentration was too low for detection by ELISA. Therefore, leaves from several branches must be sampled to determine the presence of TmRSV in a tree. The distribution of TmRSV in bark of MM 106 trees was similar to that in leaves, but TmRSV could be detected from a slightly lower proportion of the bark samples, and the infected bark samples gave lower absorbance readings on ELISA plates. Distribution of TmRSV in roots was erratic and the concentration apparently low. These findings are somewhat surprising because Stouffer and Uyemoto (26) suggested that TmRSV could be detected best in succulent terminal leaves and during the cooler months of the growing season. TmRSV distribution in MM 106 trees seems to differ from that in grapevines (10), where only a few leaves of infected plants were ELISA-positive over a summer, and also from that in peach trees (L. B. Forer, *personal communication*), where TmRSV could be detected in leaves in May but not in June and July.

The erratic distribution of TmRSV in the potted MM 106 tree in the greenhouse (Fig. 2) could be explained in various ways. Apparently, TmRSV had moved only upward from the inoculum and was confined to the single inoculated branch. Such a situation has been observed with TmRSV in grapevine (29). Of course, a downward translocation might also have occurred, but if so, the virus concentration was too low to detect by ELISA. An additional observation may have had significance: The bark of this greenhouse-grown tree was very tight on the wood and rather dry on the first two sampling dates but was slipping readily in the August sampling, when one bark sample was positive for TmRSV. It cannot be concluded which factor (the short period of time since inoculation or the greenhouse environment), if either, was responsible for the low detection rate in this tree.

In grafted trees (cultivar/MM 106), there were no leaves available from the rootstock part, and only a few from root suckers. TmRSV was detected from about the same proportion of bark and root samples from the rootstock portion of trees 6, 8, and 9. Despite the somewhat limited number of samples taken per tree and number of trees assayed in this tree group, we feel that the data support use of bark sampling as a practical method if various samples can be collected from a tree without causing too much physical damage (20). Rosenberger et al (19) detected TmRSV in bark tissue of Rome Beauty and Empire taken 4–10 cm above the graft union; however, detection of TmRSV in three of eight Golden Delicious bark samples was not expected, because Cummins and Gonsalves (4) did not detect TmRSV from this cultivar during a 6-yr study in which they had sampled only leaf tissue. Possibly, virus particles cannot move far, move only slowly, are destroyed in Golden Delicious tissue, or are destroyed by environmental conditions in the upper plant parts. Alternatively, our findings may simply reflect the uniqueness of TmRSV distribution in each tree.

Another observed trend was associated with the site of virus inoculation (high on stem or side branch vs. root) or with the mode of inoculation (bud-grafting vs. nematode transmission). TmRSV was not detected in roots of trees that had been bud-inoculated high on the stem (Fig. 2). However, 62% of all root samples were ELISA-positive in those trees (trees 7–9) that presumably had been inoculated by nematodes (Table 1) or by bud-grafting low on the stem (tree 6). Tree 5 (Fig. 1) was a special case: TmRSV was prevalent in root, bark, and leaf tissue, but this tree also was unique in that the root system was physically connected with the bud-inoculated mother tree. These observations of partial tree infection, depending on where or how the inoculation was achieved, indicate again that translocation of TmRSV within apple trees is very slow but is probably more efficient upward. A separate study comparing the efficiency of transmission of TmRSV via *Xiphinema* vectors vs. bud-inoculation to apple rootstocks under field conditions is in progress.

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