Evaluation of Tobacco Introduction 1406 as a Source of Virus Resistance

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ABSTRACT

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The resistance of Tobacco Introduction (TI) 1406 plants to infection by three potyviruses, potato virus Y (PVY), tobacco etch virus (TEV), and tobacco vein-mottling virus (TVMV) was dependent on the specific virus or virus strain present in the inoculum. Resistance of TI 1406 to TVMV and a mild strain of TEV (TEV-M) was characterized by curtailed or limited virus movement and drastically reduced virus multiplication. Infection of TI 1406 with a severe isolate of TEV (TEV-A) or PVY resulted in systemic symptoms less severe than those of the controls (cultivar Burley 21), but virus titers in T.I. 1406 were equivalent to those in Burley 21. Symptoms produced on TI 1406 infected with a number of viruses outside the potyvirus group were as severe as those on Burley 21 controls.

Tobacco etch virus (TEV) and tobacco vein-mottling virus (TVMV) cause serious diseases of burley tobacco in Kentucky. All burley cultivars are susceptible to infection, although some are more tolerant in that symptoms are less severe (17,18). Even on cultivars considered tolerant, the chemical composition of leaf tissue may be considerably modified by virus infection (16), and yield reductions may be significant (15). Because no resistance to these diseases has been found in the commonly grown burley cultivars, new sources of resistance have been sought.

One source of resistance is Tobacco Introduction (TI) 1406, also known as the "Virgin A Mutant" (8), or the Virginia Mutant (19). TI 1406 was introduced as a mutant of the cultivar Virgin A from the Tobacco Institute in Forchheim, West Germany, where it had been reported to be resistant to a European isolate of potato virus Y (PVY) (10,11). Investigations in the United States further demonstrated its resistance to PVY, on the basis of a lack of symptom production following inoculation (19,20). As the importance of TVMV in reducing yield of burley tobacco cultivars became apparent (15), tests were conducted to determine the resistance of TI 1406 to TVMV and TEV (4). Since no detectable symptoms developed in field-grown TI 1406 following inoculation with TEV or TVMV, it has been used in a breeding program to introduce resistance to TEV and TVMV into the burley cultivars grown in Kentucky (4).

This paper reports the results of studies in which enzyme-linked immunosorbent assay (ELISA) and infectivity tests were used to determine the nature and completeness of the resistance of TI 1406 to potyviruses PVY, TEV, and TVMV.

MATERIALS AND METHODS

Hosts. The tobaccos (*Nicotiana tabacum* L.) TI 1406 and Burley 21 (the latter a standard burley cultivar that develops obvious symptoms when infected by PVY, TEV, or TVMV) were used. About 8 wk after seeding, one leaf per plant was mechanically inoculated with PVY, TEV, or TVMV. Inoculum was usually applied to the entire adaxial leaf surface by using a cheesecloth pad. In some experiments, however, only 2 × 4-cm interveinal panels of a fully expanded leaf were inoculated. Leaves were rinsed 1 hr after inoculation. All experiments were done in a screened greenhouse.

Viruses. The isolates of TVMV and PVY were those used in a previous study (18). Two isolates of TEV, designated M and A, were used: Isolate M (TEV-M) was a previously described isolate (5), which had been maintained by mechanical transmission, and

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isolate A (TEV-A) was isolated in 1978 from a field-grown tobacco plant exhibiting severe etch symptoms and was maintained by successive single-aphid (*Myzus persicae* Sulz.) transfers.

The properties of the two TEV isolates are described elsewhere (T. P. Pirone and D. W. Thornbury, *unpublished*). On tobacco, the symptoms produced by TEV-A and TEV-M were the same as those previously described (1) for tobacco severe etch virus and tobacco mild etch virus, respectively. Confluent precipitin band patterns were formed in SDS-agar gel diffusion plates (6) with antiserum to TEV-M in the center well and TEV-A and TEV-M antigen in adjacent outer wells. Both TEV isolates induced wilt of Tabasco pepper (*Capsicum frutescens*) 5–10 days following inoculation; this is a characteristic response to TEV infection (7).

Viruses were purified from systemically infected tobacco leaves 2–3 wk after inoculation by using method 1 of Moghal and Francki (14). Purified PVY, TEV-M, TEV-A, or TVMV at a concentration of approximately 300 μg/ml were used as inoculum in all experiments.

ELISA. Antisera to TEV, PVY, and TVMV were produced by a combination of intravenous and subcutaneous injections of rabbits at 2-wk intervals, with 0.5–1.0 mg of purified virus. The TEV antiserum was produced to both TEV-M and TEV-A by combining purified preparations of each isolate before injection. When injected subcutaneously, the purified virus was emulsified with an equal volume of Freund's incomplete adjuvant. The rabbits were bled at 1-wk intervals after two or three injections. Antiserum titers were determined by the ring interface precipitin test; antisera with dilution end points >1:512 were collected and stored frozen in 1.0-ml portions in glass tubes.

The ELISA procedure as described by Clark and Adams (2) was followed. For each isolate tested, 10 or 15 1-cm-diameter disks were collected from the leaves of a minimum of three TI 1406 and three Burley 21 plants that had been inoculated with virus from 1 hr to 21 days previously. Since preliminary experiments showed no differences in ELISA readings with freshly harvested or frozen tissue, disks were frozen (−20 C) for up to 5 wk before processing so that disks collected from a given plant over a 2- to 3-wk period could all be assayed on a single ELISA plate (No. 1-223-29, Dynatech Laboratories, Inc., Alexandria, VA 22314).

A group of five leaf disks represented a test sample and was ground in 1.0 ml of phosphate-buffered saline containing 0.05% Tween-20 and 2% polyvinyl-pyrrolidone, MW 44,000 (PBS-Tween-PVP). The resulting extract was placed in duplicate wells of an ELISA plate previously coated with partially purified immunoglobulin (Ig) at a concentration of 1 μg per well.

Fifty microliters of enzyme-conjugated Ig was added to 20 ml of PBS-Tween-PVP, which contained 1 ml of extract from healthy tissue (filtered through two layers of cheesecloth) to decrease the background of the controls. From 1 to 2 hr after the addition of the substrate solution, reactions were arrested with 3 M NaOH. The contents of each well were diluted fivefold in distilled water and the absorbance at $A_{405\ nm}$ was measured. Control tissue from uninoculated plants was processed similarly. Absorbances greater than twice the average for healthy control samples were considered positive (12).

RESULTS

Tobacco vein-mottling virus. TI 1406 does not develop visible symptoms following inoculation with TVMV. ELISA results indicated that the TVMV content in inoculated leaves of TI 1406 after 7, 14, and 21 days was not significantly different from healthy, uninoculated TI 1406 control tissue (Fig. 1A). The high ELISA values at 1 hr and 1 day after inoculation were evidently due to residual inoculum because these disappeared by 3–7 days after inoculation (Fig. 1A). ELISA detected no movement of TVMV antigen from adjacent inoculated panels into uninoculated panels on a TI 1406 leaf (Table 1). As in Fig. 1A, ELISA values for TVMV-inoculated areas of TI 1406 decreased between 1 and 14 days (Table 1); this was again attributed to the disappearance of residual inoculum.

Because ELISA measures viral antigen, infectivity assays were used to determine relative amounts of infectious virus. No local lesion host is known for TVMV, so qualitative assays were conducted to determine if infectious virus could be detected in the uninoculated panels adjacent to TVMV inoculated panels, and in uninoculated leaves. Extracts from each leaf or panel tested were applied to three or four Burley 21 tobacco plants. After 2–3 wk none of 45 Burley 21 assay plants inoculated with leaf sap derived

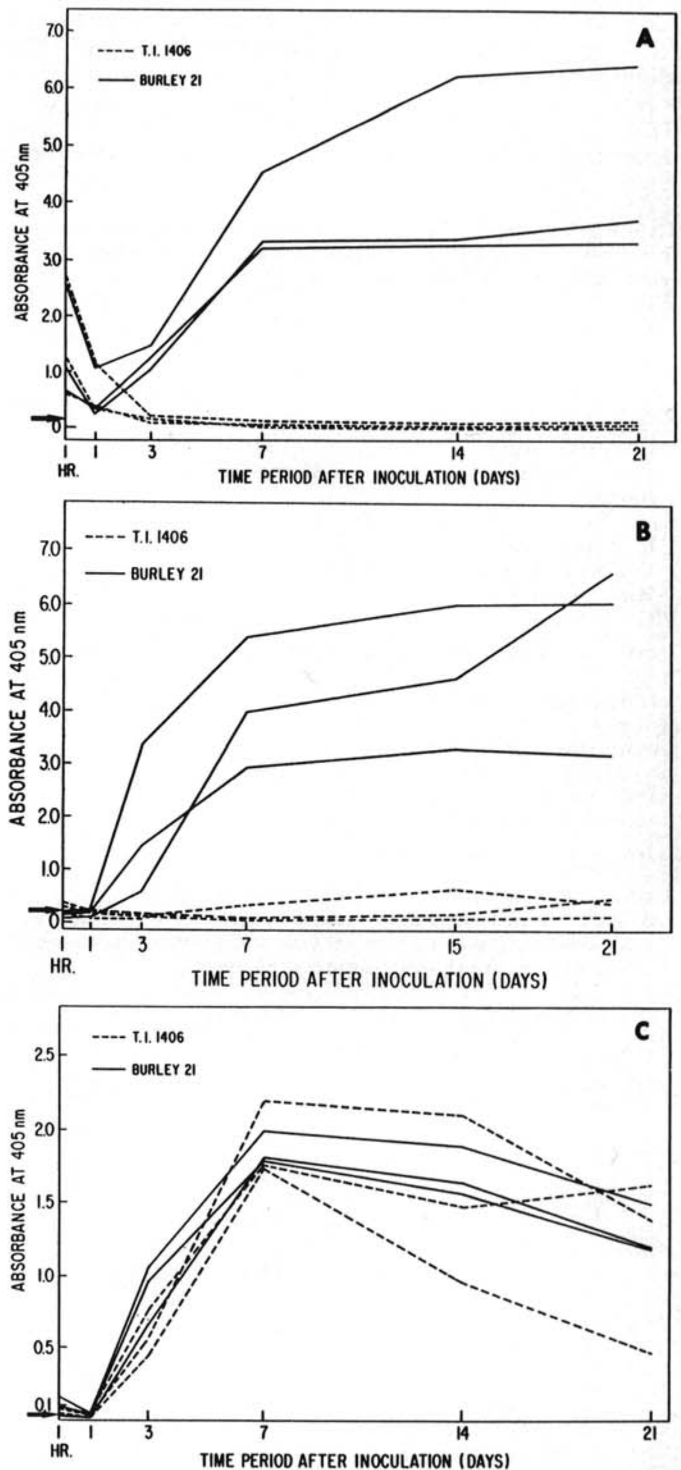

Fig. 1. ELISA absorbances ($A_{405\ nm}$) for disks from inoculated leaves of TI 1406 tobacco plants at various time periods following inoculation with: **A,** TVMV; **B,** TEV-M; and **C,** PVY. Each plotted line represents a single replicate. Absorbance values were means from two samples that were collected from each replicate at each time period tested. A sample consisted of five leaf disks. Contents of each well were diluted fivefold before reading. The arrows indicate the mean value obtained with healthy control extracts. Readings from a Burley 21 control are shown for comparison.

from uninoculated areas of inoculated TI 1406 plants had developed virus symptoms.

Tobacco etch virus. As is the case with TVMV, TI 1406 plants remain symptomless following inoculation with TEV-M. ELISA absorbances ($A_{405\ nm}$) for leaf disks from inoculated leaves of TI 1406 and Burley 21 plants at periods from 1 hr to 21 days following inoculation with TEV-M indicated that either no, or a very slight, increase in virus titer occurred in TI 1406 during the 21-day period (Fig. 1B). In another set of experiments, ELISA results showed some increase in virus titer in inoculated TI 1406 leaves 14 days following inoculation with TEV-M (Table 2). However, the ELISA absorbance readings from TEV-M inoculated leaves of TI 1406 were at least 10 times lower than the absorbance readings from TEV-M inoculated leaves of the control cultivar, Burley 21. As determined by ELISA, TEV-M does not move into uninoculated leaves of TI 1406 (Table 2).

Estimates of relative virus concentrations in uninoculated leaves of TI 1406 and Burley 21 plants inoculated 20 days previously with TEV-M were obtained by application of leaf extracts of each plant to three half-leaves of *Chenopodium amaranticolor*. Lesions were counted 20 days later. The results confirmed those of the ELISA assays shown in Table 2; no infectivity was recovered from uninoculated leaves of inoculated TI 1406 plants while an average of 75 lesions per half-leaf were produced by the control (Burley 21) extracts.

Inoculation with TEV-A resulted in moderate symptoms on TI 1406 plants and severe symptoms on Burley 21 plants. ELISA results showed that virus concentration was fairly comparable in inoculated leaves of TI 1406 and Burley 21 plants inoculated 14 days previously with TEV-A (Table 2). Similarly, ELISA results for leaf disks from uninoculated leaves of TI 1406 and Burley 21 plants 20 days following inoculation with TEV-A showed that TEV-A concentration in TI 1406 and Burley 21 was roughly equivalent (Table 2). Thus, the titer of TEV-A in both inoculated and uninoculated leaves of TI 1406 plants was comparable to the virus titer found in the Burley 21 control plants, although the symptoms were less severe on TI 1406.

It is interesting to note that the levels of TEV-A antigen in Burley 21 appear to be much lower than the levels of the TEV-M antigen in Burley 21 (Table 2). Because the same concentrations of TEV-A and TEV-M resulted in very similar ELISA absorbance readings (Fig. 2), the possibility that these differences are due to a difference in reactivity to the antiserum seems unlikely. Even though the titer of TEV-A in Burley 21 may be lower than that of TEV-M, the symptoms produced on Burley 21 in response to TEV-A infection are more severe.

TABLE 1. Enzyme-linked immunosorbent assay (ELISA) results for disks from areas of TI 1406 and Burley 21 tobacco leaves inoculated with tobacco vein-mottling virus (TVMV), and adjacent, uninoculated areas

	ELISA test result[b]			
	Day 1		Day 14	
Source of leaf disks[a]	$A_{405\ nm}$	(range)	$A_{405\ nm}$	(range)
TI 1406				
Inoculated areas[c]	1.24	(1.14–1.44)	0.06	(0.05–0.07)
Uninoculated areas	0.03	(0.03–0.03)	0.05	(0.04–0.05)
Healthy control	0.02		0.05	
Burley 21				
Inoculated areas[c]	0.67	(0.52–0.75)	1.37	(1.30–1.45)
Uninoculated areas	0.03	(0.02–0.04)	0.19	(0.07–0.27)
Healthy control	0.02		0.05	
PBS control	0.03		0.03	

[a] Disks were collected from nine inoculated areas and nine uninoculated areas of Burley 21 or TI 1406 leaves. Seventy disks were collected at 1 day and again at 14 days following inoculation. The leaf disks were assayed in groups of five leaf disks per group.

[b] Absorbance values were means of duplicate wells; the contents of each well was diluted five-fold before being read at $A_{405\ nm}$. Numbers in parentheses show range of values for three determinations.

[c] Inoculation concentration was 300 μg/ml.

TABLE 2. Enzyme-linked immunosorbent assay (ELISA) results for leaf disks from inoculated and uninoculated leaves of TI 1406 and Burley 21 tobacco plants following inoculation[a] with tobacco etch virus-isolate M (TEV-M) or tobacco etch virus-isolate A (TEV-A)

Source of leaf disks[b]		Absorbance at 405 nm[c]	
Cultivar	TEV isolate	Inoculated leaves[d]	Uninoculated leaves[d]
Inoculated			
TI 1406	M	0.11 (0.04–0.20)	0.02 (0.01–0.02)
Burley 21	M	2.06 (1.93–2.25)	1.63 (1.52–1.71)
TI 1406	A	0.35 (0.33–0.37)	0.23 (0.22–0.24)
Burley 21	A	0.50 (0.47-0.51)	0.21 (0.20-0.21)
Healthy			
TI 1406 control		0.02	0.02
Burley 21 control		0.02	0.02
PBS control		0.02	0.02

[a] Inoculum concentration was 300 μg/ml.

[b] 280 leaf disks, representing 28 determinations, were collected from inoculated and uninoculated leaves. Two samples were obtained for each determination. A sample consisted of five leaf disks.

[c] Absorbance values were means from the two samples collected for each determination. Contents of each well diluted fivefold before reading at 405 nm. Numbers in parentheses show range of readings for three determinations.

[d] Leaf disks from inoculated leaves were collected 14 days following inoculation. Leaf disks from uninoculated leaves were collected from the same plants but at 20 days following inoculation.

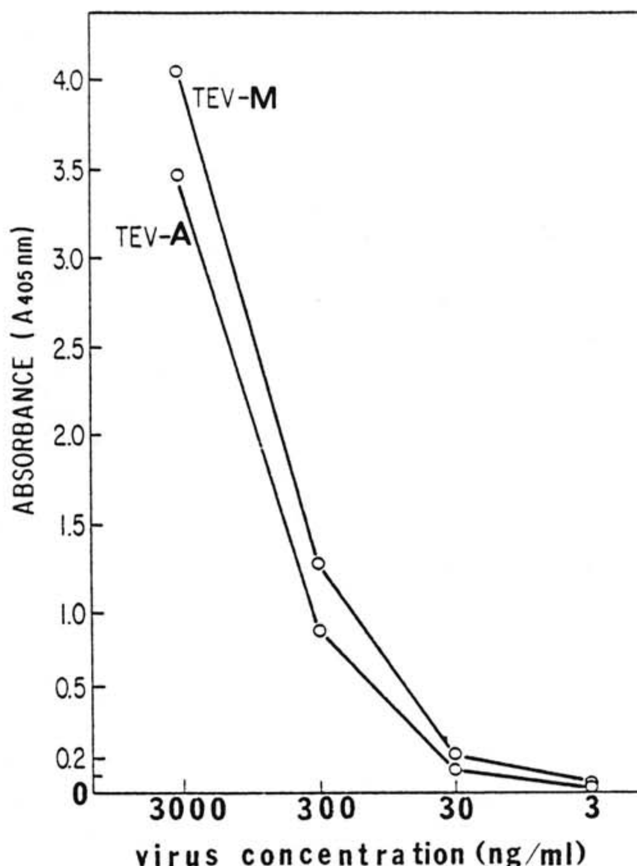

Fig. 2. ELISA absorbances ($A_{405\ nm}$) obtained for purified preparations of TEV-M and TEV-A. The coating and enzyme-conjugated immunoglobulins were prepared from a single serum produced to both TEV-M and TEV-A. Absorbance values are means from four wells for each concentration. Contents of each well were diluted fivefold before reading. The mean value for PBS control wells was 0.03.

Potato virus Y. TI 1406 plants developed mild symptoms following inoculation with potato virus Y (PVY) while symptom development on Burley 21 was more pronounced. ELISA absorbances ($A_{405\ nm}$) for leaf disks from PVY inoculated leaves of TI 1406 and Burley 21 plants at various time periods following inoculation demonstrated that concentrations of PVY in TI 1406 were similar to those in Burley 21 (Fig. 1C). Similarly, results for leaf disks from systemically infected leaves of TI 1406 and Burley 21 plants 17 days following inoculation with the virus indicated almost equivalent levels of PVY in TI 1406 and Burley 21. The ELISA absorbance values for nine test samples of systemically infected TI 1406 were from 0.46 to 0.70, mean ($\bar{X}$) = 0.57, while the range of readings for nine test samples of Burley 21 was 0.43 to 0.66, $\bar{X}$ = 0.60. The mean for healthy control sap was 0.07. Systemic spread and multiplication of PVY in TI 1406 appears to be similar to that in Burley 21.

The ELISA results suggested that, although symptoms of PVY were less severe on TI 1406 than on Burley 21, virus concentrations were probably equivalent and hence TI 1406 could serve as an effective a source of virus for spread in the field as would a cultivar such as Burley 21. Using previously described procedures (18), aphids (*Myzus persicae* Sulz.) were allowed to probe infected leaf tissue for 30 sec to 1 min and then either five or 10 aphids were placed on each of 20 assay plants. Aphids which acquired PVY from TI 1406 leaves transmitted PVY to 15/20 and 13/20 assay plants with 10 and five aphids, respectively, placed on each assay plant. Aphids that acquired PVY from Burley 21 leaves transmitted PVY to 13/20 assay plants whether 10 or five aphids were placed on each assay plant. Tests using one aphid per assay plant resulted in 8/20 assay plants infected when TI 1406 was the virus source and 2/20 when Burley 21 was the virus source. Thus, these aphid transmission studies indicated that TI 1406 is at least as efficient a source of PVY inoculum as is Burley 21.

DISCUSSION

Symptom production on TI 1406 was dependent on the specific virus or virus strain present in the inoculum. Inoculations with TEV-M or TVMV did not result in visible symptoms, while inoculation with PVY or TEV-A resulted in mild symptoms. Symptom production appeared to be correlated with the degree of virus multiplication within the TI 1406 plant. Both PVY and TEV-A multiplied in TI 1406 to levels similar to those reached in the susceptible Burley 21 tobacco (Fig. 1C and Table 2). Multiplication of TVMV and TEV-M in TI 1406 either did not occur or was drastically curtailed (Fig. 1A and B and Table 2). While the ELISA values for TVMV-inoculated TI 1406 are initially higher than controls, the fact that values are highest immediately after inoculation and decline to control levels after 3 days suggests that residual inoculum, rather than products of virus multiplication, is being measured.

TVMV and TEV-M were restricted to inoculated areas of TI 1406 plants (Tables 1 and 2). This localization did not appear to be due to structural barriers (9), but rather may be due to inhibition of virus multiplication. Localization of virus by the inhibition of virus multiplication has been suggested by other investigators (3,13).

The specificity of the virus resistance should be considered in assessing the usefulness of TI 1406 in a breeding program. TI 1406 is susceptible to viruses from several different plant virus groups. Severe systemic symptom development occurred on TI 1406 following inoculation with alfalfa mosaic, cucumber mosaic, tobacco streak, and tobacco mosaic viruses (9). Of the potyviruses tested, infection of TI 1406 with TEV-A or PVY resulted in systemic symptoms and virus titers equivalent to those in susceptible Burley 21. Thus of the viruses tested, the resistance appears to be effective only in TEV-M and TVMV infections. The fact that the level of resistance appears to be strain-specific suggests that TI 1406 may be of limited usefulness as a source of virus resistance.

LITERATURE CITED

1. Bawden, F. C., and Kassanis, B. 1941. Some properties of tobacco etch viruses. Ann. Appl. Biol. 28:107-118.
2. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
3. Cohen, J., and Loebenstein, G. 1975. An electron microscope study of starch lesions in cucumber cotyledons infected with tobacco mosaic virus. Phytopathology 65:32-39.
4. Collins, G. B., Litton, C. C., and Legg, P. D. 1975. Breeding for disease resistance in tobacco. (Abstr.) Kentucky Agric. Exp. Stn. Annu. Rep. 88:21.
5. Ghabrial, S. A., and Pirone, T. P. 1967. Physiology of tobacco etch virus-induced wilt of tabasco peppers. Virology 31:154-162.
6. Gooding, G. V., and Bing, W. W. 1970. Serological identification of potato virus Y and tobacco etch virus using immunodiffusion plates containing sodium dodecyl sulfate. (Abstr.) Phytopathology 60:1293.
7. Greenleaf, W. H. 1953. Effects of tobacco-etch virus on peppers (*Capsicum* sp.). Phytopathology 43:564-570.
8. Gupton, C. L., and Burk, L. G. 1973. Location of the factor for resistance to potato virus Y in tobacco. J. Hered. 64:289-290.
9. Johnson, M. C. 1980. The resistance of T. I. 1406 tobacco to three potyviruses. Ph.D. dissertation, University of Kentucky, Lexington. 104 pp.
10. Koelle, G. 1958. Versuche zur Vererbung der Krankheitsresistenz bei Tabak. Tabak-Forschung 24:83-84.
11. Koelle, G. 1961. Genetishe Analyse einer Y-Virus-(Rippenbräune) resistenten Mutante der Tabaksorte Virgin A. Züchter 31:71-72.
12. Lister, R. M., and Rochow, W. F. 1979. Detection of barley yellow dwarf virus by enzyme-linked immunosorbent assay. Phytopathology 69:649-654.
13. Loebenstein, G., Sela, B., and Van Praagh, T. 1969. Increase of tobacco mosaic local lesion size and virus multiplication in hypersensitive hosts in the presence of actinomycin D. Virology 37:42-48.
14. Moghal, S. M., and Francki, R. I. B. 1976. Towards a system for the identification and classification of potyviruses. Virology 73:350-362.
15. Pirone, T. P. 1974. Effect of tobacco vein mottling virus on yield of burley tobacco cultivars. Tob. Sci. 18:113-114.
16. Pirone, T. P., and Davis, D. L. 1977. Modification of the chemical composition of burley tobacco by infection with tobacco vein mottling virus. Tob. Sci. 21:83-84.
17. Pirone, T. P., and Gooding, G. V., Jr. 1973. Effect of tobacco vein mottling virus on field-grown burley tobacco varieties. Plant Dis. Rep. 57:845-847.
18. Pirone, T. P., Gooding, G. V., Jr., and Smiley, J. H. 1973. Tobacco vein mottling virus on burley tobacco in Kentucky. Plant Dis. Rep. 57:841-844.
19. Sievert, R. C. 1972. Sources of resistance to potato virus Y in the genus *Nicotiana*. Tobacco Science 16:92-94.
20. Silber, G., Heggestad, H. E., and Kahn, R. P. 1965. A tobacco with tolerance to potato virus Y. (Abstr.) Phytopathology 55:1076.