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CYTOPATHOLOGY OF MIXED INFECTIONS
WITH POTATO VIRUSES X AND Y
IN NICOTIANA TABACUM LEAVES

presented by

Harold E. Kauffman

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of the requirements for

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Pathology

W. J. Hooker
Major professor

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ABSTRACT

CYTOPATHOLOGY OF MIXED INFECTIONS WITH POTATO VIRUSES X AND Y IN NICOTIANA TABACUM LEAVES

by Harold E. Kauffman

Turkish tobacco leaves were inoculated with either potato virus X (PVX) or potato virus Y (PVY) alone or the two in combination (PVX + PVY) and placed under controlled conditions at 20°, 24°, 28°, or 32° C. At intervals after inoculation, mechanically inoculated and systemically invaded leaves of virus infected plants and comparable leaves of healthy plants, were sectioned, stained with acridine orange, and viewed under the ultraviolet light microscope.

Parietal cytoplasm of healthy cells fluoresced red to a very small extent. Tissue infected with PVY rarely contained fluorescent inclusions while in tissue infected with PVX alone, discrete red fluorescent inclusions (indicating RNA) were prevalent in certain areas of the leaf. A moderately severe strain (PVX-5) induced more inclusions than did a mild strain (PVX-8). In doubly infected leaves which were invaded during rapid expansion, large fluorescent inclusions were present in practically all epidermal and mesophyll cells.

The relative difference in fluorescence between singly infected (PVX) and doubly infected tissue was greater in

systemically invaded leaves than in inoculated leaves. Optimum fluorescence of PVX inclusions was obtained at 20° and 24° C. At 28° and 32°, size and frequency of fluorescent inclusions decreased in comparison to that at 20° and 24° but differences between singly and doubly infected tissues were marked. At 28° most cells of doubly infected tissue contained fluorescent inclusions while with PVX alone, fluorescence was very poor. At 32°, there was no fluorescence in PVX infected leaves, while in doubly infected tissue, fluorescent material was present in cells near the veins. PVX concentration as measured by infectivity of Gomphrena globosa was generally in agreement with cytological observations. PVY concentration, as estimated by dilution end-point, using the synergistic response of PVX and PVY in tobacco plants, was unaffected by the presence of PVX.

CYTOPATHOLOGY OF MIXED INFECTIONS WITH POTATO VIRUSES X AND Y IN *NICOTIANA TABACUM* LEAVES

By

Harold Eugene Kauffman

A THESIS

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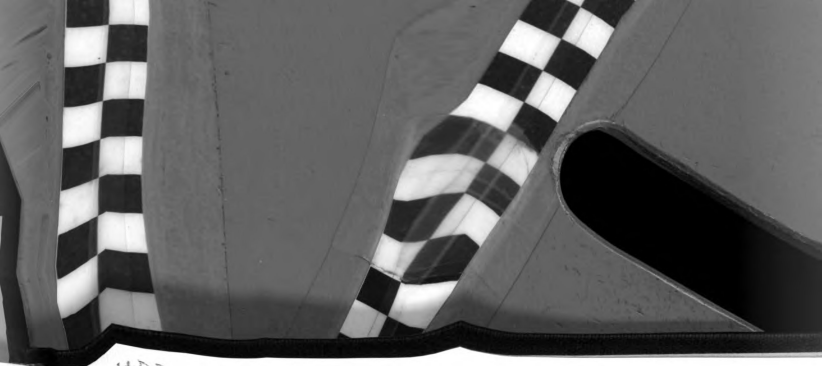

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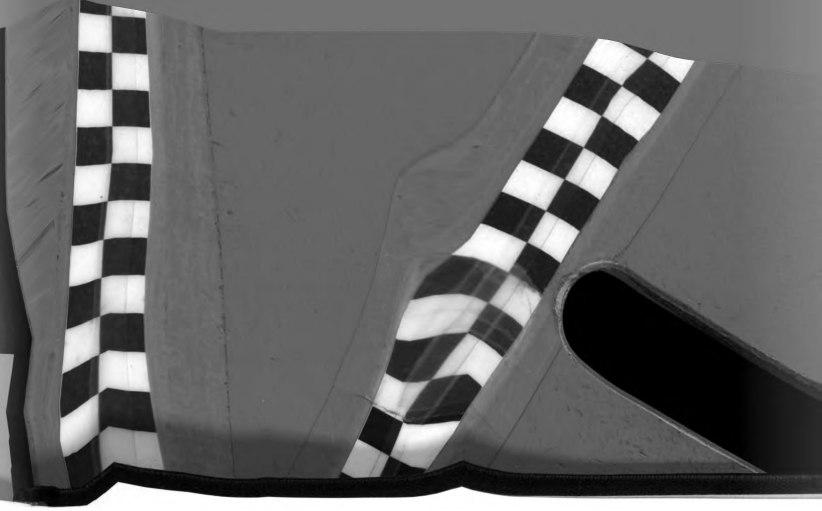

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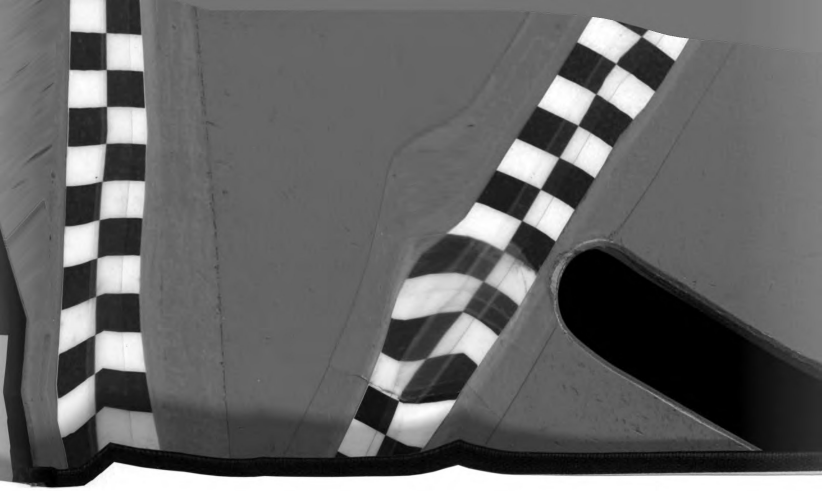

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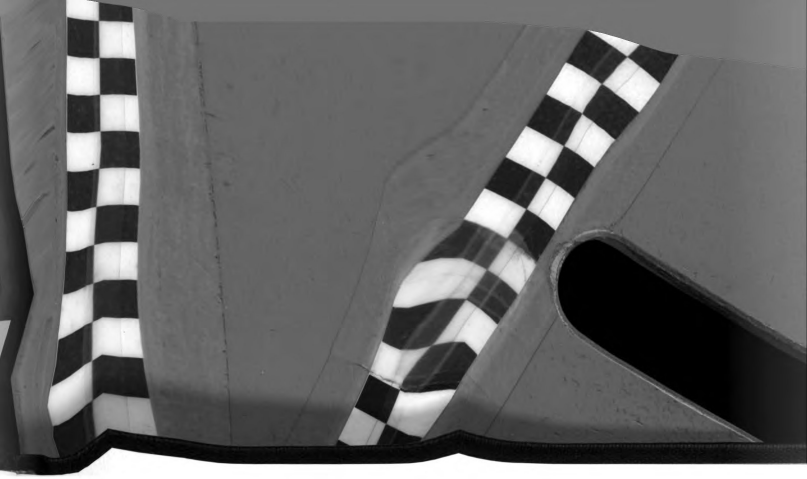
INTRODUCTION

This investigation was undertaken to determine cytological differences between single and double infections with potato virus X (PVX) and potato virus Y (PVY) in tobacco leaves and to determine if cytological aspects might be useful evidence for anticipating virus titre in infected tissue.

Intracellular inclusions in potato leaves showing mosaic symptoms, were first observed by Smith (1924). Hoggan (1927) and Clinch (1932) described amorphous intracellular inclusions in cells of virus infected potato leaves, possibly PVX, and suggested that the inclusions had the same general constitution as surrounding cytoplasm. Salaman (1938) observed that in tissue fixed in ethyl alcohol followed by treatment with formalin-chrom-acetic fixative, some inclusions of PVX were granular and others were vacuolate. Koshimizu et al. (1953) noted that inclusions of PVX varied in size, depending on the host tissue. Bawden and Sheffield (1944), using light microscopy, and Kikumoto and Matsui (1961), using electron microscopy, observed more inclusions of PVX in mesophyll cells than in epidermal cells of tobacco and of Datura stramonium respectively.



Studies of cytological changes within PVX infected epidermal cells of Datura tatula L. during virus multiplication, were made by Hooker (1964). Using a differential nucleic acid stain, pyronin Y-methyl green, RNA was shown to be aggregating near the nucleus two days after inoculation. Typical inclusions were observed by phase microscopy on the third day. Summanwar (1964), using another nucleic acid specific stain, acridine orange (AO), observed increased RNA flourescence in cytoplasm of epidermal cells of D. tatula after 24 hours. Flourescence became progressively more intense during the period up to five days and the mass of RNA (presumably virus RNA), became larger. As plant tissue became older, virus titre decreased and the mass of RNA also decreased in size.

Contrary to earlier reports (Bawden, 1939), Bawden and Sheffield (1944) found, though rarely, small inclusions in PVY infected potato and tobacco leaves. Koshimizu et al. (1953) observed that PVY inclusions in tobacco, potato, and tomato leaves were smaller and more granular than PVX inclusions. Using electron microscopy, Edwardson (1966) did not find laminated inclusions in PVY infected tobacco tissue but did find small, pinwheel inclusions.

Koshimizu et al. (1953) noted that doubly infected (PVX + PVY) potato and tobacco leaf tissue stained with giemsa contained several cells with both PVX vacuolated inclusions and PVY granulated inclusions. The large

vacuolated inclusions were present in more cells than the granulated inclusions.

Burnett's report (1934) that PVX remained infective longer in dried tomato leaves, doubly infected with PVX and tobacco mosaic virus (TMV) than in those singly infected with PVX alone, may have been the first indication that symptoms of synergism were associated with increased titre of one of the viruses. The first quantitative evidence for stimulatory action of one virus on another, was Bennett's report (1949) that the titre of dodder latent mosaic virus was stimulated in tomato plants by the presence of either TMV or tobacco etch virus. Ross (1950), using local lesion bioassay methods, noted that potato and tobacco plants doubly infected with PVX and PVY contained considerably higher titres of PVX than did singly infected plants.

Interactions of PVX and PVY in inoculated leaves of tobacco were demonstrated by Rochow and Ross (1955) in which doubly inoculated leaves contained PVX concentrations two times those found in singly inoculated leaves. Thomson (1958, 1961) reported that PVX lesions were more numerous in tobacco leaves infected with PVY than in leaves inoculated with PVX alone. Damirdagh and Ross (1967) obtained marked increases in amount of necrosis in doubly inoculated tobacco leaves by inoculating them with low concentrations of PVX mixed with high concentrations of PVY, but the increase of PVX concentration in the leaves was only 2.5 fold.

Synergistic interactions as evidenced primarily by increased PVX titre in systemic infections of tobacco were shown by Rochow and Ross (1955) but the interaction depended upon the stage of the disease. In doubly infected leaves that were invaded while rapidly expanding, strong symptoms of synergism were evident and PVX titres increased 10-fold over those in comparable singly infected leaves. In leaves that developed subsequent to those with acute symptoms of systemic infection and showed relatively mild symptoms, the PVX titre increased only 2-4 fold.

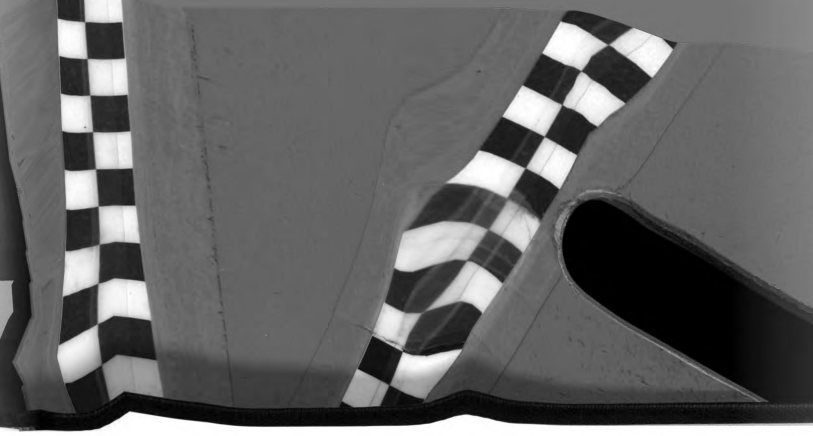
Serological methods (Rochow et al., 1955; Close, 1964), electron microscopy particle counts (Rochow et al., 1955), and electrophoretic methods (Bawden and Kleczkowski, 1957) have also been used to demonstrate increases in PVX titre in doubly infected plants. On the contrary, serological work by Bercks (1955a, 1955b) indicated that the increase in PVX titre was only transitory and that symptom severity was not correlated with PVX titre. These divergent results may be influenced in part by different virus strains or assay methods.

Ross (1950) reported that multiplication of mild strains of PVX was stimulated more by PVY than multiplication of severe strains. Rochow and Ross (1955) observed that their five isolates of PVY seemed to stimulate PVX to the same extent. Sequence of inoculation had little effect of PVX titres in inoculated leaves (Stouffer and Ross,

1961b) but in systemically invaded leaves the effect was marked. Rochow and Ross (1955) reported that in plants inoculated with PVX two weeks before a later inoculation with PVY, increases of PVX in the systemically invaded leaves were similar to those in plants simultaneously inoculated with the two viruses. On the other hand, when PVY was inoculated two weeks before inoculation with PVX, increases in PVX were very low.

Pound and Helms (1955) studied PVX concentrations in tobacco plants at various temperatures as determined by ultraviolet absorption and by infectivity methods. Concentrations of PVX were somewhat reduced at 28° C as compared to those at 20° or 24°. These results were confirmed in inoculated leaves by Ford and Ross (1962) (bioassay) and Willis et al. (1963) (bioassay and serology) and in systemically infected leaves by Stouffer and Ross (1961a) (bioassay) and Close (1964) (serology). Multiplication of PVX was considerably greater in doubly infected leaves at high temperatures than in singly infected leaves (Stouffer and Ross, 1961a; Ford and Ross, 1962). Close (1964) reported that at 31° C PVX rarely moved from inoculated leaves of tobacco when inoculated with PVX alone but when also inoculated with PVY, systemic invasion was frequently accomplished.

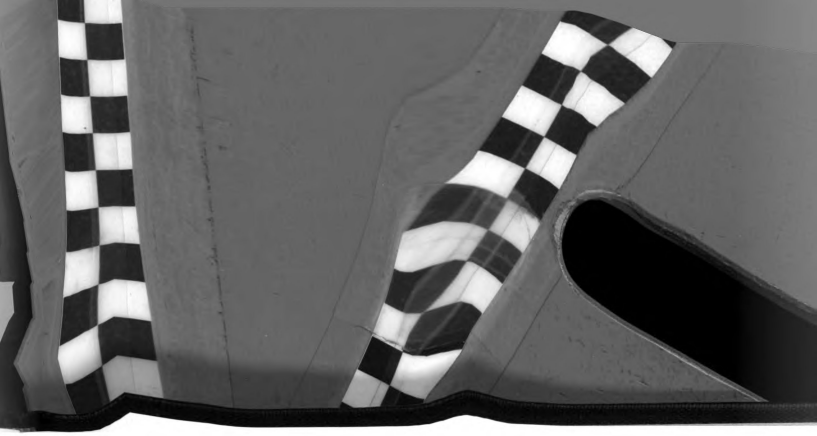
Bawden and Kassanis (1941), Bercks (1955a), and Close (1964), using serological methods, reported that PVY did not increase in doubly infected tobacco plants. The same



results were obtained by Ross (1950) and Rochow and Ross (1955), using local lesion assay methods.

Bioassay of PVY infectivity by local lesion hosts has been relatively unsatisfactory. Although several hosts have been reported to form local lesions when inoculated with PVY, only Physalis floridana Rydb. (Ross 1948, 1953; Rochow and Ross, 1955) and Chenopodium quinoa Willd. (Delgado-Sanchez and Grogan, 1966) have been used for bioassays. P. floridana, however, requires considerable time for growth to a suitable size and maturity (Ross, 1953) and Darby et al. (1951) reported erratic results with P. floridana when inoculated with certain strains of PVY. Ross (1948) reported that some strains of PVX also produce lesions on P. floridana. Delgado-Sanchez and Grogan (1966) noted that C. quinoa was easy to grow and consistently produced local lesions with three strains of PVY. C. quinoa was not a useful local lesion host for PVY in this study, since local lesions were also formed after inoculation with PVX.

In the present investigation, PVX distribution was determined and evidence concerning concentration of PVX in tobacco leaf tissue was obtained, using a differential nucleic acid stain, acridine orange. Cytological observations of singly infected (PVX and PVY alone) and doubly infected (PVX + PVY) leaf tissue were made at four different temperatures. Evidence that cytological aspects of virus multiplication may be used as further indication of virus



MATERIALS AND METHODS

PVX inocula, isolates PVX-8 and PVX-5 (Timian et al., 1955), were cultured in D. tatula in the greenhouse. Isolate PVX-8 was symptomless in D. tatula while PVX-5 induced a strong mottle in systemically infected leaves. PVY-3, an isolate originally obtained from a naturally infected potato plant in North Dakota, was cultured in Turkish tobacco (Nicotiana tabacum L.). Inoculated leaves of PVY infected plants were symptomless but a mild mottle and vein clearing developed in systemically invaded leaves.

Plants were grown in sterilized U. C. soil, mix C, a mixture of 50 per cent sand and 50 per cent peat moss (Baker, 1957), supplemented twice a week with a nutrient solution (Plant Marvel Laboratories, Chicago 28, Illinois: of 12, 31, and 14 per cent of N, P, and K respectively at the rate of one tablespoon per gallon of water). Unless otherwise indicated, plants were grown without supplemental light in the greenhouse maintained near 20° C except during summer months when daytime temperatures sometimes reached 28°.

Inocula for each mixed infection experiment were prepared from 2-4 week old infected leaves of source plants by grinding them with a mortar and pestle. Inoculum of either PVX of PVY alone was diluted 1:10 (v/v) with distilled water.

When used in combination, each virus was similarly at a 1:10 dilution in the mixture.

Turkish tobacco plants used for each experiment were selected for uniformity when the largest leaf measured 5-7 cm across. The bottom leaves were removed and the two expanded upper leaves were dusted with 400 mesh silicon carbide. Inoculations were made by gently rubbing the virus suspension over the leaf surface with a glazed, glass spatula. In each set of experiments, the two largest upper leaves of a group of plants were inoculated with PVX alone, another group with PVY alone, and a final group with a mixture of PVX and PVY. Control plants were rubbed with distilled water. After inoculation, leaves were gently rinsed with tap water and placed on a greenhouse bench. Twenty-four hours after inoculation, plants were put in chambers maintained at constant temperatures of 20°, 24°, 28°, and 32° C (±1.0). Fluorescent lights continuously supplied 500-700 ft c of light to each chamber 16 hours per day.

Inoculated and systemically invaded leaves were removed from plants at intervals after inoculation for cytological observation. Of the systemically invaded leaves, only those with distinct vein clearing symptoms in doubly infected plants and comparable ones from singly infected plants, were selected. A strip was cut lengthwise from each leaf and infiltrated with water under negative pressure (McWhorter, 1951). Epidermal sections of veinal and interveinal tissue, cut with a razor blade from the upper surface

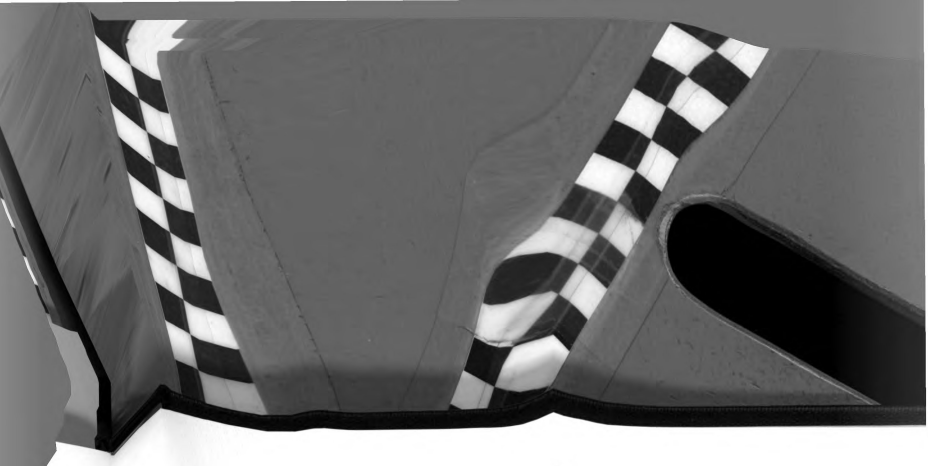
of the leaf, and cross sections of interveinal tissue, cut with an experimental microtome (Hooker, in press), were stained with acridine orange (Lot 14831, George T. Gurr, Ltd., London) by a modification of the method of Hooker and Summanwar (1964), in which fixation was reduced to 10 minutes and 6.7 per cent phenol was added in 50 per cent ethyl alcohol. Enzymatic removal of RNA from fixed tissue was accomplished with ribonuclease (RNAase), chromatographically prepared pancreatic Ribonuclease A, (Worthington Biochemical Corp., Freehold, New Jersey). Fixed tissue sections were treated in RNAase (50 µg/ml in 0.2 M phosphate buffer of pH 6.4) for one hour at room temperature, rinsed in water, and stained with AO. Controls consisted of diseased tissue treated in 0.2 M phosphate buffer for one hour at room temperature followed by a water rinse and staining with AO.

Observations of AO stained tissue were made with an American Optical achromatic objective with a General Electric mercury vapor light. Near-blue ultraviolet light with a peak between 390 and 420 mµ, was obtained by a blue filter (Corning #5113, 405 mm thick). Phase contrast observations were made with a Wild-Heerbrugg Fluotar objective, 43x, NA 0.75. Photographs were made using 35 mm Kodak Ektachrome X film. Black and white negatives (Eastman Panatomic X film) were made from color slides using a red (25 A Wratten) and a green (58 B Wratten) filter to separate red and green fluorescent substances respectively. The final color prints were

made on type R paper and black and white prints on Ansco Jet paper.

To measure PVX infectivity, both inoculated leaves from three plants were ground together and assayed 10 days after innoculation. Similarly, systemically invaded leaves were assayed after selection on the same basis as described for cytological observations. The juice was pressed through two layers of cheese-cloth, centrifuged for 15 minutes at 10,000 RPM and diluted with distilled water. The two-dilution method of Spencer and Price (1943), at log intervals of 1.0, was used to determine PVX infectivity on Gomphrena globosa L. Relative infectivity of the "unknown" was calculated by means of the formula given by Sherwood et al. (1944).

G. globosa plants were grown in a 26° C temperature room in the greenhouse. Natural daylight was supplemented during the winter by flourescent lights with an intensity of 450-700 ft c. During spring and early summer, plants were shaded to reduce light intensity of the sun. Eight uniform plants with two large upper leaves were selected for each assay and all but the upper two leaves were trimmed from the plants. The leaves were dusted with silicon carbide and inoculated by uniformly rubbing a virus suspension over a half leaf three times with a glass spatula. Following inoculation, plants were placed on a greenhouse bench and local lesions were counted six days later.



PVY concentration was estimated by dilution end-point, using the synergistic reaction of Turkish tobacco plants doubly infected with PVX and PVY. PVY assay samples from singly and doubly infected leaves were prepared by centrifuging juice for five minutes at 3,000 RPM's and diluting in ten-fold series. Frozen samples of a standard PVX-5 suspension diluted to $1/10^3$ were thawed and mixed with each sample. Uniform tobacco plants with the largest leaf 2-3 cm across, were inoculated with the virus mixtures. Plants were rinsed with tap water and placed on a greenhouse bench with 400-500 ft c of supplemental light. The percentage of plants showing the synergistic reaction ten days after inoculation was recorded.

EXPERIMENTAL RESULTS

Symptomology of Singly and Doubly Infected Turkish Tobacco Leaves

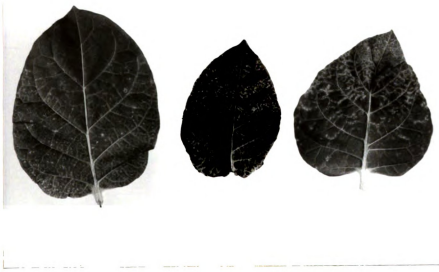
Isolate PVX-8 was symptomless in tobacco. PVX-5 induced a few necrotic ringspots in inoculated leaves but no symptoms were evident in systemically infected leaves.

Leaves inoculated with a mixture of PVX-8 and PVY usually were symptomless, while leaves inoculated with a combination of PVX-5 and PVY developed chlorotic and necrotic ringspots seven days following inoculation.

Veinal chlorosis was evident in the first systemically invaded leaves of plants inoculated seven days previously with a mixture of PVX-8 and PVY. At ten days, the leaf immediately above the inoculated leaf was symptomless but varying portions of leaves 2, 3, and 4 displayed veinal chlorosis (Fig. 1-A). Plants doubly infected with PVX-5 and PVY displayed similar but more severe symptoms. Some of the veinal cells became necrotic and the leaves were stunted (Fig. 1-B).

At 20° and 24° C, symptoms in plants doubly infected with either strain of PVX were more severe than in plants at 28°. At 32°, doubly infected leaves usually remained symptomless.

A



B

Fig. 1.--Symptoms of synergism in systemically invaded leaves 2, 3, and 4 from plants doubly infected with PVX and PVY at 28° C for 10 days. (A) mild symptoms with PVX-8 + PVY; (B) severe symptoms with PVX-5 + PVY.

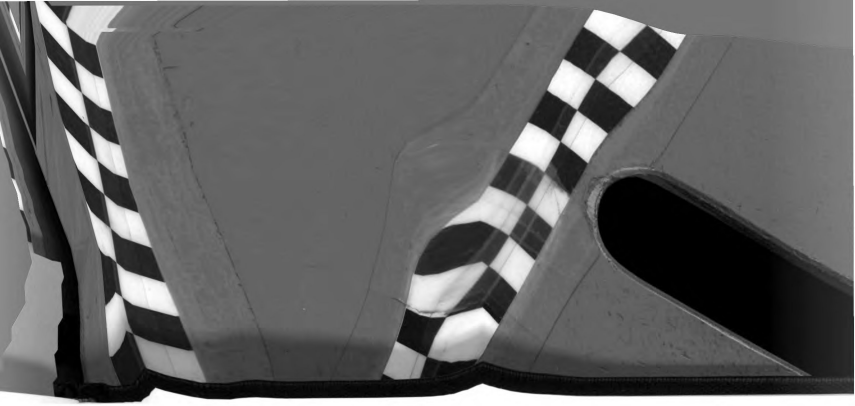
Cytology: Acridine Orange Fluorescence in Leaf Tissue

Non-Inoculated Controls

Leaf sections were fixed and stained with acridine orange (AO) as described. DNA in the nuclei fluoresced yellowish green and RNA of nucleoli fluoresced flame red. Parietal cytoplasm of healthy cells fluoresced red to a very small extent while the cytoplasm of virus infected cells usually contained brilliant red fluorescent masses. Parietal cytoplasm fluoresced more in small, immature cells than in large mature cells. Precise comparison of cells from inoculated leaves was possible since all cells were mature when observed but precise comparison of systemically invaded leaves was more difficult since not all cells were mature.

PVY Single Infections

Sections were made of both inoculated and systemically invaded PVY infected leaves. Most cells of PVY-3 infected leaves lacked fluorescent inclusions (Fig. 2-A) and the cells appeared very similar to those of non-inoculated controls. In some leaves, fluorescent inclusions were present in veinal parenchyma cells but the inclusions were small and granular and they fluoresced red with very low intensity (Fig. 2-B).

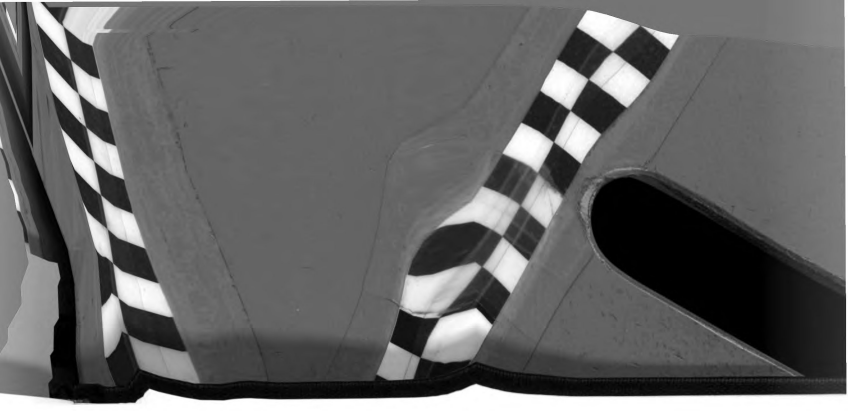


PVX Single Infections

Inoculated Leaves.--Two days after inoculation with either strain of PVX, some epidermal cells contained diffuse red, fluorescent material in the cytoplasm. At three days, the fluorescent masses were near the nucleus and they were less diffuse. At 10 days, the inclusions were about the size of the nucleus. In leaves infected with PVX-5, more than 50 per cent of the epidermal cells contained inclusions while in PVX-8 infected leaves 20 per cent of the cells contained inclusions. The red fluorescent inclusions were identified as RNA by treating fixed sections with RNAase. Since similar inclusions were not present in stained healthy cells, the red cytoplasmic fluorescence was interpreted as being virus RNA.

Small fluorescent inclusions were observed in some palisade mesophyll cells infected with PVX-5, seven days following inoculation. Inclusions were about the size of the nucleus at 10 days but cells with inclusions were more prevalent in some areas of the leaf than in others. PVX-8 infected leaves contained fewer inclusions in the palisade cells than did PVX-5 infected leaves.

Systemically Invaded Leaves.--Seven days following inoculation, epidermal cells of systemically invaded leaves contained diffuse, red fluorescent material in the cytoplasm. At 10 days, many cells contained discrete red masses near the nucleus. Inclusions in PVX-5 infected leaves (Fig. 3-A)



were brighter and more numerous than those in PVX-8 infected leaves (Fig. 2-C). Most palisade cells of PVX-5 infected leaves contained well developed inclusions but palisade cells of PVX-8 infected leaves rarely contained distinct fluorescent inclusions.

Double Infections

Inoculated Leaves.--Parietal cytoplasm of some epidermal cells of doubly inoculated leaves began to fluoresce red two days after inoculation. At three days, small, fluorescent inclusions were noticable in the cytoplasm near the nucleus and at four days, most inclusions were slightly larger than the nucleus. During early stages of infection (3-4 days), fluorescence appeared to be slightly more intense in doubly infected leaves than in those singly infected. The nucleolus was larger and stained more brilliantly in both singly and doubly infected tissue than in non-inoculated controls. Later (10-14 days), inclusions in doubly infected tissue were slightly larger than those in singly infected tissue. Furthermore, a larger number of cells contained inclusions in doubly infected leaves. There were no observable differences between inclusions of PVX-8 and PVX-5 in epidermal cells.

Many mesophyll cells in limited areas of doubly inoculated leaves contained red fluorescent inclusions at seven days. Most inclusions were slightly larger than the nucleus.

Systemically Invaded Leaves.--Cells of these leaves contained more red fluorescence in the cytoplasm than cells of any other treatment. Some necrosis was evident in tissue doubly infected with PVX-5 and PVY but cytological observations were made of non-necrotic tissue only. At seven days, large areas of the cytoplasm of all epidermal cells contained diffuse, red fluorescent material. At 10 days, cytoplasmic fluorescence was usually concentrated near the nucleus with small amounts along the cell wall (Fig. 2-D and 3-B). Most inclusions were much larger than the nucleus and some inclusions appeared granular while others appeared vacuolate. At 14 days, most inclusions were granular and the parietal cytoplasm fluoresced very lightly. All red staining substances of the cytoplasmic inclusions were removed by RNAase, indicating that the fluorescence in the cytoplasm was RNA (Fig. 3-C).

Extremely large cytoplasmic inclusions were present in veinal parenchyma cells from both the upper and lower surface of the leaf (Figs. 2-D and 2-F). Although nuclei of these cells were larger than nuclei of epidermal cells, many inclusions were 3-4 times the size of the nucleus. The nucleoli were noticeably larger and more brightly stained than those of comparative non-inoculated tissue.

Palisade cells uniformly contained fluorescent inclusions that were larger than the nucleus (Fig. 3-E). Inclusions in tissue doubly infected with PVX-5 and PVY were

larger and brighter than those in PVX-8 + PVY infected tissue.

Fluorescent, cytoplasmic inclusions were larger and more numerous in leaf areas with vein clearing symptoms than in areas with no symptoms. Association between number of inclusions and symptom severity was evident in systemically invaded leaves two and four (Fig. 1). Both leaves displayed vein clearing symptoms in half of the leaf--the basal half of leaf two and the apical half of leaf four. Nearly every cell in vein cleared areas of either leaf contained large inclusions while cells in symptomless areas rarely contained inclusions.

Influence of Temperature on Single and Double Infections

PVX-8 Inoculated Leaves.--Differences in red fluorescence in leaves singly infected with PVX-8 and those doubly infected with PVX-8 and PVY were relatively minor. At the two lower temperatures, PVX-8 alone induced flourescent inclusions in less than five per cent of the cells as compared to inclusions in 20-40 per cent of the cells in doubly infected leaves. At 28°, singly infected leaves rarely contained inclusions while inclusions were present in a few limited areas of doubly infected leaves. At 32°, no inclusions were present in singly infected leaves while cells along the veins contained inclusions in doubly infected leaves.

PVX-8 Systemically Invaded Leaves.--Differences were marked between singly and doubly infected leaves at all temperatures. Leaves of singly infected plants at 20° and 24° rarely contained fluorescent inclusions. Those present were small and were usually located near the veins (Fig. 2-C). Comparable doubly infected tissue, on the other hand, contained large inclusions in most epidermal and mesophyll cells in practically all areas of the leaf (Fig. 2-D). In plants at 28° or 32°, PVX-8 failed to produce inclusions in singly infected leaves (Fig. 2-E) while in doubly infected leaves, inclusions were present in most cells of leaves at 28° (Fig. 2-F), and in limited areas near veins in leaves at 32°.

PVX-5 Inoculated Leaves.--Inoculated leaves infected with PVX-5 at 20° and 24° contained inclusions about the size of the nucleus in most epidermal cells. Leaves doubly infected with PVX-5 and PVY at the same temperatures contained inclusions that were slightly larger than those in singly infected leaves. At 28°, inclusions in singly infected leaves were limited to fewer areas and they were smaller than inclusions in doubly infected leaves. At 32°, PVX-5 alone rarely induced red fluorescent inclusions in epidermal cells while leaves doubly infected, contained inclusions in epidermal cells near veins.

PVX-5 Systemically Invaded Leaves.--Differences between singly and doubly infected systemically invaded leaves were marked. At 20° and 24°, inclusions similar in

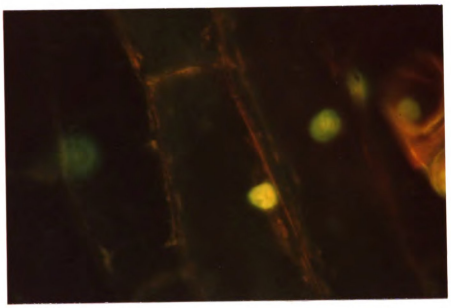
size to the nucleus were present in many epidermal cells of singly infected leaves (Fig. 3-A). Fluorescent inclusions in comparable doubly infected leaves were several times larger than the nucleus and in many cells the parietal cytoplasm also fluoresced red (Fig. 3-B). At 28°, inclusions were present in limited areas in the singly infected leaves while in doubly infected leaves, inclusions were present in most cells. At 32°, singly infected leaves contained no cytoplasmic inclusions. Doubly infected leaves at the same temperature, contained fluorescent inclusions in epidermal cells near the veins.

Cytology: Phase Microscopy

Epidermal sections of systemically invaded leaves were mounted in water for observation. Cells in non-inoculated tissue did not contain masses of cytoplasm which appeared similar to virus inclusions (Fig. 4-A).

Although no cytoplasmic inclusions were evident in PVY-3 infected cells with phase microscopy, active cytoplasmic streaming was observed in many cells (Fig. 4-B).

There was little evidence of active cytoplasmic streaming in most cells of tissue infected with PVX-8. Some epidermal cells appeared similar to those of non-inoculated leaves and other cells appeared to contain small amounts of granular material near the nucleus (Fig. 4-C). Many cells of PVX-5 infected tissue, on the other hand, contained small granular inclusions, about the size of the nucleus.



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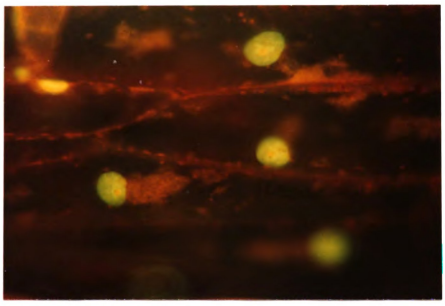
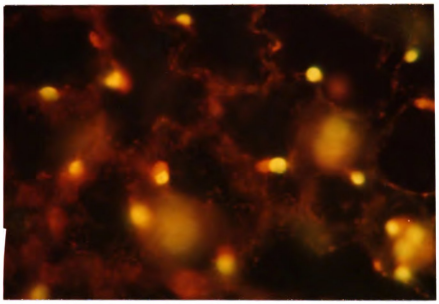
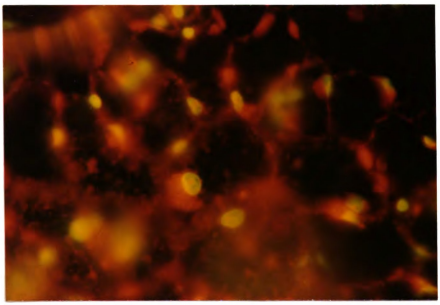


B

Fig. 2.--Epidermal cells near main vein of systemically invaded leaves of Turkish tobacco 10 days after inoculation. (A) typical PVY infected cells; (B) an inclusion rarely present in PVY infected cells.

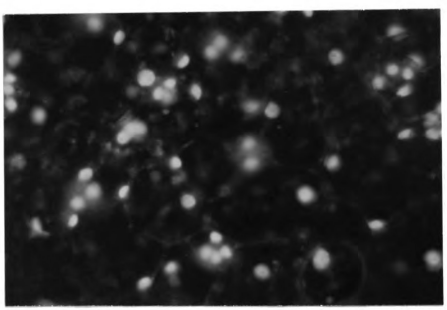


A

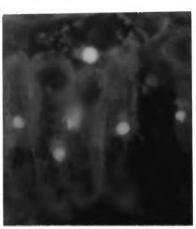


B

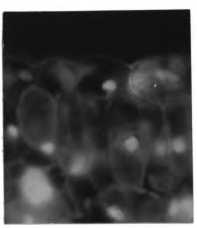
Fig. 3.--Cells of systemically invaded leaves of Turkish tobacco. (A) epidermal cells in a plant inoculated 10 days previously with PVX-5, 20° C; (B) similar to A, but doubly infected with PVX-5 + PVY, 20° C.



C



D



E

Fig. 3 (cont.).--(C) similar to B, but treated with RNAase; (D) palisade cells in cross section in a plant inoculated 14 days previously with PVX-5, 20° C; (E) similar to D, but doubly infected with PVX-5 + PVY, 20° C.

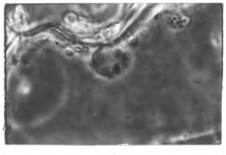
In systemically invaded leaves, doubly infected with either PVX-5 + PVY or PVX-8 + PVY, most cells contained large cytoplasmic inclusions (Fig. 4-D). Most inclusions were granular, although a few were vacuolate.

Phase contrast observations of AO stained tissue showed that inclusions in doubly infected tissue appeared to contain RNA throughout the inclusion. The same inclusion which fluoresced flame red with AO, appeared to be of similar size when observed with phase contrast (Figs. 5-A, 5-B, and 5-C).

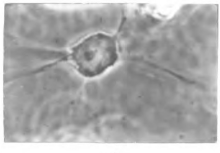
Bioassay of PVX in Singly and Doubly Infected Leaves

PVX infectivity was measured in juice from singly and doubly infected tobacco leaves to determine if differences in cytological detail were associated with differences in PVX titre over the range of temperatures. Leaves were assayed 10 days following inoculation since cytological and preliminary bioassays indicated that virus titre was at a high level at that time. Of the systemically invaded leaves, only those with distinct vein clearing symptoms were assayed from doubly infected plants. These were compared with leaves of similar position from singly infected plants.

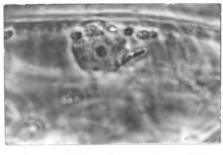
PVX-8 infectivity was determined in leaves from plants kept at the four temperatures. A standard inoculum was used for each assay within an experiment and infectivity at each temperature is expressed relative to the standard. Figure 6 shows the average results of two experiments. PVX-8



A



B



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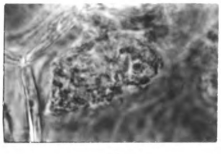
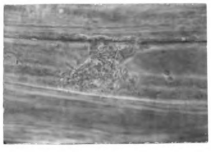
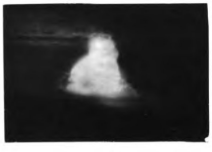


D

Fig. 4.--Living nuclei of epidermal cells in systemically invaded leaves of Turkish tobacco 10 days after inoculation. (A) non-inoculated control; (B) PVY infected cell; (C) PVX-8 infected cell; (D) PVX-8 + PVY infected cell.



A



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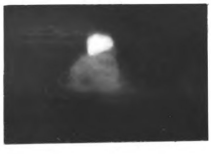


C

Fig. 5.--Similarity of inclusions in the same cell fixed and stained with AO as observed by: (A) phase contrast, and B and C by ultra violet fluorescence microscopy. B and C color separation by red and green filters, respectively.

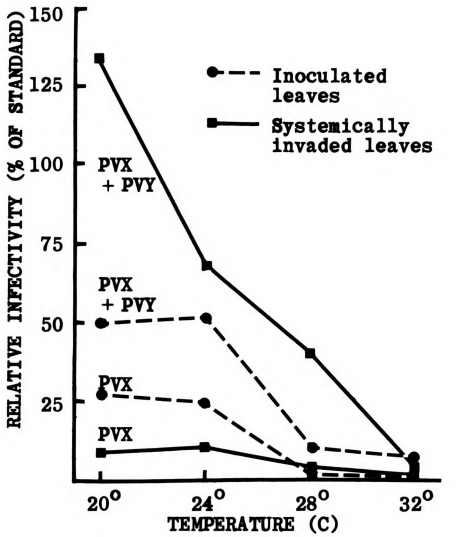
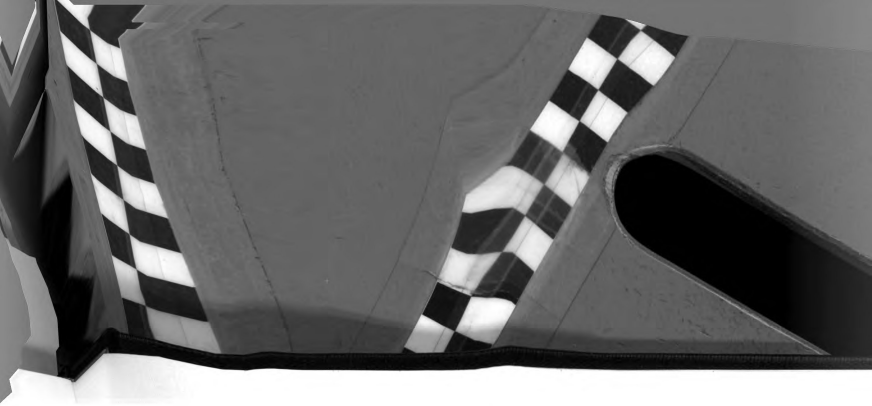


Fig. 6.--Relative infectivity (local lesion assay on G. globosa) of PVX-8 in singly and doubly infected leaves of N. tabacum at various temperatures.



concentrations were high in all singly and doubly infected leaves at 20° and 24° while at 28° and 32°, concentrations were much lower. In all tests, PVX-8 concentrations were higher in doubly infected leaves than in comparable singly infected leaves. At each temperature except 32°, PVX-8 concentrations were highest in doubly infected, systemically invaded leaves. At 32°, the doubly infected, inoculated leaves contained the highest relative titre of PVX-8.

For PVX-5 infected plants, direct comparisons were made between singly and doubly infected leaves to determine relative increases in PVX titre at each temperature. Ratios of infectivity in doubly infected leaves to that in singly infected ones (Xxy/Xx) were determined at each temperature. Figures 7 and 8 compare average infectivity ratios of two experiments of PVX-5 infected plants at each temperature with infectivity ratios of PVX-8. Infectivity ratios of leaves at 32° were disproportionately high due to extremely low titres in singly infected leaves. Because of this, interpretation of the ratios at 32° should be made with reservation. Only the ratios from 20°, 24°, and 28° will be discussed in greater detail.

Infectivity ratios of PVX-8 were greater than those of PVX-5 in all inoculated and systemically invaded leaves at all temperatures. In inoculated leaves, the largest increase of PVX-8 concentration in doubly infected leaves over those in singly infected leaves, was 10-fold at 28°

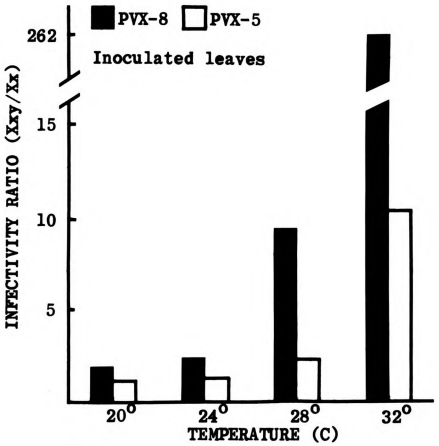


Fig. 7.--Ratios of infectivity of two strains of PVX in doubly infected/singly infected (Xxy/Xx), inoculated leaves of N. tabacum at various temperatures.

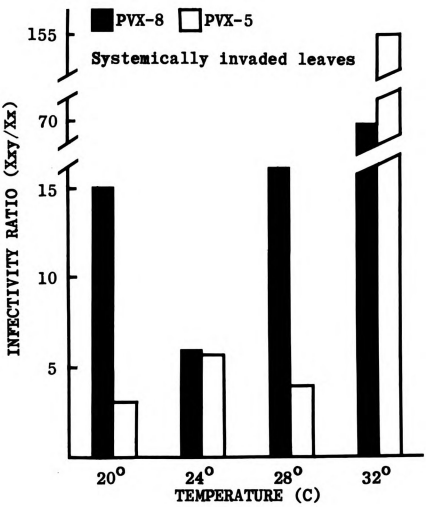


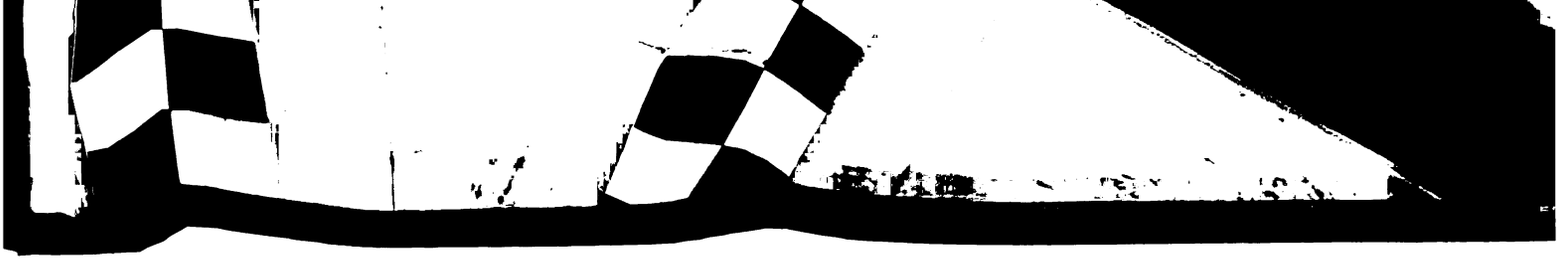
Fig. 8.--Ratios of infectivity of two strains of PVX in doubly infected/singly infected (Xxy/Xx), systemically invaded leaves of N. tabacum at various temperatures.

compared to the largest PVX-5 increase, 3-fold, at the same temperature. The greatest increase of PVX-8 concentrations in doubly infected, systemically invaded leaves, was at least 15-fold at 20° and 28° compared to the greatest increase of PVX-5, 6-fold, at 24°. These results indicate that the mild strain (PVX-8) is stimulated to a greater extent by PVY than the more severe strain (PVX-5).

In inoculated leaves, PVY stimulated greater relative increases of PVX concentrations as the temperature increased. In systemically invaded leaves, on the other hand, no direct association was obtained between relative increases of PVX titre and temperature.

Bioassay of PVY in Singly and Doubly Infected Leaves

Local lesion assay of PVY is relatively unsatisfactory because suitable hosts are not available and response is variable. In these tests, PVY infectivity was determined by dilution end-point using the synergistic reaction of PVX and PVY in N. tabacum plants. Preliminary trials indicated that when a series of PVY dilutions were mixed with a standard concentration of PVX and inoculated to tobacco plants, a constant end-point was reached at which plants no longer responded with the typical synergistic reaction of doubly infected plants. Greatest uniformity in results was obtained when vigorously growing, small N. tabacum plants with the largest leaf measuring between 2-3 cm across,



were used for assays. Symptoms were clearly expressed in doubly infected plants 10 days after inoculation (Fig. 9).

To determine if the concentration of PVX influenced the dilution end-point of PVY, PVX-8 infected tobacco leaf juice was clarified, diluted in 10-fold series, and mixed with samples from a PVY dilution series. Ten days after inoculation to small tobacco plants, mild symptoms of the doubly infected plants were evident. At PVX-8 dilutions of $1/10^1$ - $1/10^3$ (v/v), essentially similar responses were obtained over the dilution series with PVY (Fig. 10). Thus, a wide range of PVX dilutions appeared to react similarly with PVY at different concentrations. At PVX-8 dilutions of $1/10^4$, the number of doubly infected plants was very low. These results suggested that concentrations of PVX, as are commonly found in doubly infected leaves of tobacco, do not influence the synergistic response so long as the supply of PVX is adequate.

Since PVX-5 + PVY produced more severe symptoms than did PVX-8 + PVY in tobacco plants, PVX-5 from clarified D. tatula leaf juice was tested similarly. Fewer doubly infected plants were obtained with PVX-5 dilutions of $1/10^1$ and $1/10^2$ than with dilutions of $1/10^3$ or $1/10^4$ (Fig. 11). This suggested that an inhibitor may have been interfering with infectivity of PVY at low dilutions of PVX and that the inhibitor was diluted out at higher dilutions. Since the titre of PVX is considerably higher in D. tatula than in

Fig. 9.--Synergistic reaction of a doubly infected, small N. tabacum plant used for determining the dilution end-point of PVY. The plant was inoculated 10 days previously with a mixture of an assay sample of PVY and a standard PVX suspension.

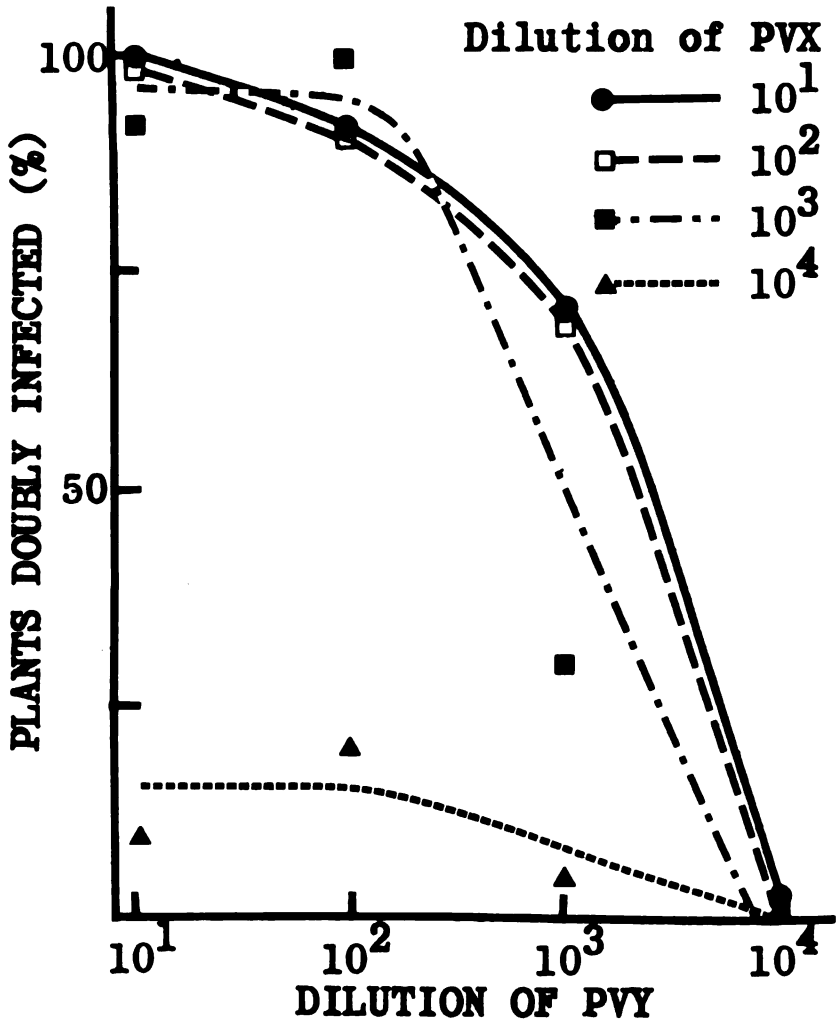


Fig. 10.--Influence of PVX concentration (PVX-8 in clarified juice of N. tabacum) on PVY infectivity in a dilution series. Data based on N. tabacum plants showing synergistic response from double infections.

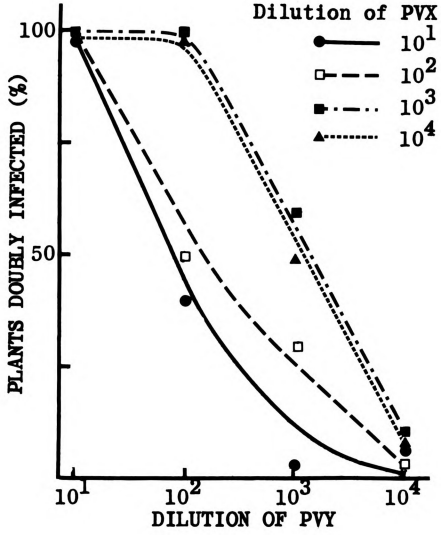


Fig. 11.--Influence of PVX concentration (PVX-5 in clarified juice of D. tatula) on PVY infectivity in a dilution series. Data based on N. tabacum plants showing synergistic response from double infections.

tobacco, dilutions of $1/10^3$ of D. tatula juice may approximate the infectivity of juice from N. tabacum of $1/10^1$. In subsequent experiments with PVX-5 at $1/10^3$, results were consistent and symptoms were clearly defined. Leaves of the small tobacco plants inoculated with high concentrations of PVY and low concentrations of PVX did not become necrotic as Damirdagh and Ross (1967) reported in mature inoculated leaves. Standard samples of PVX-5 from D. tatula were frozen and used in all PVY bioassays.

Comparative PVY bioassays were run on the systemically invaded leaves of doubly and singly infected tobacco test plants at the various temperatures. Forty small N. tabacum plants were inoculated with each suspension mixture (Fig. 12).

At 20°, 24°, and 28° C, infectivity of PVY in both doubly and singly infected leaves was essentially similar. At 32°, infectivity of PVY in singly infected leaves was slightly higher than the infectivity in doubly infected leaves. This suggests that at the three lower temperatures, the presence of PVX in the tissue did not influence the multiplication of PVY while at 32° the presence of PVX may have slightly inhibited multiplication of PVY. Although the infectivity of PVY was not directly compared at the four temperatures, PVY titre appeared essentially similar at all temperatures.

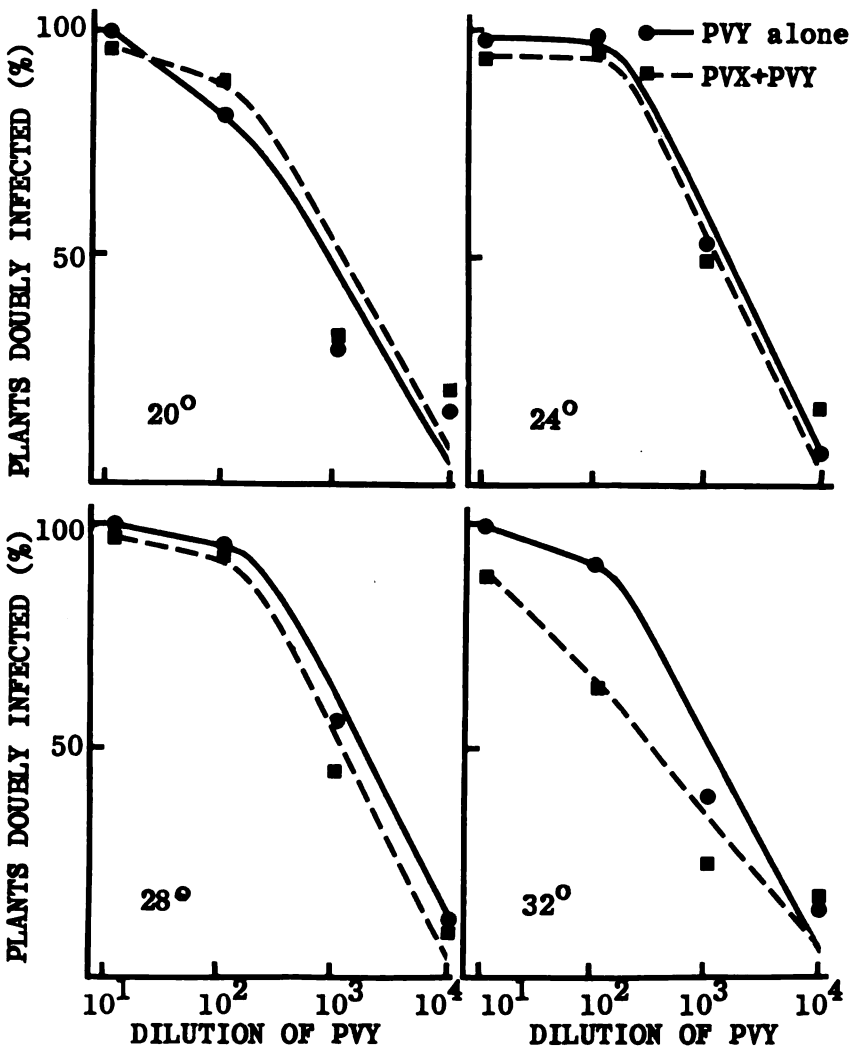


Fig. 12.--PVY infectivity in N. tabacum leaves singly infected with PVY or doubly infected with PVX-8 + PVY and kept at various temperatures. PVY infectivity was determined by dilution end-point of the synergistic response of PVY and PVX in N. tabacum.

DISCUSSION

Cytological observations with a nucleic acid stain are believed to provide an indication of virus concentration within an entire tissue, between cells of such tissue, and distribution of virus within the cells. In certain respects this may provide as much or more information as bioassays which give some composite information of over-all virus concentration within the various tissues of the organ or part of organ, used for the triturates.

In this study of tobacco leaves, diffuse, fluorescent material (RNA) in cytoplasm was evident in single PVX infections two days following inoculation and a few cells contained small inclusions at three days. Later, many, but not all, cells of the tissue contained fluorescent inclusions. In another host, PVX inoculated epidermal cells of D. tatula cotyledons, fluorescent material was evident within 24 hours after inoculation and at 4-5 days, practically all cells contained inclusions (Hooker, 1964; Summanwar, 1964). Synthesis of PVX in tobacco tissue is apparently slower and less uniform from cell to cell than in D. tatula cotyledons.

The isolate of PVY used in this study was similar to the strain studied by Bawden and Sheffield (1944) as it formed inclusions but rarely in infected leaves. The AO stained inclusions were usually small and they flouresced

red with very low intensity, indicating low levels of RNA in the inclusions. Cells lacking cytoplasmic fluorescence may have been infected but virus concentrations were so low that virus inclusions were not distinguishable.

Cytoplasmic fluorescence in doubly infected tissue was much greater than in tissue singly infected with either PVX or PVY. Pronounced and clearly defined virus inclusions in doubly infected tissue were believed to be those of PVX for the following reasons: (1) the isolate of PVY used in this study rarely formed inclusions in single infections; (2) PVY infectivity titres did not increase in doubly infections; (3) PVX infectivity titres were much higher in double infections than in single infections; and (4) inclusions were correspondingly much more pronounced and fluoresced with greater intensity in double infections.

Rubio (1956) reported that virus content of cellular inclusions varied greatly, depending upon virus-host systems. Some inclusions, such as X-bodies induced by cabbage black ring in cauliflower, were composed of flexous strands similar to virus particles. Other inclusions, such as X-bodies of the common strain of TMV in tobacco, contained few if any virus particles. Although similar systems have been studied in phase contrast microscopy and acridine orange staining, direct comparison of the same PVX inclusion by ultraviolet and phase microscopy have not previously been made. In this study, doubly infected tissue stained with AO contained

inclusions of similar size and shape when observed both under ultraviolet and phase contrast microscopy, suggesting that RNA was distributed throughout the inclusion.

Increased titres of PVX in double infections with PVY is reflected in increased size of inclusions of PVX within most doubly infected cells and increased uniformity of distribution of inclusions in leaf tissue. In leaves inoculated with both viruses, inclusions were slightly larger and they were prevalent in more areas of the leaf than those in singly inoculated leaves. In doubly infected, systemically invaded leaves, inclusions were very large and were prevalent in practically all palisade and epidermal cells while those in singly infected leaves were small and were unevenly distributed.

PVX concentrations were very high in parenchyma cells surrounding the main veins in doubly infected, systemically invaded leaves as evidenced by high infectivity titres reported by Rochow and Ross (1955) and by extremely large fluorescent inclusions observed in this study. Rochow and Ross also reported that the concentration of PVY near large veins was approximately double that in interveinal tissue. Since this high concentration of PVY near veins occurred both in singly and doubly infected leaves, the large inclusions present in veinal tissue of doubly infected plants is believed to be primarily that of PVX. The presence of high concentrations of PVX in these doubly infected veinal cells

suggests that PVY may have entered these cells at precisely the right time to stimulate optimum multiplication of PVX as is suggested by Damirdagh and Ross (1967).

PVY is reported to stimulate a greater relative increase in concentration of mild strains of PVX than of severe strains (Ross, 1950; Rochow and Ross, 1955; Close, 1964). The mild strain used in this study (PVX-8), induced fewer inclusions in single infections than did the moderately severs strain (PVX-5). In double infections, similar inclusions were induced by both strains in the epidermal cells while PVX-5 induced slightly larger inclusions in mesophyll cells. Differences appeared greater, however, between single and double infections with PVX-8 than with PVX-5. Infectivity assays in this study also demonstrated greater increases with PVX-8 as infectivity of PVX-8 was increased up to 15-fold in double infections while PVX-5 titre was increased up to 6-fold.

As shown cytologically, low titres of PVX-8 in single infections may allow a greater increase of PVX multiplication in double infections than is possible with relatively high titres of PVX-5. Willis et al. (1963), using infectivity and serological methods, reported that a mild strain of PVX, the yellow strain--a mutant of PVX-8 (Hansen and Larson, 1959), reached much lower titres in tobacco tissue than did the ringspot strain tested. Thus, when titres of PVX are low in single infections, possibly most cells can sustain a large increase in PVX concentration in double infections before virus multiplication is limited.

Symptom severity in doubly infected plants has been directly correlated with PVX concentration by Close (1964). Rochow and Ross (1955) report a similar association between PVX concentration and symptom severity with the reservation that the tissue must not have become necrotic. Bercks (1955a), however, noted that correlation of symptom severity and concentration of PVX is not always obtained. Visual symptoms of doubly infected, systemically invaded leaves, were most pronounced in these trials in chlorotic areas along the veins. Cytologically, when veins were chlorotic, veinal parenchyma cells usually contained large fluorescent inclusions and when chlorosis was not evident, inclusions were rare. Thus, severity of chlorotic symptoms and PVX concentration in veinal tissue appeared to be positively associated.

Results of the present cytological and infectivity studies at various temperatures, are generally in accord with the infectivity assays of Ford and Ross (1962) and Stouffer and Ross (1961a) and with the serological assays of Close (1964). The relative increase of PVX in doubly infected, systemically invaded leaves at 32° in this study is higher than the 11.5 ratio reported by Stouffer and Ross (1961). Close (1964) reported that PVX rarely moved out of inoculated leaves at 31° C unless the leaves were also infected with PVY. In the present study, PVX moved out of singly inoculated leaves at 32°, but the concentration of

PVX in systemically infected leaves was extremely low. In both singly and doubly infected plants at 32°, the inoculated leaves had greater concentrations of PVX, as measured by bioassay, than the systemically invaded leaves. Fluorescent inclusions with the mild strain of PVX were present only in double infections in either inoculated or systemically invaded leaves. Thus, at 32°, PVX apparently is dependent upon the presence of PVY in order to reach a sufficient titre to form inclusions.

The dilution end-point assay used in this study to measure PVY infectivity appeared to be accurate. The use of uniform, small tobacco plants, uniform inoculation procedures, and uniform environmental conditions was extremely important in obtaining consistent results with this method.

Duggar and Armstrong (1925) reported extensive inhibition of infectivity of TMV by leaf juice of D. stramonium. Köhler (1957), working with both PVX and PVY, and Blaszczak et al. (1959), working with PVX, also reported the presence of inhibitors in D. stramonium leaf juice and the removal of the inhibitors by dilution of the juice. Inhibition of PVY infectivity in this study by clarified D. tatula juice may have been associated with a similar inhibitor and the inhibitor was similarly removed by dilution.

Infectivity of PVY as measured by the method described in the present paper, did not increase in the presence of PVX. This is in agreement with local lesion assays of Rochow and

Ross (1955) and serological assays of Bercks (1955a) and Close (1964). Close (1964) observed slight reduction of PVY in doubly infected plants as compared to singly infected plants, at 28° and 31° C. Although this may be a relatively minor difference, in the present study there was indication of a similar response at 32° C.

SUMMARY

Cytopathology of PVX and PVY in singly infected (PVX or PVY alone) and doubly infected (PVX + PVY) tobacco leaves was studied by the use of acriding orange, a nucleic acid specific stain. Appearance, size, and distribution of red fluorescent, cytoplasmic inclusions in virus infected tissues was correlated with virus infectivity as determined by local lesion assay of PVX on G. globosa.

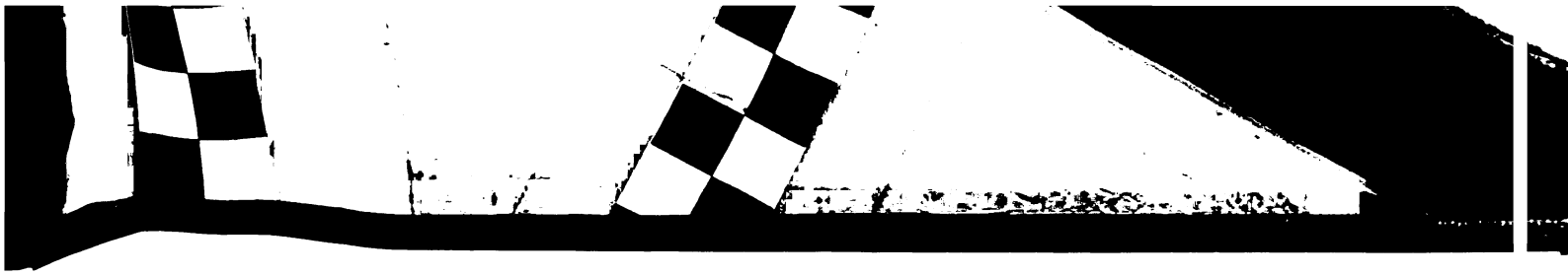
PVX infected tissue contained discrete, red cytoplasmic inclusions in many, but not all cells. A moderately severe strain (PVX-5) induced more inclusions in singly infected tissue than did a mild strain (PVX-8).

The few fluorescent inclusions present in PVY infected tissue fluoresced red with very low intensity. PVY concentration, as estimated by dilution end-point, using the synergistic response of tobacco plants doubly infected with PVX and PVY, was not influenced by the presence of PVX in the tissue.

In leaves inoculated with both viruses, cytoplasmic inclusions were present in more cells than in leaves inoculated with PVX alone and the titre of PVX was correspondingly higher in the doubly infected leaves. In doubly infected, systemically invaded leaves, large inclusions were present in practically every cell. Extremely large inclusions, 4-5

times the size of the nucleus, were present in parenchyma cells surrounding the veins. PVX titre in the doubly infected, systemically invaded leaves was up to 15 times as high as in comparable singly infected leaves.

Fluorescence was greatest in PVX infected tissue at 20° and 24° C. At 28°, fluorescence in PVX singly infected tissue was poor and at 32°, PVX alone failed to induce fluorescent inclusions. Doubly infected tissue at 28°, contained inclusions in most cells and at 32°, inclusions were prevalent in cells near the veins. Although infectivity of PVX in both singly and doubly infected leaves, was highest at 20° and 24°, the relative increase of PVX concentration in doubly infected leaves over that in singly infected leaves, was greatest at 28° and 32°.



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