CYTOPATHOLOGY OF POTATO VIRUS X IN COTYLEDONS OF DATURA TATULA L.

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY
Arvind S. Summanwar

1964

This is to certify that the

thesis entitled

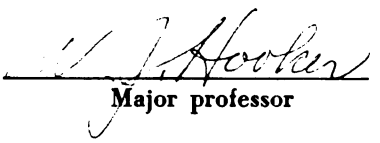
CYTOPATHOLOGY OF POTATO VIRUS X IN COTYLEDONS OF DATURA TATULA L.

presented by

Arvind S. Sunnanwar

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Plant Pathology



Major professor

Date August 6, 1964

O-169

CYTOPATHOLOGY OF POTATO VIRUS X IN COTYLEDONS OF *DATURA TATULA* L.

By

Arvind S. Summanwar

AN ABSTRACT OF A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1964

ABSTRACT

CYTOPATHOLOGY OF POTATO VIRUS X IN COTYLEDONS OF *DATURA TATULA* L.

by Arvind S. Summanwar

Cotyledons were mechanically inoculated with potato virus X (PVX) on the upper surface and later thoroughly washed with water. After inoculation, plants were grown under controlled conditions at 20° C in continuous fluorescent light of 800-900 FC. Slices of upper epidermal tissue from inoculated cotyledons and from controls similarly rubbed with water were stained with acridine orange and viewed under ultraviolet light. Ribonucleic acid in the nucleoli and in the cytoplasm fluoresced flame red. Deoxyribonucleic acid in the nuclei fluoresced yellowish green. During early virus synthesis (1-3 days after inoculation), as determined by local lesion assay on *Gomphrena globosa* L., flame red fluorescence was diffused in the cytoplasm. With increased virus titre (4-5 days), the red mass became larger and was usually situated near the nucleus. As virus infectivity decreased (9-13 days), the fluorescing material became more dense, reduced in size and often situated near the nucleus. The mass of red staining material in the cytoplasm and the more lightly staining substance in the nucleolus were consistently removed by RNAase treatment. In cross sections, palisade cells contained a similar red mass near the nucleus 3 days after inoculation. This inclusion became larger

on the fifth day when the virus titre reached its peak. More fluorescing material seemed to be present in the palisade cells than in the epidermal cells.

Lability of PVX to different concentrations of RNAase was determined after 2 and 24 hour incubation periods by bio-assay.

CYTOPATHOLOGY OF POTATO VIRUS X IN COTYLEDONS OF *DATURA TATULA* L.

By

Arvind S. Summanwar

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1964

ACKNOWLEDGMENT

I am greatly indebted to Dr. W. J. Hooker for his valuable guidance, help and encouragement during the course of this investigation and in the preparation of the manuscript.

Thanks are also due to Dr. W. B. Drew, Dr. D. J. deZeeuw, Dr. H. H. Murakishi, Dr. J. L. Lockwood, and Dr. N. R. Thompson for their interest and assistance. I also wish to extend my thanks to Dr. E. S. Beneke and Dr. J. H. Hart for their critical evaluation of the manuscript and to Mr. P. Coleman for the preparation of black and white photographs. Co-operation extended by the colleagues in the laboratory is also highly appreciated.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure | Page

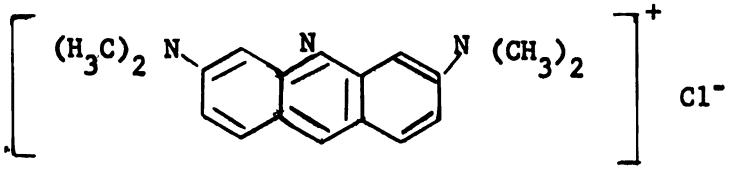
INTRODUCTION . - Relatively little is known concerning the cytopathology of potato virus X (PVX) in infected plant cells during the period of virus multiplication. Intracellular inclusions in PVX infected leaves have been reported by earlier workers. Specific differentiation of nucleic acids and the changes in nucleic acid distribution at periodic intervals in infected cells have not been previously studied. Since additional information concerning the cytology of PVX infected host cells was desired, virus multiplication in inoculated Datura tatula cotyledons was determined periodically by bio-assay of virus infectivity in a local lesion host, Gomphrena globosa L. and by ultraviolet microscopy with a nucleic acid specific stain, acridine orange.

REVIEW OF LITERATURE . - Smith (1924) using Heidenhain's iron-haematoxylin and Flemming's triple stain observed intracellular inclusions in potato leaves showing mosaic symptoms. Salaman (1938) reported intracellular inclusions in infected tobacco and in Datura stramonium leaves with all PVX strains tested. Tissues, momentarily immersed in absolute alcohol, were fixed in formalin chrom-acetic under reduced pressure and stained either with gentian violet, methylene blue, or safranin. Virus inclusions in the epidermal and palisade cells were larger than the nucleus and those in the palisade cells of Datura were usually elongated. Bawden and Sheffield (1944), in their cytological studies of Salaman's strains of virus X, observed that intracellular inclusions differed in size with different hosts. All strains of PVX produced elliptical inclusions only slightly larger in size than the nucleus in tobacco. In potato (President and Majestic), inclusions were larger than the nucleus and occupied as much as half the volume of the cell.

Kikumoto and Matsui (1961), by means of electron microscopy, observed in PVX infected leaves of D. stramonium intracellular masses composed of fibrous and flexuous particles believed to be PVX. They frequently found more fibrous masses of PVX in the cells of the palisade and spongy mesophyll than in the epidermal cells. Fibrous masses of PVX particles gradually increased in size with advance of infection.

Bukatsch and Haitinger (1940) and Strugger (1940) independently introduced acridine orange (AO) in fluorescence microscopy. AO is,

2, 8 bis-dimethylamino acridine (Gurr, 1960), an unusual compound of polychromatic fluorochroming properties, the structure being



AO staining is important in cytochemical studies because of its differential affinity for nucleic acids permitting simultaneous differentiation of ribonucleic acid (RNA) and deoxyribonucleic acids (DNA). Armstrong (1956) and von Bertalanffy and Bickis (1956) independently demonstrated usefulness of AO as a fluorescent stain for differentiation of nucleic acids in animal tissues.

von Bertalanffy and Bickis (1956) studied the influence of hydrogen-ion concentration on AO fluorescence of rat liver tissue, over the range from pH 2.6 to 11.2. Fluorescence of nuclei was more bluish-green in acid, and yellowish-green in basic AO. Red aggregates of RNA in the cytopolasm appeared at all pH values, except at the highly basic range, pH 11.2. Color of the stained sections was darker (brownish red) in acid, and brighter (more orange) in basic pH levels. They considered optimum conditions of staining for fluorescence studies to be pH from 6.0 to 6.5 and AO concentration 1:10,000 for 15 minutes. A short rinse of 0.5 M (or less) $CaCl_2$ improved differentiation of RNA and DNA. Red aggregates of RNA in the cytoplasm were removed in both fresh frozen and frozen Carnoy-fixed sections by ribonuclease treatment

prior to staining with AO. They considered the advantages of AO fluorescence technique over the histochemical methods as (1) simplicity, (2) high level of sensitivity as compared to other cytochemical methods, (3) simultaneous differentiation of RNA and DNA, and (4) fluorochromed specimens are readily stained by other methods after first being studied with the fluorescence microscope.

Armstrong (1956) also studied the influence of pH range and optimal AO concentrations for staining animal tissues. Intense red fluorescence was obtained at pH 6.0 and longer staining time was required for the more dilute (1:20,000 or 1:1000,000) solutions of AO for differential staining. Armstrong and Niven (1957), with ultraviolet (UV) microscopy using a darkfield condenser and both an exciter filter and barrier filter observed characteristic fluorescence of nucleic acids. They suggested that cytochemical staining techniques be applied to studies of nucleic acid metabolism of host-parasite systems at the cellular level because of the specific interaction of amino acridines with cell contents.

Schümmelfeder et al. (1957) reported the importance of concentration of the dye in the development of fluorescence and showed that the color reaction shifts from green to yellow to orange to red as more dye is attached. They postulated that the highly polymerized conditions of the DNA molecule prevented intracellular DNA structures from developing a red color, even under conditions of favorable pH. They also noted, that DNA structures tended to form reddish-flourescing complexes after acid hydrolysis.

Schümmelfeder et al. (1957) reported that when stained tissues with AO fluorochrome were viewed under ultraviolet light, RNA complexes fluoresced flame red in color and that DNA complexes fluoresced yellowish green. The differences in fluorescence (Schümmelfeder, 1958) were attributed to the fact that RNA and DNA have different degrees of polymerization. RNA forms polymers of low molecular weight in contrast to DNA which is highly polymerized. Depolymerized DNA in 1 N HCl when stained with AO fluoresced similar to that of RNA. Schümmelfeder et al. (1958) confirmed the specificity of AO for nucleic acid by comparative staining with gallocyanine-chromalum which has a high specificity for nucleic acids.

Anderson et al. (1959) in a review, discussed the application of AO fluorescence techniques in plant, animal, and bacterial cytology. They observed that AO technique offers a particular advantage in the study of viruses or bacteriophages containing double stranded DNA. These forms of DNA are resistant to deoxyribonculease (DNAase) prior to fixation, while the normal cellular DNA is not resistant. Viral DNA is susceptible to DNAase after prior exposure to pepsin. In contrast, viral RNA cannot be distinguished enzymatically from cytoplasmic host cell RNA.

Mayor and Diwan (1961), in their studies of AO staining of two purified RNA viruses : poliovirus and TMV, believed that since alcoholic fixation is necessary, AO staining is not a surface phenomenon. They further observed that alcoholic fixation denatured the surface of the virus particles in such a way to make it completely penetrable to the fluorochrome and also to expose sufficient reactive sites within

the virus particles to enable the development of brilliant red RNA staining. In their studies, concentrations of methanol greater than 70 percent were necessary for complete poliovirus fixation and penetration of AO for brilliant red fluorescence. They concluded that no staining took place below 70 percent methanol because denaturation of protein coat was not sufficiently extensive to permit penetration of the fluorochrome. The end point of methanol for TMV fixation was slightly lower than poliovirus but the general effect was similar. Mayor and Hill (1961) employed AO staining for a single-stranded DNA bacteriophage, which fluoresced flame red under ultraviolet light. Substances fluorescing flame red were removed when smears were treated with DNAase while RNAase had no effect. This staining property was believed to serve as a simple and direct means for staining single-stranded DNA.

There are different views concerning the differential flouresence mechanism of cellular nucleic acids. DeBruyn et al. (1953) in their in vivo and in vitro studies of diaminoacridines for nucleoprotein found that cell nuclei can be stained if killed by fixation by some means. The basic dyes failed to stain unfixed cell nuclei. They explained that killing of the tissue displaced the protein moiety from the nucleoprotein, resulting in binding between a basic dye and nucleic acid by way of the free phosphatic acid groups. In a spectrophotometric study of the interaction of nucleic acids with several aminoacridines, Morthland, et al. (1954) found a significant shift of the absorption peak of the dyes when DNA or RNA was added. Their findings suggested the formation of a complex bond consisting partly

of an electrostatic attraction between positively charged ions of the dye and negatively charged components of the nucleic acid molecule. The same authors noted that DNAase substantially impaired the formation of an acid-dye complex.

Joshi and Korgaonkar (1959) observed fluorescence developed by tissue culture cells stained with AO prior to, and after, exposure to different dosages of X-rays. These investigators considered that the increased uptake of dye after irradiation could be caused by some intramolecular disorientation of DNA, involving internucleotide bonds, thus exposing additional sites for subsequent binding of AO.

Mayor and Diwan (1961) stressed the importance of the steric arrangement of DNA and RNA molecules and suggested that RNA should have more exposed sites than DNA for the attachment of AO molecules, thus favoring the development of red fluorescence. Mayor and Hill (1961) concluded that under favorable conditions, AO nucleic acid was a matter of steric fit, and that the color reactions developed depended on concentration of attached dye, and not on chemical differences between nucleic acids.

Recently AO staining of tobacco mosaic virus in infected hair cells of tomato has been reported by Hirai and Wildman (1963). Cytochemical identification of RNA and DNA was by fixing the cells in Carnoy's solution and staining in pH 7.0 phosphate buffer containing 0.1 percent AO for 10-15 minutes. Brick red fluorescence indicated the presence of RNA, and DNA fluoresced yellow. Apparently, the differentiation of RNA and DNA was not complete as they considered pyronin-methyl green superior as a DNA, RNA stain.

Hooker and Summanwar (1964) used AO stain techniques for other plant virus infections namely potato virus X, tobacco necrosis virus, and tobacco etch virus. Differentiation of RNA and DNA was obtained with a method modified from von Bertalanffy and Bickis (1956).

MATERIALS AND METHODS . - A non-necrotic variant of potato virus X (PVX), X 5 (Timian et al., 1955) was cultured in Nicctiana glutinosa L. until virus infectivity titre was high. Clarified sap was prepared by grinding systemically infected leaves in a mortar and pestle, filtering the juice through a double layer of cheese-cloth, and centrifuging at low speed (1000 g. for 10 minutes). The supernatant was heated in a water-bath at 45°C for 10 minutes, cooled quickly, again centrifuged at low speed, diluted 1:5 with distilled water and frozen in small vials each containing 1.0 ml.

Cotyledons of Datura tatula were selected for cytopathological examination because the virus reaches a high titre in the tissues, cotyledons are thick, free from hairs, and either epidermal or transverse sections could be readily obtained.

Vigorous seedling plants of D. tatula grown in UC soil mixture C (Baker, 1957) were selected for uniformity when one week old. Cotyledons were dusted with 400 mesh carborundum, supported with a paper towel, and inoculated on the upper surface with clarified PVX inoculum using a smooth flat surfaced glass spatula. Approximately equal volumes of virus suspension were used on each cotyledon and after inoculation, cotyledons were thoroughly washed with water. Control cotyledons were similarly rubbed with water. Plants were grown under controlled conditions at 20°C in continuous fluorescent light of 800-900 foot candles.

Infectivity titres in inoculated cotyledons of D. tatula were determined by local lesion assay on the uppermost pair of fully

expanded leaves of Gomphrena globosa plants which had been selected for uniformity of plant type and maturity. Each assay involved 6 inoculated cotyledons from randomly selected plants of D. tatula. One cotyledon from each plant was excised, triturated on a glass slide, diluted to 1:2 (w/v, equal parts by weight of leaf tissue and by volume of distilled water), and inoculated to half leaves of G. globosa plants using uniform inoculating pressure. The remaining half leaf of G. globosa was rubbed with standard inoculum diluted 1:10, which produced from 70 to 110 local lesions per half leaf of G. globosa. Local lesions were counted on the sixth day.

Fluorescence microscopy. - Cotyledons from inoculated and non-inoculated control plants similarly rubbed with water were examined on 0-, 1-, 2-, 3-, 4-, 5-, 7-, 9-, and 13-days after inoculation. To obtain uniformity in all experiments, cotyledons were inoculated in the morning around 10:00 a.m. and samples were taken at the same time on each observation. Epidermal slices from the upper surface were made with a razor after aqueous infiltration (removal of intracellular air) of cotyledon tissue by an aspirator. Epidermal tissues were stained with acridine orange (AO) by the following schedule (Hooker and Summanwar, 1964): fixation in 50 percent ethyl alcohol, 1 hr.; followed unless otherwise indicated by 10 minutes changes in distilled water; AO (lot 14831, George T. Gurr, Ltd., London) 0.1 percent stock solution in distilled water diluted to 0.01 percent with pH 6.0 M/15 phosphate buffer (M/15 $Na_2HPO_4.7H_2O$ and M/15 KH_2PO_4); pH 6.0 phosphate buffer; short distilled water rinse; $CaCl_2$, 0.1 M of pH 7.3; short

distilled water rinse; 2 changes of phosphate buffer; mount in buffer.

Cross sections of cotyledons from inoculated as well as from non-inoculated control plants were treated in a similar manner for certain comparisons.

Ultraviolet light was supplied by a General Electric, AH-6 high pressure, water cooled, mercury vapor lamp. A blue filter (Corning #5113, 4.05 mm. thick) transmitted ultraviolet and violet light between 350 and 480 mμ with a peak between 390 and 420 mμ. A yellow barrier filter (Kodak Wratten K2 #8) consisting of 2 layers of gelatin was placed between the microscope (Microstar, Spencer, American Optical Company) objective and the prism. Photographs were made using Kodak Ektachrome-X daylight film. Color separation of red and green fluorescing substances respectively in black and white negatives (Eastman Panatomic X film) was accomplished from color slides by a red (25 A Wratten) and a green (58 B Wratten) filters. The final black and white prints were made on Ansco Jet paper and color prints on type R paper.

Nucleic acid identification by enzymatic treatment. - Deoxyribonuclease (DNAase) obtained from the Worthington Biochemical Corporation, Freehold, New Jersey, was used for specific removal of deoxyribonucleic acid (DNA) before AO staining. Tissue sections were fixed in 50 percent ethyl alcohol for 1 hour, rinsed twice in distilled water and treated at room temperature for 4 hours in DNAase (50 μg/ml in distilled water) of pH 6.4. For controls, diseased tissues were treated with

water. After 4 hours, tissues were rinsed in water, and similarly stained with AO.

Ribonuclease (RNAase) purchased from the same source, was similarly used for the specific removal of ribonucleic acid (RNA). Tissue sections were fixed in 50 percent ethyl alcohol for 1 hour, rinsed twice in distilled water and treated at 36°C in a constant temperature water-bath for 4 hours in RNAase (50 µg/ml in 0.2 M phosphate buffer of pH 6.4). For controls, diseased tissues were treated with 0.2 M phosphate buffer of pH 6.4. After 4 hours, tissues were rinsed in water and similarly stained with AO.

EXPERIMENTAL RESULTS . - Bio-assay of PVX infectivity in inoculated cotyledons. - D. tatula plants were inoculated as described earlier. At given intervals, 6 inoculated cotyledons from 6 randomly selected plants were ground and diluted 1:2 and assayed on 3 half leaves (Test 1) and on 8 half leaves (Test 2) of G. globosa plants.

Virus infectivity titres in inoculated cotyledons on the 0-, and 1-day were not sufficient to produce local lesions in G. globosa (Fig. 1). After this latent period of at least 24 hours, virus titres in the inoculated cotyledons increased rapidly from the 2- to 4-day periods. Virus titres in the inoculated cotyledons increased to the maximum 5 days after inoculation, and gradually decreased through the 13-day observation. Similar results were obtained in 2 previous trials, data of which are not presented. In other trials, involving periods of observation beyond 13 days, virus titres in inoculated cotyledons dropped as tissue became senescent.

Comparison of fixatives . - In preliminary trials, 1 hour fixation of tissues in 50 percent ethyl alcohol was satisfactory for AO studies. Less fixation time resulted in incomplete staining and incomplete differentiation of cell contents. When tissue was fixed in 95 percent ethyl alcohol, fluorescence was similar to that obtained with 50 percent ethyl alcohol. Fixation with 95 percent caused severe plasmolysis of tissues. In modified Carnoy's fixative (50 per cent ethyl alcohol--3 parts, and 1 percent acetic acid--1 part),

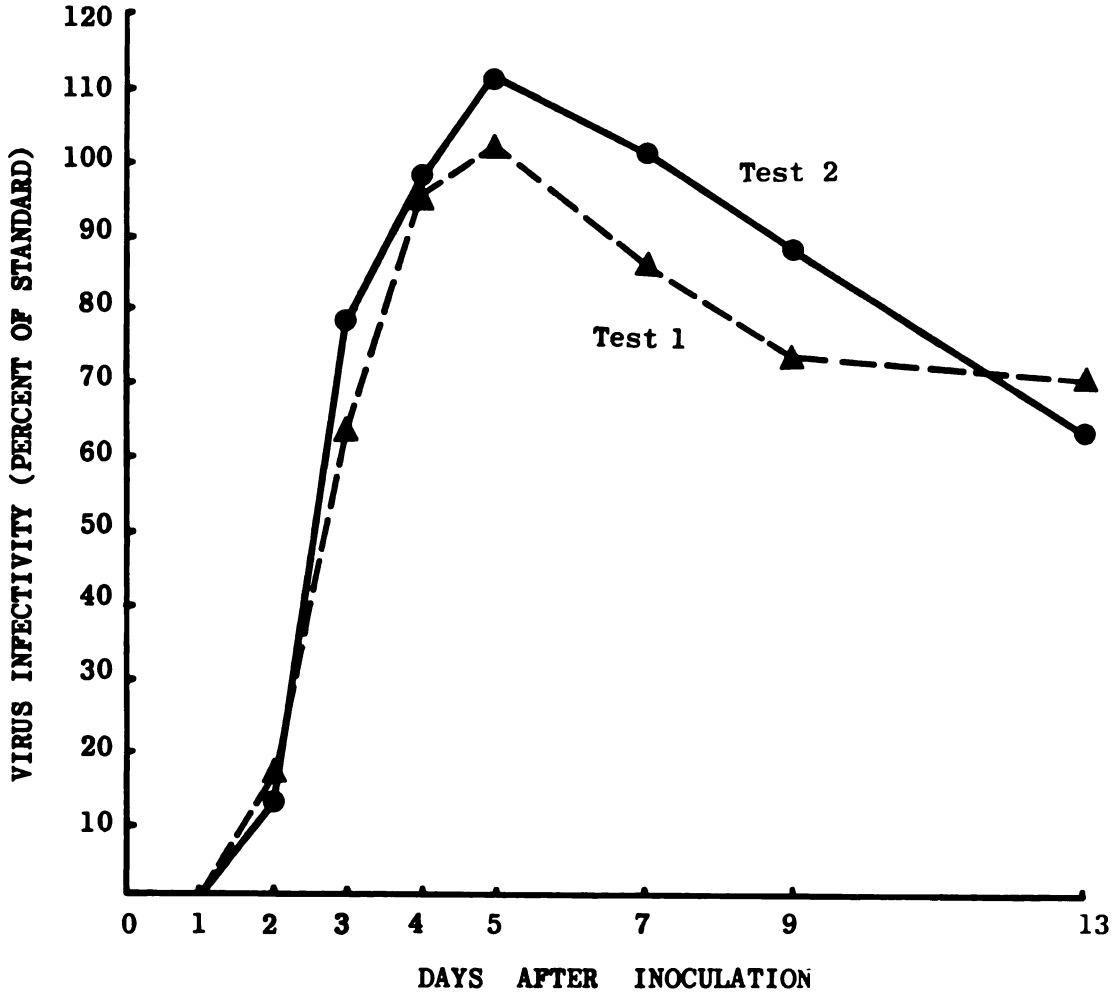


Fig. 1.--Infectivity of PVX in inoculated cotyledons of Datura tatula as determined by bio-assay in Gomphrena globosa.

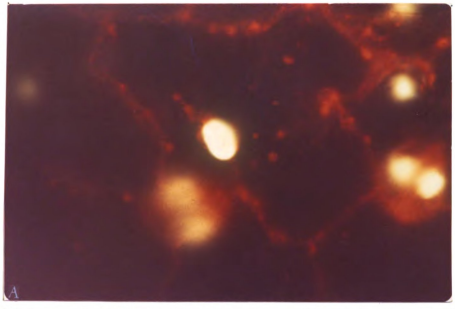
plasmolysis was even greater. For these reasons fixation in 50 per cent ethyl alcohol seemed advisable.

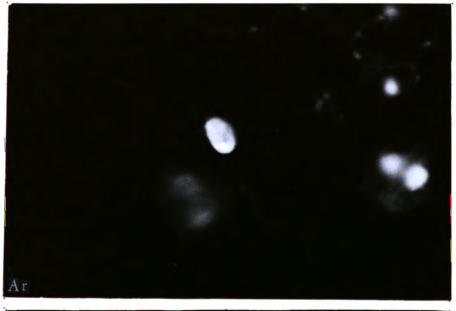
Intracellular acridine orange fluorescence in inoculated cotyledons . - Epidermal slices from the upper surface of inoculated and non-inoculated cotyledons were made during the active period of virus synthesis (3- to 5-days) and stained with AO as described. DNA of the nuclei, exclusive of nucleoli, fluoresced yellowish green in both infected and in healthy tissues. The nucleoli of healthy cells fluoresced red to a much lesser extent (Fig. 2-A, Ar)[1] than did the more prominent nucleoli of virus infected tissue (Fig. 2-H, Hr and K,Kr). Parietal cytoplasm of healthy tissue fluoresced red to only a small extent (Fig. 2-A,Ar). Virus infected tissues were characterized by prominent, discrete red fluorescing masses which were situated usually near the nucleus and differed in size from cell to cell. At the 4-, and 5-day periods (Table 1), the majority of the cells contained inclusions, either approximately equal in size (Fig. 2-D,Dr) or larger than the nucleus (Fig. 2-E,Er).

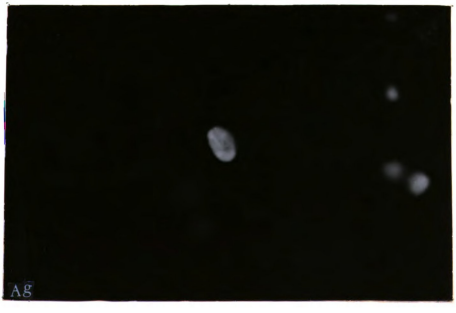
The identity of the discrete red fluorescing masses as RNA (presumably virus RNA) was determined by ribonuclease treatment of inoculated epidermis. In RNAase treated sections, the red staining substance of the cytoplasmic inclusion and in the nucleoli was removed (Fig. 2-F,Fr). Control sections from diseased tissue incubated with 0.2M PO_4 buffer of pH 6.4 without enzyme fluoresced similarly to tissues stained in the usual procedure (Fig. 2-E,Er). This indicates that the red fluorescent cytoplasmic inclusions were chiefly RNA

[1]Differences in nucleolar appearance were lost in reproduction but were distinct in the specimens and in the original colored slides.

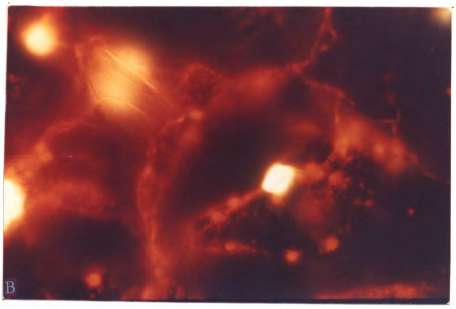
Fig. 2.--Photographs of typical cells in color, and in color separation (black and white) enlargement 750X, with red fluorescing material shown in photographs prepared using a red (r) filter and green fluorescing material shown similarly using a green (g) filter. Upper epidermis of D. tatula cotyledons : A), Ar), and Ag), from non-inoculated plants.

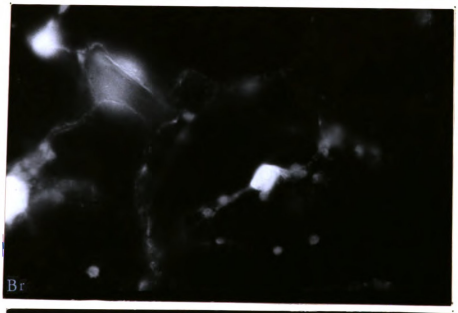

A


Ar


Ag

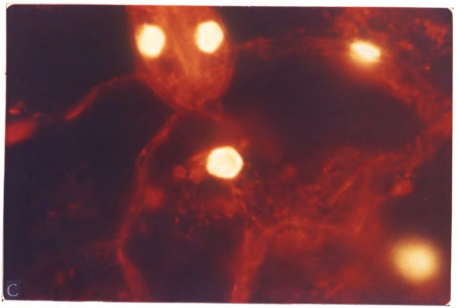
B-H, upper epidermis from PVX inoculated cotyledons : B), Br), and Bg), 1-day after inoculation, apparently virus infected cell with a thick red strand in the cytoplasm and noticeably increased diffused red fluorescence in the parietal cytoplasm;

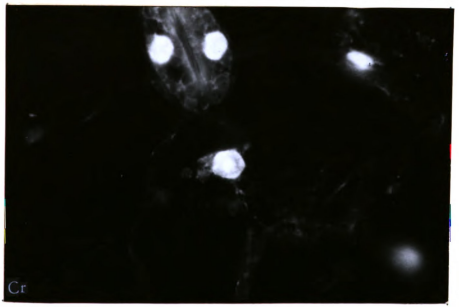

B


Br


Bg

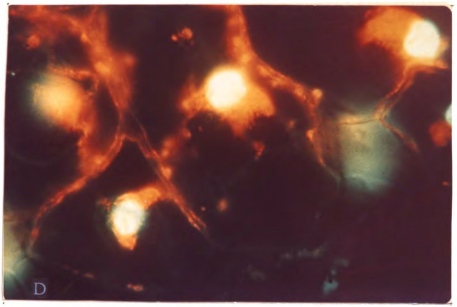
C), Cr), and Cg), 2-days, cell with discrete red fluorescing cytoplasmic inclusion, smaller in size than the nucleus;

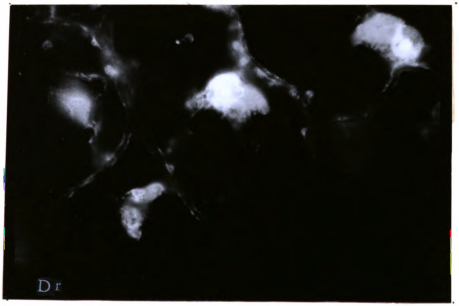

C


Cr


Cg

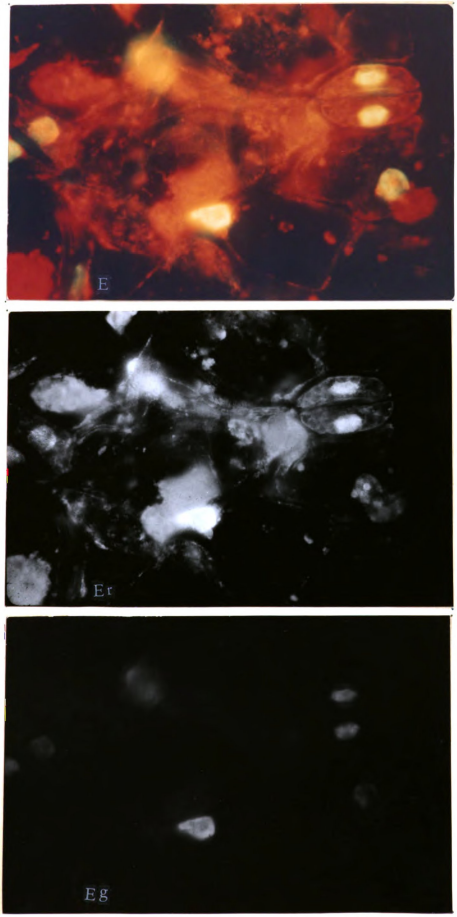
D), Dr), and Dg), 3-days, cell with discrete red fluorescing cytoplasmic inclusion, approximately equal in size to the nucleus;


D

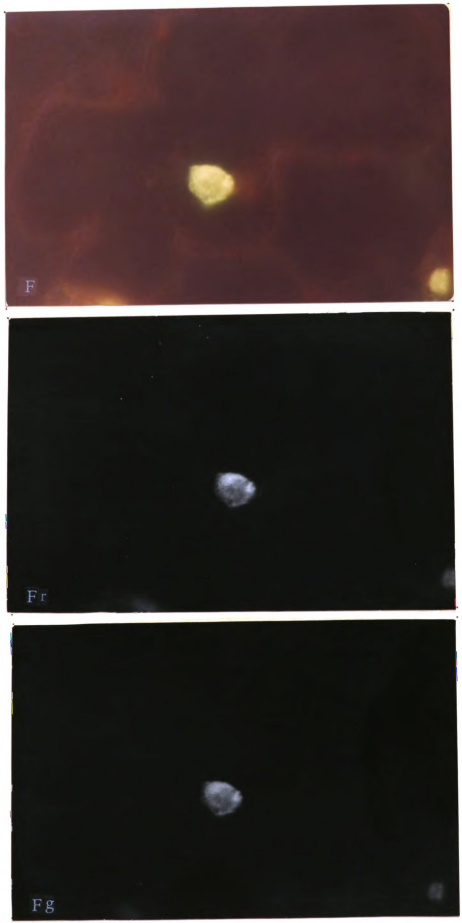

Dr


Dg

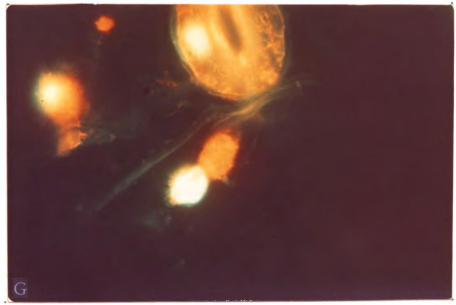
E), Er), and Eg), 5-days, cell with discrete red fluorescing cytoplasmic inclusion, larger than the nucleus;

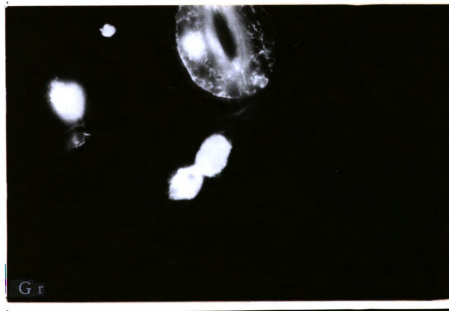

E
Er
Eg

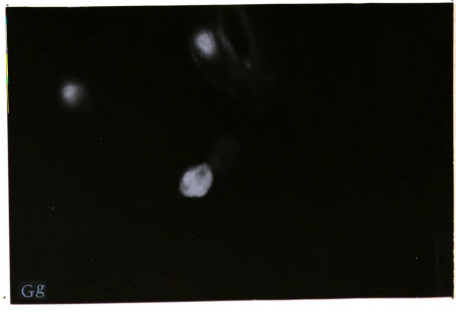
F), Fr), and Fg), 5-days, inoculated as E but treated with RNAase;


F
Fr
Fg

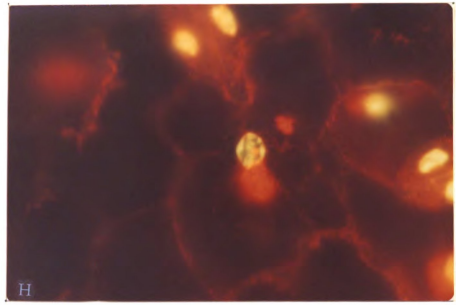
G), Gr), and Gg), 11-days, cell with discrete red fluorescing cytoplasmic inclusion, approximately equal in size to the nucleus;

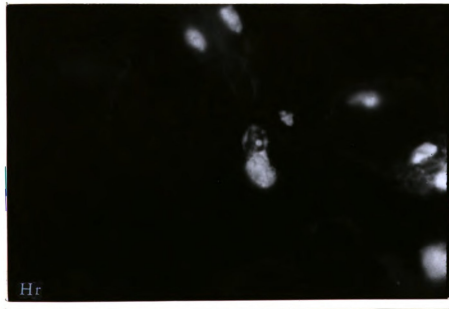

G


Gr


Gg

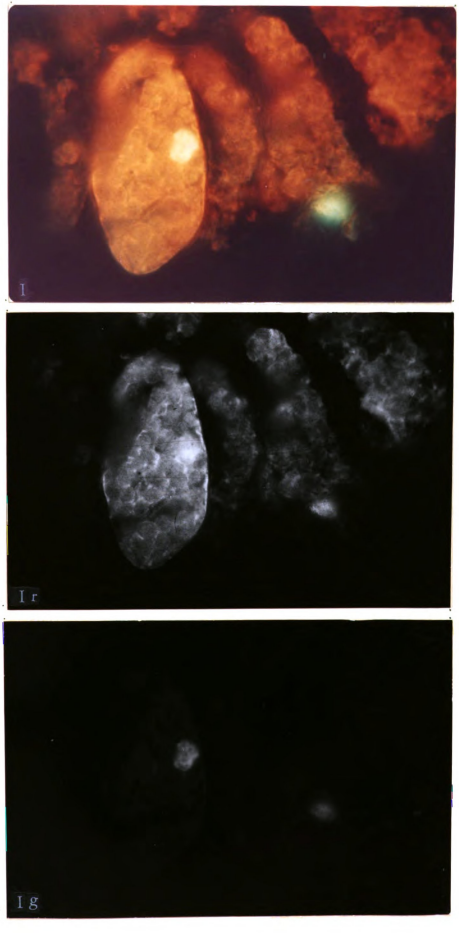
H), Hr), and Hg), 13-days, cell with discrete red fluorescing cytoplasmic inclusion, approximately equal in size to the nucleus. Note the prominent nucleolus;


H

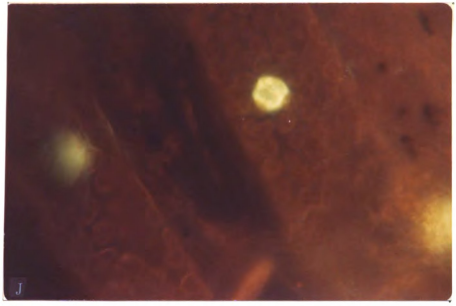

Hr

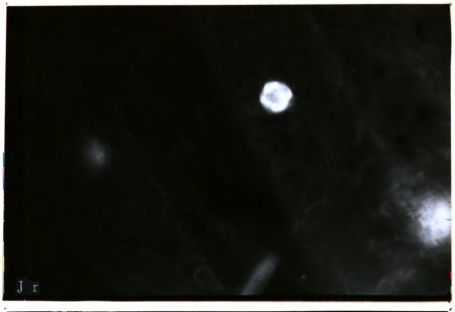

Hg

I-L, palisade cells in cotyledon cross sections: I), Ir), and Ig), non-inoculated;


I
Ir
Ig

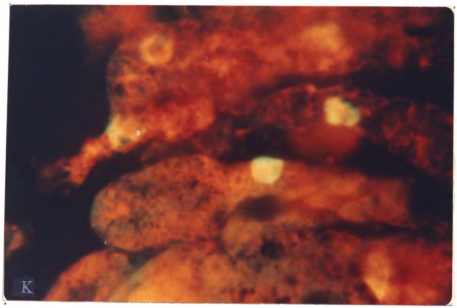
J), Jr), and Jg), non-inoculated as I but treated with RNAase;

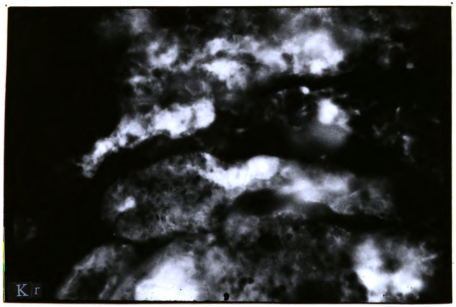

J

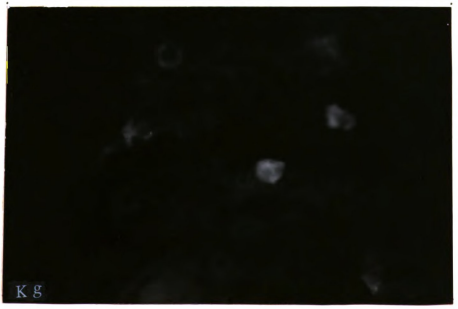

J r


J g

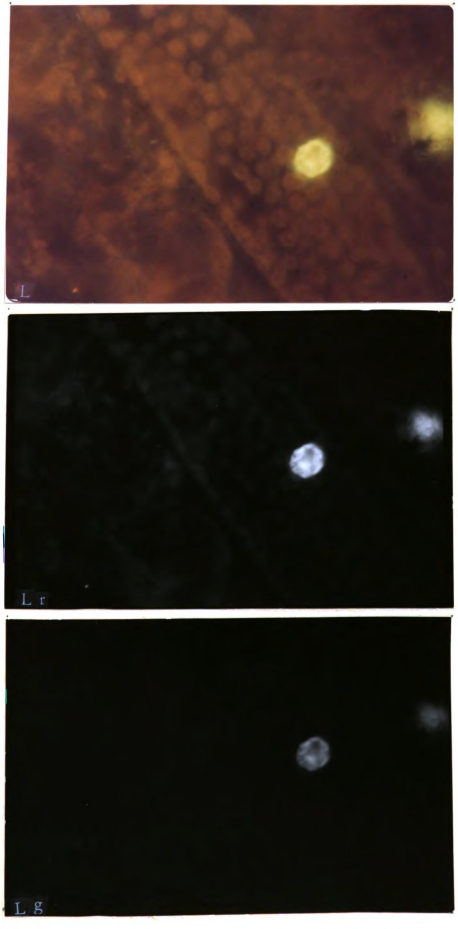
K), Kr), and Kg), inoculated 5-days previously with PVX. Notice the elongated red fluorescing cytoplasmic inclusions which contrast in color with the normal fluorescence of the chloroplasts;


K


Kr


Kg

L), Lr), and Lg), inoculated as K but treated with RNAase.


L
L r
L g

(presumably virus RNA) since similar red inclusions were not observed in stained healthy tissues at any time. Similarly the red fluorescing substance in the nucleoli of healthy non-inoculated control cotyledons was removed by enzyme treatment.

The identity of the yellowish green fluorescing material as DNA was determined by deoxyribonuclease treatment of inoculated and non-inoculated epidermis. Yellowish green fluorescing substances were consistently removed from the nucleus of inoculated and non-inoculated controls by the enzyme.

Intracellular red fluorescence in upper epidermal cells of inoculated D. tatula cotyledons was determined at intervals after inoculation. Over 200 AO stained epidermal cells were observed and classified at each interval in 2 different sets of experiments. Since results of both trials were in good agreement, combined data are presented (Table 1). AO stained epidermal cells from healthy cotyledons served as controls.

It is generally accepted that when a plant leaf is mechanically inoculated with virus inoculum, virus is not introduced equally into all cells, and therefore, the cells, initially, are not uniformly infected. Presumably virus or a virus precursor is later transferred from cell to cell through plasmodesmata.

When AO stained epidermal cells of inoculated D. tatula cotyledons were examined under fluorescent microscopy at the 0-day period (i.e. 1-2 hours after inoculation) the nuclei exclusive of the nucleoli, fluoresced yellowish green in both infected and healthy tissues. In some of the epidermal cells of inoculated cotyledons more diffused red

TABLE 1.--Intracellular acridine orange fluorescence in upper epidermis of PVX inoculated *D. tatula* cotyledons

Days after inoculation	Condition of cells[1]	Cells without discrete red inclusion		Cells with discrete red fluorescing cytoplasmic inclusion						Total number of cells observed
				Inclusion smaller than the nucleus[2]		Inclusion approx. equal to the nucleus[3]		Inclusion larger than the nucleus[4]		
		No.	%	No.	%	No.	%	No.	%	
0	a	91	34							271
	b	180	66							
1	a	76	33							231
	b	147	64							
				8	3					
2	a	5	1							260
	b	48	19							
				153	59	50	19	4	2	
3	a	3	1							217
	b	13	6							
				61	28	90	42	50	23	
4	b	4	2							
				22	9	79	32	140	57	245
5				17	6	91	32	180	62	288
9				29	11	146	53	98	36	273
13				67	25	131	49	68	26	266

[1]a: Apparently non-infected cells similar to healthy cells of non-inoculated epidermis as in Fig. 2-A,Ar.

b: Apparently virus infected cells with noticeably more diffuse red fluorescence in the parietal cytoplasm than in the non-inoculated controls as in Fig. 2-B,Br.

[2]Cells with discrete red fluorescing cytoplasmic inclusion, smaller than the nucleus as in Fig 2-C,Cr.

[3]Cells with discrete red fluorescing cytoplasmic inclusion, approximately equal in size to the nucleus as in Fig. 2-D,Dr and H,Hr.

[4]Cells with discrete red fluorescing cytoplasmic inclusion, larger than the nucleus as in Fig. 2-E,Er.

fluorescence was present than in healthy stained tissues. These cells on the basis of intense diffused red fluorescence were possibly infected cells, i.e. the cells into which virus was introduced when the cotyledons were mechanically inoculated. At 0-day period, out of 271 cells observed, 66 percent exhibited increased red fluorescence in the parietal cytoplasm and 34 percent of the cells fluoresced similarly to those of cells from healthy tissues (Table 1).

In the early period of virus synthesis before infectivity could be demonstrated (1-day after inoculation), nuclei of certain cells, presumably those virus infected, were characterized by prominent and apparently enlarged nucleoli which fluoresced red and seemed to contain more RNA than did the nucleoli of healthy cells which fluoresced to only a small extent. In some cells, red fluorescing substance was diffused throughout the cell, some cells fluoresced similar to healthy stained tissues, and others were characterized by a thick red strand in the cytoplasm (Fig. 2-B, Br). At the 1-day period, cells had either increased amounts of diffuse red fluorescing material and/or a thick red strand in the cytoplasm (Table 1).

At the 2-day period, when virus infectivity titres were just beginning rapid increase, cytoplasmic RNA was abundant at the periphery of the cell and was commencing to aggregate near the nucleus (Fig. 2-C, Cr). At this period almost all cells contained some red fluorescing material (Table 1). The red inclusion next to the nucleus present in 80 percent of the cells differed in size from cell to cell, although the majority of the cells contained discrete red inclusions smaller than the nucleus (Fig. 2-C, Cr; Table 1).

At the 3-day period, cytoplasmic RNA was massed near the nucleus and at the edge of the cell there was a reduction in red fluorescence (Fig. 2-D,Dr). Most of the cells appeared to be infected but the relative size of the inclusion (Table 1) differed from cell to cell. Approximately 65 percent of the cells had a discrete red mass or inclusion approximately equal in size to (Fig. 2-D,Dr) or larger than the nucleus (Table 1).

When virus infectivity was highest, 4-5 days after inoculation, infected tissues were characterized by prominent masses which fluoresced a brilliant red color and were usually situated near the nucleus. The size of the red inclusion in the cytoplasm differed from cell to cell but the majority of the cells either had a discrete red inclusion approximately equal in size to or larger than the nucleus. No cells without red inclusions were present at the 5-day observation.

As the inoculated cotyledons approached maturity, red fluorescing material became increasingly dense, considerably reduced in size (Table 1), and usually situated near the nucleus. At the 9-day period, higher percentages of cells had red inclusions approximately equal to the size of the nucleus than at the 5-day period (Table 1). At the 13-day period, a higher percentage of cells had red inclusions approximately equal in size to the nucleus (Fig. 2-H,Hr) than at the 9-day period (Table 1).

Red fluorescing substances photographed in the color separation series as white in black and white photographs when a red filter (r) was used. When a green filter (g) was used, green fluorescent substances were shown. The nucleus appeared green in the color photographs

and in the slides at the time of photographing. Red fluorescence in the nucleus was apparent only in the nucleolus. Comparisons of photographs in the color separation series, r and g, suggest that at least some RNA was present outside the nucleoli in the nuclei of all cells photographed.

Cotyledons 16-20 days after inoculation turned yellow and dropped. Non-inoculated cotyledons remained attached to the plants for another 8-10 days before turning yellow and dropping off because of senescence.

Cumulative data from Table 1 are graphed (Fig. 3). A very small percentage (3 percent) of infected cells contained virus inclusions at the 1-day period (Fig. 3). At the 2-day period, virus infected tissues were characterized by the presence of inclusions differing in size. Although almost 80 percent of the infected cells contained inclusions, 21 percent of them had inclusions approximately equal to or larger in size than the nucleus and 2 percent virus infected cells had inclusions larger in size than the nucleus.

At the 3-, and 4-day observations, inclusions although differing in size from cell to cell, appeared in almost all cells. Over half of the cells had inclusions approximately equal to or larger in size to the nucleus. At the 5-day period, all cells contained inclusions. Inclusions larger than the nucleus were present in approximately 62 percent of the cells.

At the 9-day period, although all the cells had virus inclusions, the inclusions were reduced in size and less than 40 percent of the cells had inclusions larger in size than the nucleus. The size of

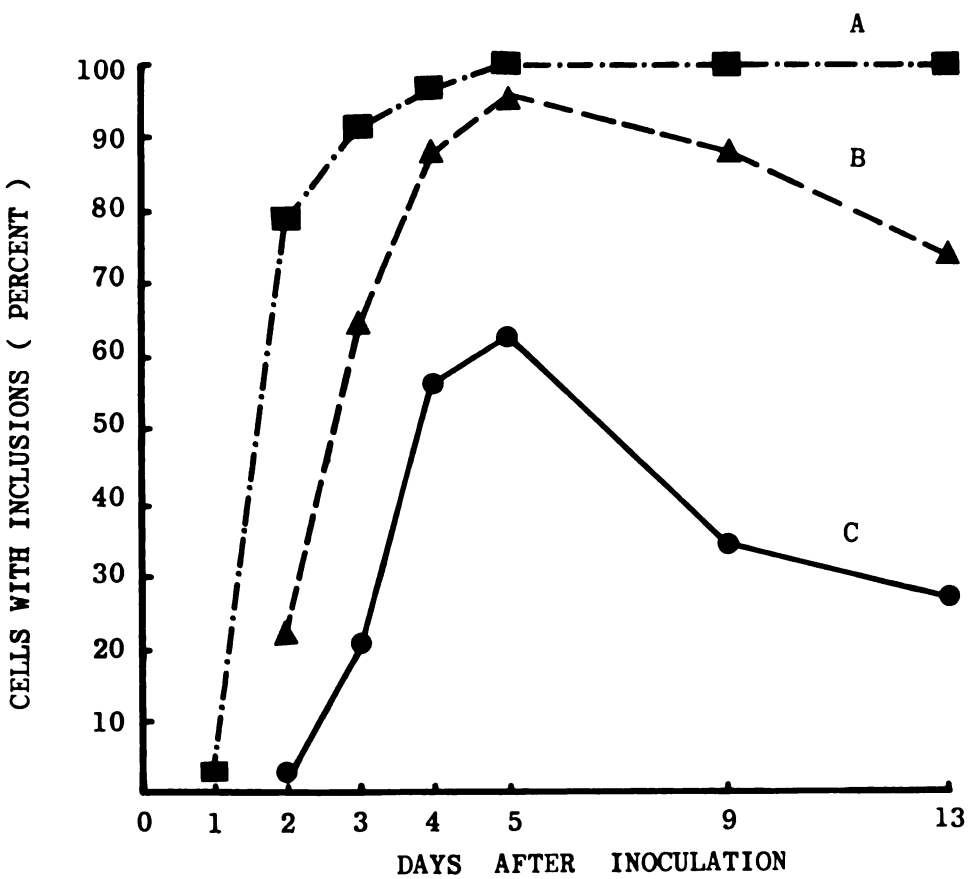


Fig. 3.--Presence and relative size of virus inclusions at intervals after inoculation: (A) inclusion present; (B) inclusion approximately equal in size to or larger than the nucleus; (C) inclusion larger than the nucleus.

virus inclusions in the infected cells was reduced further on the 13-day observation.

Fresh epidermal slices cut as described and fresh leaf cross sections cut with a special microtome from inoculated and non-inoculated cotyledons were compared on 2-, 3-, 5-, 9-, and 13-days after alcohol fixation and by the usual staining method.

At the 2-day period, red fluorescing material was abundant in the parietal cytoplasm of the epidermal cells and was beginning to aggregate near the nucleus. Although red cytoplasmic fluorescence in inoculated and non-inoculated palisade cells was apparently similar, nuclei of palisade cells of virus infected tissues contained nucleoli which were more prominent and apparently enlarged as compared to the nucleoli of palisade cells of non-inoculated tissues. At this stage, infection of mesophyll cells apparently lagged behind epidermal cells by approximately 1 day.

At the 3-day period, virus infected epidermal cells were characterized by a red mass near the nucleus and, at the edge of the cell there was little or no fluorescing material. In infected palisade cells, elongated red fluorescing cytoplasmic inclusions smaller in size than the nucleus, were formed next to the nucleus.

At the 5-day period, most virus infected epidermal cells contained inclusions either approximately equal in size to or larger than the nucleus. Palisade cells of infected tissues were characterized by elongated flame red fluorescing inclusions larger in size than the nucleus (Fig. 2-K,Kr). Non-inoculated tissue never contained discrete red inclusions (Fig. 2-I,Ir). Palisade cells containing

chlorophasts in either healthy or diseased tissue fluoresced orange. This orange fluorescence was reduced but not completely removed by RNAase treatment. Palisade cells of inoculated and non-inoculated cotyledons treated with RNAase fluoresced to approximately the same intensity (Fig. 2-L,Lr, and J,Jr). The color of fluorescence of virus inclusions and chloroplasts was distinct.

The red cytoplasmic inclusions at 9-, and 13-day periods seemed to fluoresce more intensely in infected palisade cells than infected epidermal cells. At these periods, in both the tissues, inclusions were considerably reduced in size and situated usually near the nucleus.

Elongate flame red fluorescing inclusions occupied relatively more volume in infected palisade cells than in infected epidermal cells. More intensity of the red fluorescent substance and larger size inclusions in infected palisade cells suggested that relatively more RNA was present in palisade cells than in the virus infected epidermal cells.

Lability of PVX to ribonuclease . - Lability of clarified PVX inoculum to different concentrations of RNAase after 2 and 24 hours incubation at 4°C was determined by infectivity assay in G. globosa (Table 2). Infectivity of PVX was progressively reduced as RNAse concentration was increased. Infectivity was further reduced by increasing the incubation period from 2 to 24 hours.

TABLE 2.--Lability of PVX to different concentrations of ribonclease as determined by infectivity assay in G. globosa

RNAase (µg/ml)	Infectivity[1] After	
	2 hours	24 hours
50.0	0.10	0.0
5.0	0.95	0.11
0.5	5.08	1.76
0.05	22.12	7.86
0.005	66.57	67.31
0.0005	79.02	83.39
0.00005	95.24	94.09

[1]Infectivity expressed as number of local lesions in the treatment compared to an untreated control and calculated as percent of standard.

DISCUSSION . - During the latent period following inoculation, although transfers from inoculated D. tatula cotyledons were not infectious to G. globosa, approximately 66 percent (0-day) of the epidermal cells of the inoculated cotyledons exhibited noticeably more diffuse red fluorescence than did cells of similarly healthy stained tissues. This remained relatively unchanged during the 1-day period and still infective virus could not be demonstrated. Possibly the presence of a thick red strand in some of the cells could be considered the next stage of virus synthesis but infectivity could not be associated with this stage. Bawden and Harrison (1955) and Harrison (1956) in their studies on the multiplication of tobacco necrosis virus in inoculated leaves of French bean plants concluded that virus was not detectable during the latent period because: (a) the first formed virus was in some way immature, able to invade and infect neighboring cells of the inoculated leaf, but too unstable to provide infective inoculum, (b) it may become too firmly attached to infection sites in neighboring cells to be released in leaf extracts. The increase in number of cells with red fluorescence observed at the 2-day periods (Table 1) probably involved both processes of PVX multiplication-(1) the multiplication of virus within individual cells, and (2) the spread of virus into other cells, together with virus multiplication in these cells. Evidence of the step (1) may have been observed on the 0-,and 1-day as a diffused red fluorescence in the cytoplasm. The red fluorescence of cells on the 0-day may be attributable to virus introduced into the cell. That there was little

change in cell cytology on the 0-, and 1-day may be significant in this interpretation. The first infective virus was obtained with considerable inclusion formation on the second day but active virus infectivity was not obtained until a day later, the 3-day period, when inclusions were present in practically all cells. Spread from cell to cell was not evident until the 2-day period, and when the number of apparently healthy cells began to drop. All epidermal cells were not visibly infected until the 5-day period.

Possibly the presence of inclusions of different sizes in infected cells at 3- to 4-day periods, may have been associated with different rates of virus multiplication within different cells. Cells with inclusions larger than the nucleus may have permitted more rapid multiplication than cells where inclusions were smaller. Inclusion size may also have been influenced by time of infection particularly after the 2-day period.

At the 5-day period, the infection involved 100 percent of the epidermal cells in inoculated tissue, as determined on the basis of inclusions in infected cells. Benda (1959) postulated that a large percentage of cells in the inoculated leaf were never infected. His statement was based on the maximum number of local lesions produced by U 2 strain of tobacco mosaic virus in Nicotiana sylvestris. Since the present studies are made at the cell level and necrosis is not a criteria of infection, it would appear that in this host virus combination essentially all the cells of the epidermis may be infected. Since fluorescence of nucleic acids between the non-inoculated and the inoculated tissues are easily differentiated, these data provide direct

cytological relationship of the host cells and the virus. Thus the data are at least as reliable as those obtained using local lesion criteria.

Salaman (1938), as well as Bawden and Sheffield (1944) reported that inclusions in virus X infected leaves of tobacco and Datura were larger than the nucleus. They studied the epidermal and palisade cells of systemically infected leaves, apparently of high virus titre. In the present studies, the size of the inclusions was positively associated with virus infectivity titres. The highest titre being obtained when the maximum number of inclusions were larger than the nucleus. Inclusions were more or less round in shape but differed in size from cell to cell.

Shalla (1959) made electron microscopic studies of epidermal and mesophyll cells of systemically infected tobacco and tomato leaves with tobacco mosaic virus (TMV). He observed the presence of rod-shaped TMV like particles in crystalline inclusion bodies and interpreted the particles as being TMV. The presence of inclusions in PVX infected epidermal or palisade cells were reported by Salaman (1938), and Bawden and Sheffield (1944). Later Kikumoto and Matsui (1961) using the electron microscope observed fibrous particles. Although circumstantial evidence has suggested that protoplasmic inclusions were infective virus, this work presents additional evidence that the development of cytoplasmic inclusions are of PVX-RNA and the appearance and the development of the inclusions are positively related to virus infectivity, as determined by bio-assay.

Reasons for believing that the cytoplasmic inclusions of PVX are actually virus are: (1) constant removal of discrete red cytoplasmic inclusions from the epidermal and palisade cells and red fluorescing

material from the nucleolus by RNAase treatment suggested that the red fluorescent substances were mainly RNA; (2) development of cytoplasmic inclusions was associated with increased virus infectivity; (3) no inclusions were observed in epidermal and palisade cells of non-inoculated tissues at any time; and (4) RNAase destroyed infectivity of clarified PVX and prevented red fluorescence of cytoplasmic inclusions.

Welkie and Pound (1958) studied the influence of temperature on the rate of passage of cucumber mosaic virus through the epidermis into the mesophyll of cowpea. Immediately after inoculation, leaves were exposed to different temperatures (16, 20, 24, and 28°C) and stripped off epidermis at hourly intervals and the number of local lesions counted on unit areas devoid of epidermis. They reported that migration of cucumber mosaic virus into mesophyll of cowpea was dependent on temperature of incubation and that under optimum temperatures for local lesion production (20°C), 5 hours were required for the first evidence of virus to pass through the epidermis.

Fry and Matthews (1963), studied the time required for migration of tobacco mosaic virus from epidermis into mesophyll of N. glutinosa. Detached leaves were inoculated on the under surface with TMV-RNA and epidermis was stripped off at hourly intervals. Penetration of first infective virus into mesophyll tissue was detected 8 hours after inoculation.

Dijkstra (1964) also studied the rate of passage of tobacco mosaic virus from the epidermal cells into mesophyll cells of N. glutinosa leaves after inoculation of lower leaf surface. She found that 10 to 10.5 hours were required after inoculation for the infectious entities to invade mesophyll cells.

On the basis of cytopathological differences between the palisade cells of diseased and healthy tissues, it is probable that PVX migrates from epidermis into mesophyll cells in the 2-day period but more intense red fluorescence and the appearance of small red fluorescing cytoplasmic inclusions in the diseased tissues appeared on the third day. This time for mesophyll penetration is much longer than that reported in the case of cucumber mosaic virus and tobacco mosaic virus. Differences could be attributed to the fact that other workers determined the migration on the basis of infectivity. Flourescence presupposes that a sufficient mass of material be present to provide a color and presumably this would be at a higher concentration than that required for infectivity. Furthermore, hosts and viruses studied are different and precise comparisons are not possible. Worley and Schneider (1963) studied the distribution of southern bean mosaic virus antigen in bean leaves by means of a flourescent antibody stain. They observed more intensity of the stain in the palisade cells of diseased tissues at the 2-, and 3-day periods.

Comparisons of photographs in the color separation series in black and white using a red and a green filters suggested that at least some RNA was present in the nucleus outside the nucleolus. Difference in intensity of the nucleus which photographed in shades white with either the red or the green filter, suggest that even though the nuclei appeared green to the eye, considerable red fluorescence was also present. It could well be interpreted that some RNA is present in the nuclei of both diseased and healthy tissues.

Sirlin (1962) stated that the nucleolus is apparently an important source of cell RNA. RNA is first formed in the nucleolus and then moves

out of the nucleolus into the nucleus. The definite functions of the nucleolar RNA are not known. Bald and Solberg (1961) have presented evidence obtained by phase contrast observation of living cells that virus RNA may be released from the nucleolus into the nucleus. Bald (1964) gave further cytological evidence, by the use of different stains, for release of plant virus RNA from the nucleolus into the nucleus. Johnson and Jones (1962) have reported release of a substance from the nucleolus of tobacco cells in tissue culture.

Hirai and Wildman (1963) reported the use of acridine orange in their cytological studies of infected tomato hair cells by tobacco mosaic virus. RNA was characterized by the presence of brick red fluorescence and yellow fluorescence indicated the presence of DNA. In this study with PVX the fluorescence of RNA was flame red and DNA was yellowish green. Differences may be attributable to different virus-host systems. It could also be that in the case of tobacco mosaic virus, the protein sheath over the RNA core was not as easily denatured and uptake of the dye was not as complete. It could be that with tobacco mosaic virus, the use of relatively high concentration of AO and lack of $CaCl_2$ differentiation in the staining procedure resulted in incomplete differentiation of nucleic acids.

Mayor and Diwan (1961), in their studies of AO staining of two purified RNA viruses: poliovirus and tobacco mosaic virus, found that alcoholic fixation is necessary and is an indication that staining was not a surface phenomenon. The present studies also indicated that alcoholic fixation (50 percent ethyl alcohol) was necessary for staining to occur; unfixed tissues did not absorb the stain. These results are in

essential agreement with those of Mayor and Diwan (1961) and could be interpreted in the same way that alcoholic fixation denatures the surface of the virus particle in such a way to make it completely penetrable to the fluorochrome and also to expose sufficient reactive sites within the virus particles to enable the development of brilliant red RNA staining. No staining developed in unfixed tissues possibly because denaturation of protein coat did not take place. It was also found in the present studies that tissues fixed in 95 percent ethyl alcohol were plasmolysed. The plasmolysis of the tissues was still greater in modified Carnoy's fixative, i.e. 50 percent ethyl alcohol--3 parts and 1 percent acetic acid--1 part. One hour alcoholic fixation was necessary since shorter fixation times resulted in incomplete staining and differentiation of the cell complexes.

SUMMARY . - The cytopathology of PVX during virus multiplication in inoculated D. tatula cotyledons was studied by the use of ultraviolet fluorescence of nucleic acids using acridine orange stain. The appearance and the gradual increase and decrease in size of discrete red cytoplasmic inclusions in the virus infected tissues was positively correlated with different phases of virus multiplication as determined by infectivity assay in G. globosa.

At the 1-day period, in some cells, red fluorescing material was diffused throughout the cell, some cells fluoresced similar to healthy stained tissues, and others were characterized by a thick strand in the cytoplasm. Virus infectivity titre in inoculated cotyledons was not sufficient to produce local lesions in G. globosa at this period.

Gradual increase of virus infectivity titres in inoculated cotyledons from the 2- to 4-day period paralleled the appearance and gradual increase in size of discrete red fluorescing cytoplasmic inclusions. At 5-day period, virus infectivity titres in the inoculated cotyledons increased to the maximum in G. globosa. This increase in virus infectivity was associated with the appearance of red fluorescing cytoplasmic inclusions which in most cells were larger in size than the nucleus.

The gradual decrease of virus infectivity in G. globosa, i.e., 7-, 9-, and 13-day periods was positively correlated with a gradual decrease in size of the red fluorescing cytoplasmic inclusions. Inclusions appeared as dense masses.

The identity of discrete red fluorescing mass of RNA (presumably virus RNA), in the epidermal and palisade cells, was confirmed by an enzymatic removal (RNAase).

On the basis of relative red fluorescence, infected palisade cells contained more RNA than did infected epidermal cells.

Lability of clarified PVX to different concentrations of RNAase was demonstrated after 2 and 24 hours treatment using infectivity assay in G. globosa. There was a direct correlation between the RNAase concentration and reduction in virus infectivity.

The use of this specific stain, acridine orange, and the relation of red fluorescence to virus multiplcation was demonstrated. At least some relation exists between the intensity of red fluorescence within the cell and virus infectivity titres.

LITERATURE CITED

Anderson, E. S., J. A. Armstrong, and J. S. F. Niven. (1959). Fluorescence microscopy: observations of virus growth with aminoacridines. Symposia of Soc. Gen. Microbiol. Number IX, Virus growth and variation, Great Britain, 224-255.

Armstrong, J. A. (1956). Histochemical differentiation of nucleic acids by means of induced fluorescence. Exptl. Cell Res. 11, 640-643.

Baker, Kenneth F. (1957). Chemical ingredients for U. C. soil mix C. The U. C. system for production of healthy container-grown plants, Division of Agricultural Sciences, University of California, Fertilizer I (C), 73.

Bald, J. G. (1964). Cytopathological evidence for the production of plant virus ribonucleic acid in the nucleus. Virology 22, 377-387.

Bald, J. G. and R. A. Solberg. (1961). Apparent release of tobacco mosaic virus in living infected cells. Nature 190, 651-652.

Bawden, F. C. and F. M. L. Sheffield. (1944). The relationship of some viruses causing necrotic diseases of potato. Ann. Appl. Biol. 31, 33-40.

Bawden, F. C. and B. D. Harrison. (1955). Studies on the multiplication of a tobacco necrosis virus in inoculated leaves of French bean plants. J. gen. microbiol. 13, 494-508.

Benda, G. T. A. (1959). Concerning: Mutual exclusive of strains of tobacco mosaic virus. Virology 9, 712-714.

Bertalanffy, L. von and I. Bickis. (1956). Identification of cytoplasmic Basophila (RNA) by fluorescence microscopy. J. Histochem. Cytochem. 4, 481-493.

Bukatsch, F. and M. Haitinger. (1940). Beiträge zur fluoreszenzmikropischen Darstellung des Zellinhaltes, insbesondere des Cytoplasmas und des Zellkernes. Protoplasma 34, 515.

DeBruyn, P. P. H., R. S. Farr, Hilda Banks, and F. W. Morthland. (1953). In vivo and in vitro affinity of diaminoacridines for nucleoproteins. Exptl. Cell Res. 4, 174-180.

Dijkstra, J. (1964). The early events of tobacco mosaic virus infection in N. glutinosa. Mededelingen Landbouwhogeschool Wageningen, Nederland, 64-2.

Fry, P. R. and R. E. F. Matthews. (1963). Timing of some early events following inoculation with tobacco mosaic virus. Virology 19, 461-469.

Gurr, E. (1960). Encyclopaedia of microscopic stains. 13-15. Leonard Hill Ltd., London, England.

Harrison, B. D. (1956). The infectivity of extracts made from leaves at intervals after inoculation with viruses. J. gen. microbiol. 15, 210-220.

Hirai, T. and S. G. Wildman. (1963). Cytological and cytochemical observations on the early stage of infection of tomato hair cells by tobacco mosaic virus. Plant and Cell Physiol., 4, 265-275.

Hooker, W. J. and A. S. Summanwar. (1964). Intracellular acridine orange fluorescence in plant virus infections. Exptl. Cell Res. 33, 609-612.

Johnson, J. M. and L. E. Jones. (1962). Phase contrast observations of the nucleolus and nucleoli during growth and aging of tobacco cells in microculture. Amer. Jour. Bot. 49, 660 (Abstract).

Joshi, U. N. and K. S. Korgaonkar. (1959). Fluorescence of tissue culture cells stained with acridine orange. Nature 183, 400-401.

Kikumoto, T. and C. Matsui. (1961). Electron microscopy of intracellular potato virus X. Virology 13, 294-299.

Mayor, H. D. and A. D. Diwan. (1961). Studies on the acridine orange staining of two purified RNA viruses: poliovirus and tobacco mosaic virus. Virology 14, 74-82.

Mayor, H. D. and N. O. Hill. (1961). Acridine orange staining of single stranded DNA-nucleic acid of bacteriophage Ø X 174. Virology 14, 264-266.

Morthland, F. W., P. P. H. DeBruyn and N. H. Smith. (1954). Spectrophotometric studies on the interaction of nucleic acids with amino acridines and other basic dyes. Exptl. Cell Res. 7, 201-214.

Salaman, R. N. (1938). The potato virus 'X', its strains and reactions. Physiol. Trans. Roy. Soc. London, Ser. B. 229, 137-217.

Schümmelfeder, N. and K. J. Ebschner. (1957). Die Grundlage der differenten fluorochromierung von Ribound Desoxyribonucleinsaure mit Acridinorange (The principle of fluorochromic staining of ribonucleic and deoxyribonucleic acids with acridine orange). Naturwissenschaften 44, 467-468.

Schümmelfeder, N., R. E. Krogh, and K. J. Ebschner. (1958). Staining analysis with acridine orange fluorechromation. Comparative histochemical and fluorescence microscope investigation of the mouse cerebellum with the help of acridine orange and gallocyanine-chrome alum stains. Zeit. fur Zelloforschung und Mikroskopische Anatomie, Abt. Histochemie. 1, 1-28.

Shalla, T. A. (1959). Relations of tobacco mosaic virus and barley stripe mosaic virus to their host cells as revealed by ultrathin tissue-sectioning for the electron microscope. Virology 7, 193-219.

Sirlin, J. L. (1962). The nucleolus. Progress in Biophysics and Biophysical chemistry 12, 25-66.

Smith, K. M. (1924). On a curious effect of mosaic disease upon the cells of potato leaf. Ann. of Botany 38, 385-388.

Strugger, S. (1940). Fluoreszenzmikroskopische Untersuchungen ueber die Aufnahme und Speicherung des Acridinorange durch lebende und tote Pflanzenzellen. Jenaische Ztschr. F. Naturwiss 73, 97.

Timian, R. G., W. J. Hooker, and C. E. Peterson. (1955). Immunity to virus X in potato: Studies of clonal lines. Phytopath. 45, 313-319.

Welkie, G. W. and G. S. Pound. (1958). Temperature influence on the rate of passage of cucumber mosaic virus through the epidermis of cowpea leaves. Virology 5, 362-370.

Worley, J. F. and I. R. Schneider. (1936) Progressive distribution of southern bean mosaic virus antigen in bean leaves determined with a fluorescent antibody stain. Phytopathology 53, 1255-1257.