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Title: TOBACCO RATTLE VIRUS: WEED HOSTS, DIAGNOSIS, CHARACTERIZATION, PURIFICATION, AND INTERACTION WITH POTATO VIRUS X

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Abstract approved Redacted for Privacy

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A series of studies was conducted to determine the presence of tobacco rattle virus (TRV), potato virus X (PVX), and other viruses in wild plants and to evaluate the efficacy of the detection methods; to characterize and purify an isolate of TRV; and to study the interaction between TRV and PVX.

Symptoms on Nicotiana tabacum L. cv. 'Samsun NN' and Phaseolus vulgaris L. cv. 'Bountiful' permitted detection of TRV, PVX, and other viruses. Identification was confirmed by comparison of these symptoms with those from standard cultures of TRV and PVX, by electron microscopy, and by serology. Portulaca oleracea L., Solanum sarrachoides Sendt., and Amaranthus retroflexus L. were carriers of TRV; the latter two also carried PVX. Most TRV carriers were found adjacent to infected potato tubers. Naturally infected S. sarrachoides contained TRV in the roots at relatively high concentration. Mechanical inoculation of TRV to A. retroflexus and S. sarrachoides produced variable symptoms. These

included red spots on the former and chlorosis and stunting on the latter. TRV moved systemically in both species. Methods of detection were adequate but electron microscopy and serology required increased TRV concentration to confirm identification.

The isolate from S. sarrachoides was characterized by symptoms, serology, and electron microscopy. To relate TRV concentration to infectivity, several purification methods were carried out. The isolate behaved similarly to other TRV strains in symptomatology and serology. Most purification procedures provided good virus yield and/or infectivity. Purity (260/280 nm) was higher when butanol was used. Precipitation with PEG provided good purity (260/280 = 1.17-1.18) typical of low RNA rod-shaped viruses. Sucrose rate-zonal density gradient reduced yields and provided imperfect separation of modal lengths. Particle breakage was excessive when using butanol, freezing, and thioglycolate, and was reduced somewhat by using only chloroform. However, particle breakage was mainly an artifact of staining and thus not affecting the correlation virus concentration-infectivity. Fixation with glutaraldehyde reduced breakage and provided two modal lengths comprising particles; 85.4 and 206.7 nm for long and short particles, respectively. Histograms differed from those reported in the literature. The proportion of long to short particles, the width of particles, and the length of short particles were within values reported in literature. In addition, long particles were longer than previously reported.

The interaction between TRV and PVX on 'Samsun NN' tobacco plants was studied using the half-leaf method on 'Bountiful' bean

plants. Eleven-day-old bean plants which were 1 cm long from primary leaves to tip of trifoliate leaf were the most sensitive. Evaluation was done by comparing extraction from singly (TRV=A) and doubly (TRV + PVX=B) inoculated tobacco plants on opposite halves of bean leaves, or by comparing A or B against a standard TRV preparation on opposite halves of bean leaves. When the standard was used, concentration was estimated by referring to a regression equation relating concentration to infectivity. Using this method, a consistent depressive effect of PVX on TRV was measured which reached the maximum at 96 hrs of incubation. At this time, TRV concentration in extracts from A was about 3 times as great as that from B.

The depression was somewhat independent of the time lapse between the entry of one virus and the other. It was higher when TRV was inoculated first than when PVX preceded TRV. However, this depression was not larger than that obtained by simultaneous inoculation at 96 hrs of incubation.

Symptoms caused by double inoculation on tobacco were remarkably different and developed faster than those induced by single inoculation with TRV or PVX. Symptoms from double inoculation were sunken lesions and, in some instances, severe stunting. TRV-inoculated tobaccos often were symptomless but their extracts were more infective than those from doubly inoculated plants.

TRV replication in symptomless plants, increased severity of symptoms due to simultaneous presence of TRV and PVX, and depression of TRV triggered by PVX could explain some of the problems encountered when detection of TRV is attempted in potato tubers and

potato plants showing conspicuous symptoms.

If concentration of TRV in the field is lower than in plants used in this thesis, the model we used might predict an even higher depression of TRV under natural conditions in potatoes.

TOBACCO RATTLE VIRUS: WEED HOSTS, DIAGNOSIS, CHARACTERIZATION, PURIFICATION, AND INTERACTION WITH POTATO VIRUS X

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

TOBACCO RATTLE VIRUS: WEED HOSTS, DIAGNOSIS, CHARACTERIZATION, PURIFICATION, AND INTERACTION WITH POTATO VIRUS X

GENERAL INTRODUCTION

Potato stem mottle, potato sprain, potato ringspot, potato knobby tuber, are some of the diseases caused by tobacco rattle virus (TRV). TRV affects potato production in Europe, Great Britain, The Netherlands, and North and South America. The economic damage is variable, difficult to quantify, and dependent on environmental conditions. Low temperature may cause severely stunted plants, or symptoms may be completely masked above 20 C.

The disease poses a threat to all potato areas where Trichodorus spp., the nematode vector of TRV, dwells. It is also a potential threat to new areas opened to potato production. In many instances, it is very difficult to identify or to confirm TRV identification. Much is unknown about epidemiological aspects that could be essential in obtaining efficient and environmentally safe control methods.

To help to parametrize the virus, a series of studies were organized in three major sections: (I) diagnosis of TRV and potato virus X (PVX) in weeds and rotation crops in Oregon potato fields, (II) purification and characterization of a TRV isolate, and (III) study of an interaction between TRV and PVX.

I. Diagnosis

Vectors and reservoirs of viruses play an important role in

transmission and perpetuation, respectively. TRV has a known nematode vector and a large host range. Some of the wild hosts of TRV have been proposed as possible bait plants. Davis and Allen (1975) indicated Solanum nigrum L., and Beemster and Rozendaal (1972) mentioned Stellaria media (L.) Vill. as possible bait plants. Viola arvensis Murr. should also be considered as a possible diagnostic plant (Cooper and Harrison, 1973).

On the other hand, the presence of the virus in wild hosts suggests the importance of weeds as reservoirs and as a source of virus for the vectors. Because of these reservoirs, eradication of TRV is almost impossible. The existence of wild hosts diminishes the efficacy of rotations as a possible remedy (Cooper and Thomas, 1971). Chemical treatments to control Trichodorus generally proved effective only within the year of application, probably because of the depth the nematode occurs when the water table moves down. Consequently, a residual nematode population could quickly regenerate and have a readily available source of virus in various weeds. The possibility of seed transmission of TRV within weed species (Cooper and Harrison, 1973) increases the importance of determining wild host carriers of the virus. For these reasons, the objectives of the first phase of the thesis were to assess (a) potential TRV reservoirs and the approximate virus content of wild hosts, (b) the efficacy of detection methods, and (c) symptoms and virus recovery from mechanically inoculated hosts. A survey was made of several Oregon fields under potato crops or including potatoes on the rotation which had a previous history of TRV. The

survey used methods that also detected PVX and other viruses.

(II) Purification and characterization

Knowledge of the characteristics of the pathogen is necessary to understand the disease. By parametrizing the pathogen, we could also have an idea of the variability of it within a certain area when compared to other strains of the same entity. TRV is quite variable and different strains can occur, even in adjacent fields (Van Hoof, 1972).

To parametrize an isolate of TRV obtained from wild hosts in Oregon potato fields, symptoms and serological reactions were studied and different purification methods were tried to estimate particle size. A certain number of intact virus particles is necessary to determine modal lengths and particle size. Also, since we needed to relate virus concentration to infectivity to obtain a dilution curve for use in further work, assurance that particles were intact was necessary. Breakage of particles results in higher than normal absorbancy readings. Infectivity of these broken particles may be reduced, thus relating a high absorbancy reading to low infectivity, establishing a wrong correlation. Because of this, a standard purification method and preparatory electron microscopy techniques were modified in order to reduce particle breakage and properly correlate concentration to infectivity measured as local lesions on Phaseolus vulgaris L. cv. 'Bountiful' plants.

(III) Interaction

Another important biofactor determining the characteristics of a disease epidemiology is the relationship with other pathogens. The effects of the interaction among different pathogens in the same plant could be synergistic, additive, or antagonistic for expression of symptoms and for virus replication.

Several facts indicated the possibility of an interaction between TRV and PVX which could affect TRV detection in potato tubers. Symptoms varied and were difficult to differentiate from each other on doubly infected tobacco plants. Electron microscope observation of doubly infected tobaccos was extremely time consuming, even when the plants showed abundant TRV symptoms. Frequently potato plants reported as virus-free contained PVX, which further shows the feasibility of both TRV and PVX being together in potato plants. In addition, wild hosts were commonly infected with both viruses. To study the possible effect of this relationship on TRV detection from infected potato plants and tubers, a system was established on Nicotiana tabacum L. cv. 'Samsun NN' plants. It was assumed that such a model might help to predict the effect of the interaction in potatoes.

To study the TRV-PVX interaction, the investigation was divided into two phases. The first phase was preliminary work aimed at perfecting bioassay of infectivity on Phasesolus vulgaris L. cv. 'Bountiful' by (a) determining the effect of age of the plants on bioassay sensitivity, and (b) development of a standard

dilution curve of pure virus suspensions to be used in estimating virus concentration. The second phase involved quantification of the interaction between TRV and PVX by determining (a) whether TRV concentration was influenced by sequence or time of entry of TRV or PVX, (b) studying the effect of initial TRV concentration on the interaction, and (c) studying the kinetics of the TRV-PVX interaction during various periods of incubation.

DIAGNOSIS OF TOBACCO RATTLE VIRUS (TRV) AND POTATO VIRUS X (PVX) ON WEEDS AND ROTATION CROPS IN OREGON POTATO FIELDS

INTRODUCTION

Tobacco rattle virus (TRV) affects potato fields in Great Britain, Europe, and North and South America, causing a disease known as corky ringspot and/or potato knobby tuber. It also is a serious problem in potato fields of the Pacific Northwest and a potential threat to new areas opened to potato production.

TRV has an extensive host range and is transmitted by nematodes, Trichodorus sp. TRV persisted in Trichodorus for three years without access to plant roots (Van Hoof, 1970). Cooper and Harrison (1973) speculated that the short term propagation of TRV can be explained because the nematodes retain infectivity at least during the growing season. However, weeds also may be involved in the virus disease cycle. The nematode may feed preferentially on weeds which will insure virus perpetuation in a permanent source of inocula. There have been indications of higher incidence of TRV in fields heavily infected with weeds. Cooper and Thomas (1971) showed evidence that fallowing for nine months decreased the disease incidence.

Detection of TRV in potatoes is difficult since transmission from infected tubers has proved erratic. Diagnosis is usually attempted at harvest time and is based on internal and external tuber symptoms. However, only a low titer virus may be present at harvest since the infectivity period is very short (Van Hoof, 1964), as may be the period of transmissibility. Probably for this reason,

utilization of electron microscopy and serology at harvest has proved to be of relatively little value in the direct identification of TRV in potato.

The use of bait plants in a field is an alternative way to identify the virus. Davis and Allen (1975) reported several hosts of TRV in Idaho. They found Solanum nigrum L. and Erodium cicutarium (L.) L'Her. to be carriers of the virus. S. nigrum was indicated as a suitable bait plant for direct detection of TRV in the field.

Unidentified hosts of TRV may be one of the main factors in the outbreak and persistence of the virus. On the other hand, wild hosts of the virus, if suitable, could be utilized in the detection of the pathogen by serving as indicator plants. Knowledge of such species would be valuable for epidemiological studies of TRV and for improving control methods and identification.

The objectives of this investigation are (1) to identify naturally infected hosts of TRV in fields of Oregon under different crops which had a previous history of TRV or that were infected at the time of the survey, (2) to evaluate methods of detecting TRV in wild hosts and rotation crops, and (3) to study symptoms and TRV recovery from mechanically inoculated hosts.

MATERIALS AND METHODS

The survey was carried out on fields with either a previous history of TRV or presently infected with TRV. These fields were either in potatoes at the time of the survey or had included potatoes in the rotation within a three-year period.

Sample size

A preliminary survey, conducted in 1973, was restricted to central Oregon fields (Prineville-Madras), and the sample collected was small. Most fields were treated with herbicide and/or fumigated to control nematodes. As a consequence, weeds were not abundant, especially in mint fields. Sampling was carried out at random on areas where incidence of TRV was or had been high, as indicated by the county agent or the grower.

The main survey was carried out in 1974, expanding the sampling to eastern Oregon fields. The seven locations surveyed in fields of central and eastern Oregon had been fumigated with either dazomet (tetrahydro-3,5-dimethyl-2H-thiadiazine-2-thione) or dichloropropene (1,3-dichloropropene) when potatoes were grown. In spite of fumigation to control Trichodorus, TRV was frequently reported by county agents. Thus, samples from these areas were harvested. The area sampled was as small as possible. Growers and county agents indicated the area in a particular field where TRV incidence was highest.

Except for cases where a very low number of weeds was present and/or where infected potato tubers were found, the sampling

procedure was based on a probability table. This table was established from findings of Cooper and Harrison (1973) which showed that when the virus was present in the field, the minimum percentage of plants of one species carrying the virus was 5%. A sample size of 50 (92% probability of at least one infected) was chosen as a feasible number of weeds to be collected per predominant species within each field. When 50 plants were not available, all specimens were collected. A field in location 7 (eastern Oregon) was actually in potatoes ready to be harvested. Numerous areas within this field had potato tubers showing brown specks and corky arcs typical of TRV symptoms. In this case, samples of weed species were obtained adjacent to potato tubers showing TRV symptoms. In addition to weeds, plants from the present crop (wheat, barley, and potatoes) also were collected.

Handling of samples

Specimens collected were placed in air-tight plastic bags which were kept in ice-cooled styrofoam boxes or metal garbage cans. After 24 hrs they were transferred to a cold room at 4 C and stored until indexing. Keeping plants dry and clean was important to avoid decay when indexing was delayed. When the number of samples exceeded the handling capacity, they were frozen until they were indexed.

Detection methods

Bioassay. Assay plants used in 1973 were Nicotiana tabacum L. cv. 'Samsun NN', and Vinca rosea L. 'Purity', N. tabacum cv.

'Samsun NN', Phaseolus vulgaris L. cv. 'Bountiful', Gomphrena globosa L., Tropaeolum majus L., and Petunia hybrida hort. ex Vilm., were used in 1974.

All composite samples were ground in a sterilized mortar with 0.05 M phosphate buffer, pH 7.0. The extracts were rubbed on leaves of assay hosts previously dusted with Carborundum (400 mesh). Leaves then were washed with tap water. When a sample was positive for TRV, roots, stems, and shoots were independently indexed to determine location and dilution end point of the virus in the plant donor. To determine dilution end point, inoculum was serially diluted from 10^{-1} to 10^{-6} with buffer.

Forty-eight hrs before inoculation, indicator plants were moved to growth chambers at 19 C. After inoculation, all indicator plants or those inoculated with TRV or PVX were maintained at 19 C and 14 hrs light until symptoms were easily identifiable on control plants. TRV isolate PV-73 from the American Type Culture Collection (ATCC) was used as a standard to compare symptoms and to verify that conditions were favorable for symptom expression. Other standards were TRV-G, an isolate from central Oregon, and I-2 from Idaho supplied by T.C. Allen and J.R. Davis, respectively. Controls consisted of one representative of each assay species, each of which was dusted with Carborundum and rubbed with the buffer used for extraction for every 10 plants inoculated from wild hosts. One out of 10 leaves of G. globosa plants was inoculated in the same manner. A PVX standard was obtained from R.J. Shepherd of the University of California (Davis). All standards were maintained by serial

transfer on plants of 'Samsun NN' tobacco.

TRV symptom expression and recovery were studied on plants of species shown by the survey as carrying TRV. Plants of such species were raised from seeds obtained from TRV-free fields, then later inoculated with TRV. Systemic movement of TRV in a plant was checked by inoculating sap from uninoculated leaves to 'Samsun NN' tobacco and 'Bountiful' bean plants.

Alfalfa mosaic virus (AMV), white clover mosaic virus (WCMV), and tomato spotted wilt virus (TSWV) were partially identified by characteristic symptoms on 'Bountiful' bean and 'Samsun NN' tobacco plants.

When samples induced TRV- and PVX-like symptoms on indicator hosts, extracts from inoculated tobacco were checked serologically and with the electron microscope to confirm the identification of both viruses. Serial transfers to increase TRV concentration were performed on 'Samsun NN' tobacco plants.

Electron microscopy. Negatively-stained dip preparations from tobacco inoculated with extracts from wild hosts were made with 2% sodium phosphotungstate, pH 7.0. A drop of stain was placed on a Formvar-coated grid and an epidermal strip and pieces of infected tobacco leaves were touched to the drop. When the samples examined originated from Medicago sativa L., dip preparations were made by fixing with 2% formaldehyde, evacuating, and staining as indicated. When crude sap was used, a drop from an infected tobacco plant was placed on a Formvar-coated grid for 30

seconds and removed with blotting paper. The grid was stained and the excess stain was blotted off. Observations were made with a Philips EM 300 electron microscope and photomicrographs were taken.

Serology. Serological identification was carried out by using the Ouchterlony agar double diffusion test, a formula adapted from methods developed at Braunschweig, West Germany (R.H. Converse, personal communication). TRV antiserum No. PV AS 75 was obtained from the ATCC. The antigen used for serology and electron microscopy was obtained by serial transfer on tobacco plants of virus originating from wild hosts. The antigen was diluted 1:2 with buffer for serology. Normal serum, sap from healthy tobacco plants, and antihealthy antiserum served as controls in the tests. The remaining extract was used to bioassay for infectivity. Antigen wells were recharged up to four times. Readings were made 8 to 14 hrs later. After the second reading, D L-3,4-dihydroxyphenylalanine (DOPA) (Madhosingh and Wood, 1971) was added to detect weak reactions.

PVX also was assayed with serology. An antiserum from R. Stace-Smith (Agriculture Canada) was diluted from 1:64 to 1:4096 and used in microprecipitin tests. Checks were PVX (PV-54-B-3B) from the ATCC and a PVX isolate from R.J. Shepherd, all propagated in 'Samsun NN' tobacco plants. The virus from wild hosts had been propagated at least once in 'Samsun NN' tobacco plants that then served as the virus source.

RESULTS

Virus identification

Symptoms characteristic of TRV on N. tabacum, and which were regarded as diagnostic, were chlorotic and necrotic arcs, flecks, vein necrosis, and leaf distortion. These symptoms were followed by stem mottling and splitting when the infection was severe. Diagnostic symptoms on P. vulgaris were small yellow to deep brown spots. The diagnostic symptoms for PVX were neat, chlorotic to white target spots on N. tabacum. G. globosa reponded similarly to both TRV and PVX by forming purple rings with chlorotic-yellow centers about five days after inoculation. AMV and WCMV caused purple rings with chlorotic centers and chlorotic rings, respectively, on P. vulgaris.

TRV symptoms on N. tabacum began to appear from 48 to 72 hrs after inoculation; stem mottle and splitting appeared 18 days after inoculation. Symptoms in P. vulgaris were evident about 24 hrs after inoculation for TRV and somewhat delayed for the others. Symptom expression and color in P. vulgaris were dependent on age of the plants. Faster symptom development and darker spots appeared on greener and younger beans.

Known TRV and PVX isolates produced symptoms similar to isolates from naturally-infected plants.

An unidentified virus caused lesions which closely resembled those induced by TSWV on N. tabacum. However, no symptoms were induced in G. globosa, P. vulgaris, T. majus, and P. hybrida by extracts from infected tobacco plants or from a culture of TSWV

provided by K.S. Derrick (Louisiana State University, Baton Rouge).

Electron microscopy

Detection of TRV in dip preparations of 'Samsun NN' demanded a large amount of time. Even when symptoms were evident, detection was difficult. Serial transfers from tobacco to tobacco was necessary to increase virus concentration before TRV from wild hosts could be detected. In contrast, identification of PVX was easily confirmed after the first transfer to tobacco since it was present in high concentration in almost all preparations.

Bacilliform particles, characteristic of AMV were not found in the electron microscope, so identification of AMV was not confirmed.

Serology

All immunodiffusion plates with Casper As 17 antiserum showed positive precipitation reactions when tested against TRV-infected plant material originating from wild hosts and standards. Precipitation lines at 1:16 dilution of the antiserum were the clearest. No specific reaction was incited by normal serum or by extracts from healthy 'Samsun NN' tobacco plants. When antigen wells were recharged several times, repeatability was excellent and lines developed in about eight hrs. Precipitation end point of PV-73 was 1:64.

In contrast, the microprecipitin test for PVX proved to be very sensitive. An antiserum titer of 1024 provided the clearest reaction with most inocula. No non-specific reaction occurred in

the control.

Naturally-infected species

In the exploratory survey carried out in 1973, TRV identification was achieved even when a small sample was collected from the four fields surveyed in central Oregon. A total of 98 specimens of 11 species were collected. In two out of four locations that were in peppermint and spearmint, three of the species were naturally-infected with TRV: Portulaca oleracea L., Solanum sarrachoides Sendt., and Amaranthus retroflexus L. and/or Amaranthus powellii Wats. Quantitative aspects of occurrence are shown on Table 1.

In 1974, a total of 673 specimens of 16 species were indexed for all seven locations surveyed. However, only in one of these seven locations were naturally-infected species detected. Species collected at this location all were obtained adjacent to TRV-infected potato tubers. Two species, A. retroflexus and S. sarrachoides, were naturally-infected with TRV in this location. A higher percentage of S. sarrachoides (50%) carried TRV as compared to A. retroflexus (11%) (Table 2). Both species also carried PVX.

M. sativa plants were infected with AMV and WCMV. Two other species, Solanum triflorum Nutt. and Tribulus terrestris L., were infected with an unidentified virus whose symptoms on N. tabacum plants resembled those of TSWV (Table 2).

Extracts from S. sarrachoides plants induced more severe symptoms on N. tabacum and more local lesions on P. vulgaris than comparable extracts from A. retroflexus. When inocula from different

parts of the plant were tested, TRV was obtained from the roots of S. sarrachoides. Serial dilution of the inoculum from this plant showed a dilution end point of 10^{-2} on plants of N. tabacum.

TRV was mechanically inoculated to A. retroflexus and S. sarrachoides. Extraction from inoculated and non-inoculated leaves of both species induced marked symptoms on N. tabacum and P. vulgaris. Therefore, TRV did go systemic in these weeds under growth chamber conditions. A. retroflexus showed red spots (Figure 1-C) and S. sarrachoides was clearly stunted and chlorotic compared to checks (Figures 1-A, B). However, symptom expression was variable and results were not always repeatable.

Table 1. Plant species collected and naturally-infected with viruses in central Oregon (Jefferson and Crook counties) in 1973, as determined by symptomatology on Nicotiana tabacum L. cv. 'Samsun NN'.

Species	Locations 1 TRV	2 TRV	3 TRV	4 TRV	Total TRV
1. Amaranthus albus L.	0/5[1/]	-	0/5	-	0/10
2. Amaranthus retroflexus L. and/or powellii Wats.	-[2/]	3/11	0/14	0/15	3/40
3. Chenopodium album L.	-	-	-	0/2	0/2
4. Euphorbia esula L.	0/5	-	-	-	0/5
5. Malva rotundifolia L.	-	-	-	0/1	0/1
6. Panicum capillare L.	-	0/2	-	-	0/2
7. Portulaca oleracea L.	1/2	1/5	0/1	0/5	2/13
8. Salsola kali L.	0/2	-	0/3	-	0/5
9. Solanum sarrachoides Sendt.	-	2/5	0/5	0/7	2/17
10. Tragopogan pratensis L.	0/2	-	-	-	0/2
11. Verbascum thapsus L.	0/1	-	-	-	0/1
Total	1/17	6/23	0/28	0/30	7/98

1/ Number of samples infected over the total number indexed/location.

2/ Not found in location

Table 2. Plant species collected in 1974 in eastern Oregon (Malheur county) and naturally-infected with viruses as diagnosed by symptomatology and confirmed by serology and electron microscopy.

Species	Location 7					Locations[4]
	TRV	PVX	AMV	WCMV	Other[3]	1 - 6
1. *Amaranthus retroflexus* L. and/or *powellii* Wats.	2/18[1]	4/18	0/18	0/18	0/18	0/130
2. *Capsella-bursa-pastoris* (L.) Medic.	--[2]	--	--	--	--	0/25
3. *Chenopodium album* L.	--	--	--	--	--	0/10
4. *Cuscuta campestris* Yunck.	0/15	0/15	0/15	0/15	0/15	--
5. *Erodium cicutarium* L.	--	--	--	--	--	0/9
6. *Malva rotundifolia* L.	--	--	--	--	--	0/5
7. *Medicago sativa* L.	0/14	0/14	3/14	2/14	0/14	--
8. *Polygonum aviculare* L.	--	--	--	--	--	0/60
9. *Polygonum persicaria* L.	--	--	--	--	--	0/70
10. *Portulaca oleracea* L.	--	--	--	--	--	0/5
11. *Sisymbrium altissimum* L.	--	--	--	--	--	0/50
12. *Solanum sarrachoides* Sendt.	7/14	7/14	0/14	0/14	0/14	0/70
13. *Solanum triflorum* Nutt.	0/5	0/5	0/5	0/5	2/5	0/55
14. *Tribulus terrestris* L.	0/18	0/18	0/18	0/18	2/18	--
15. *Trifolium pratense* L.	--	--	--	--	--	0/30
16. *Xanthium strumarium* L.	--	--	--	--	--	0/70
Total	9/84	11/84	3/84	2/84	4/84	0/589

[1] Number of samples infected over the total number indexed/location.

[2] Not found in location.

[3] Symptoms similar to those induced by tomato spotted wilt virus (TSWV) on *N. tabacum* 'Samsun NN')

[4] Locations 1-3 Crook county, locations 4-6 Malheur county.

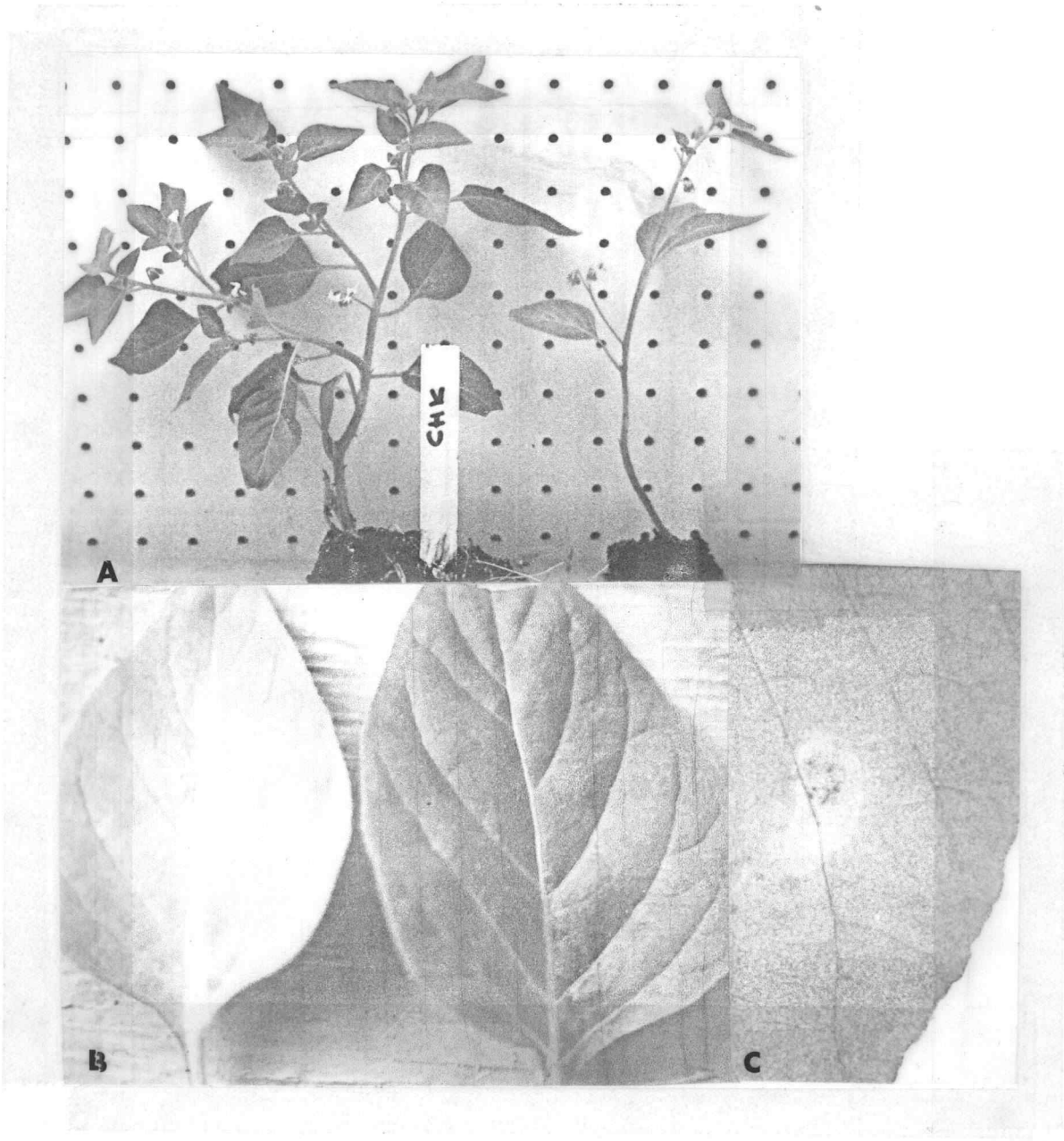


Figure 1 (A to C). Solanum sarrachoides and Amaranthus retroflexus plants showing symptoms induced by mechanical inoculation with tobacco rattle virus (TRV). (A) Left, S. sarrachoides non-inoculated; right, inoculated. (B) Leaves of S. sarrachoides, left inoculated, right non-inoculated. (C) A. retroflexus leaf showing spot produced by TRV inoculation.

DISCUSSION

Methods used to collect, transport, and store the samples proved satisfactory. Bruckart and Lorbeer (1976) used a similar method of storage with success.

Use of 'Samsun NN' tobacco and 'Bountiful' bean as assay hosts, and known virus standards for comparison provided a reliable TRV detection method. Symptoms in potatoes (brown specks, corky arcs) observed at the time of collecting the samples provided additional verification of TRV identification. A detailed study of factors affecting 'Bountiful' beans, including age, source, light, temperature, shading before and after inoculation, and study of the lowest concentration that would produce lesions, may substantially improve this already reliable assay.

Detection of TRV by serology proved reliable but, as with electron microscopy, this method required higher concentration of the inocula compared to a study of expressed symptoms. Detection by electron microscope was erratic. When PVX was also present in the preparations, TRV identification became extremely difficult. Even when tobacco leaves showed marked symtpoms, electron microscopic detection often was difficult. PVX was too abundant and when a particle suspected to be TRV was located, it was difficult to differentiate it from broken PVX particles.

We have shown that certain weeds acted as reservoirs of TRV, PVX, and other viruses (AMV, WCMV, and probably TSWV). Preliminary identification of TRV in P. oleracea, A. retroflexus, and S.

sarrachoides in 1973, indicated probable weed carriers. This was confirmed in 1974 when S. sarrachoides and A. retroflexus were found to carry TRV at relatively high frequency, 50 and 11%, respectively, in one field. The occurrence of TRV in wild hosts and in adjacent (or nearby) potato plants were highly correlated in this field. This also indicated the reliability of supporting the weed bioassay with TRV symptoms in tubers.

Virus was not detected at any of the other locations in 1974, even when the number of specimens per species indexed was high (i.e., 130 for A. retroflexus). This probably indicates that there was no TRV in those fields, or in the area sampled.

Species such as Polygonum aviculare L. and E. cicutarium reported as carriers of TRV (Cooper and Harrison, 1973 and Davis and Allen, 1975), were not found to contain virus in our survey, even when a large sample was collected in 1974. Both plant species, however, were collected from fields where neither S. sarrachoides nor A. retroflexus were found to be infected. Also, P. aviculare and E. cicutarium were not found in location 7 where all detections of TRV were achieved in 1974.

TRV was located in the roots of the indexed S. sarrachoides plants, as Davis and Allen (1975) reported for S. nigrum and E. cicutarium. The concentration of TRV in the roots of one S. sarrachoides specimen was relatively high as determined by symptoms on tobacco plants. TRV survived the extra handling imposed when the same specimen was resampled to assess the location and concentration of the virus.

Mechanical inoculation of S. sarrachoides and A. retroflexus with TRV obtained from S. sarrachoides induced symptoms such as chlorosis and stunting in the former and red spots in the latter. However, identifying these symptoms in the field may be difficult. Field temperatures above 20 C are generally unfavorable for symptom expression of TRV in the upper portions of the plants (Beemster and Rozendaal, 1972) and these conditions were quite different from the controlled environment under which TRV symptoms were induced in S. sarrachoides and A. retroflexus. Appearance of symptoms was not consistent throughout all experiments and both species in the field may show the same symptoms from many factors other than TRV infection, such as nutrient deficiencies or insect feeding. Recovery of TRV was easily achieved (when mechanically inoculated) from non-inoculated portions of the plant which indicated movement of the virus from inoculated leaves.

The identification of carriers of viruses can aid in understanding the ecological relationships that cause disease outbreaks. Consequently, better and more environmentally safe control methods could be devised. Plant carriers, as such, play a role in these ecological relationships. Potato tubers, when artificially inoculated (Van Hoof, 1964), are susceptible only during a short period of time when they have grown to a considerable size. Therefore, late-season weeds such as S. sarrachoides and A. retroflexus may play an important role in TRV epidemiology by providing a readily available source of inoculum. They may account for perpetuation of the inoculum in the long term, and for failure when using rotation

as a means of control. Also, they may be responsible for higher incidence of TRV in fields heavily infected with weeds.

PURIFICATION AND CHARACTERIZATION OF A TOBACCO RATTLE VIRUS (TRV) ISOLATE

INTRODUCTION

Characterization of tobacco rattle virus (TRV) is difficult because of its variability. TRV-induced symptoms vary significantly among TRV strains and even within the same strain under different environmental conditions (De Zoeten et al., 1966). In addition, degrees of infectiousness can vary (Sänger, 1968).

Serological variation among TRV strains can also be extreme. Harrison and Woods (1966) classified the different isolates in three categories: 1) European, 2) North American, and 3) South American, according to their response to antisera prepared against isolates from Swaffham, England (SP) and Campinas, Brasil (CAM). Isolates from the U.S. did not react to SP antiserum, and reacted with CAM only when undiluted or slightly diluted.

Purification procedures for TRV have been reported by Harrison and Nixon (1959, 1960), Allen (1963), Harrison and Woods (1966), Semancik (1966), Frost et al. (1967), Sänger (1968), and Lister and Bracker (1969). Precipitation of plant proteins with chloroform-butanol and differential centrifugation are common to most methods. Procedural amendments and modifications are numerous, such as precipitation of the long particles by polyethylene glycol (PEG) as reported by Huttinga (1973). Yields were variable depending on the method and the propagation species. Harrison and Nixon (1959)

reported 50 mg/l; De Zoeten et al. (1966) reported 50 mg/kg as being a low recovery; and Harrison (1970) reported from 20 to 100 mg/kg of leaves of systemically infected Nicotiana clevelandii. Regarding the 260/280 ratio, Semancik (1966) indicated a 1.15 ratio, and Lister and Bracker (1969) 1.14 to 1.15 uncorrected for light scattering.

In 1963, Allen characterized a strain of TRV from Oregon potato fields. This isolate was purified and particle size was determined by several authors with remarkably different results. The predominant short and long particle size reported were 79 and 194 nm by Harrison and Woods (1966), 57 and 183 nm by Lister (1968), 105 and 195 nm by Sänger (1968), and 90 and 195 nm by Lister and Bracker (1969). The corresponding long to short ratios were 2.46, 3.21, 1.86, and 2.17, respectively. This indicated either significant methodological variations, changes in the isolate, or a mixture of strains.

In a survey carried out in Oregon potato fields (1974), a strain of TRV was isolated from Solanum sarrachoides Sendt. plants. Since there are many strains of TRV and to obtain an idea of TRV variation in the Pacific Northwest when compared to other isolates, we characterized this isolate by determining symptoms, particle size, and serological reaction.

One of the main concerns of our investigation was to obtain the closest relationship possible between optical absorbance and infectivity. Breakage of the particles may increase the U.V. absorbance because of disruption of secondary and tertiary structure of proteins. More significantly, uncoated viral RNA produced

higher U.V. absorbance as a consequence of particle breakage. Therefore, absorbancy readings will not properly reflect infectivity in bioassays. Silberschmidt et al. (1971) reported that mild purification methods resulted in a higher proportion of long particles than when the samples deriving from the same virus source were submitted to greater mechanical stresses. Hampton et al. (1974) found that butanol caused characteristic breakage of pea seedborne mosaic virus (PSMV) particles and that glutaraldehyde fixation prevented breakage of the virus particles.

To reduce breakage of the virus particles, the basic TRV purification method described by Lister (1966), and Lister and Bracker (1969) was modified, and different electron microscope preparatory techniques were tried.

MATERIALS AND METHODS

Inoculation

The TRV isolate was obtained from potato fields in eastern Oregon. The virus was recovered from *S. sarrachoides* and was propagated on plants of *Nicotiana tabacum* L. cv. 'Samsun NN'. The plants carrying the TRV isolate, as well as those utilized as hosts, were kept in growth chambers at 19 C and 14 hrs light (7560 lux, cool white lamps). TRV was extracted from infected *N. tabacum* leaves in 1:1.5 (w/v) 0.05 M phosphate buffer, pH 7.0. Inoculation was done with pipe cleaners dipped into filtered inocula that contained Celite. Plants inoculated were *Chenopodium quinoa* Willd., *Cucumis sativus* L., *Gomphrena globosa* L., *N. tabacum* L., cv. 'Samsun NN', *Phaseolus vulgaris* L. 'Bountiful', *Phaseolus lunatus* L. 'Henderson's Bush', and *Vinca rosea* L. 'Purity'.

Purification and extraction

Common to all procedures, *N. tabacum* leaves showing necrotic and chlorotic arcs, vein necrosis, flecking, and leaf distortion were harvested and placed in airtight polyethylene bags in a refrigerator at 4 C for several hrs. The leaves were then homogenized in a blender with 0.01 M citric acid-phosphate buffer, pH 7.4 (1:1.5 w/v). On one occasion, borate buffer was used. The homogenate was filtered through cheesecloth and subjected to centrifugation at 10.3 G for 10 min in a Sorvall RC 2B centrifuge.

The crude extract was then submitted to one or more of the

following treatments (Table 3):

Treatment 1: Sodium thioglycolate was added to crude extract to a concentration of 0.0088 M. The supernatant was clarified by slowly adding half volume of n-butyl alcohol-chloroform mixture (v/v). The resultant emulsion was allowed to settle for 1 to 2 hr before removing the aqueous supernatant phase. This liquid was centrifuged at 10.3 G for 10 min and the supernatant phase was frozen. It then was thawed, maintained at 4 C, and centrifuged at 70.1 G in a Beckman L2-65B ultracentrifuge for 2 hr. Pellets were resuspended in 1 ml of 0.01 M phosphate buffer, pH 7.0. Each tube containing the pellets was shaken 30 seconds and the liquid was clarified by centrifugation at 10.3 G for 10 min.

Treatment 2: The supernatant was clarified by slowly adding one-half volume of chloroform. The supernatant was removed and centrifuged at 10.3 G for 10 min. It then was centrifuged at 70.1 G for 2 hr. The pellets were resuspended for 20 hr in 1 ml of 0.01 M phosphate buffer, pH 7.0, and clarified at 10.3 G for 10 min.

Treatment 3: The virus was pelleted at 269.2 G for 45 min.

Treatment 4: The virus was layered on a 0.29-1.17 M sucrose (RNAse-free) density gradient. The gradient was centrifuged for 2 hr at 75.9 G. The gradient was then fractionated with an ISCO density gradient fractionator (Instrumentation Specialties Co., Lincoln, Nebraska) and monitored at 254 nm by an ultra-violet analyzer.

Treatment 5: Fractions were layered on sucrose-polyethylene glycol (6000) columns of 7.5 g sucrose, 1.0 g PEG_{6000}, and 0.175 g sodium chloride brought to 25 ml with distilled water, pH 7.0. The 2 ml virus preparation layered on the column was centrifuged at 70.1 G for 2 hr and the pellets resuspended overnight in 0.01 M phosphate buffer, pH 7.0. Pellets were then shaken and clarification was done at 10.3 G for 10 min.

Treatment 6: Precipitation of the TRV particles was made in PEG_{6000} (2%) 1:2 (v/v) and the non-suspended precipitate was removed by centrifugation at 10.3 G for 10 min.

Table 3. Treatments (described above) combined in the different purification methods.

	Treatments					
	1	2	3	4	5	6
Method A	+		+			
Method B	+		+	+		
Method C		+	+			
Method D		+	+		+	
Method E		+	+			+
Method F		+	+		+	+
Method G					+	

Electron Microscopy

Virus particle suspensions were (a) sprayed on Formvar-coated grids, (b) placed as a drop on a grid, or (c) mixed (1:1 v/v) with 5% glutaraldehyde and placed on a grid. After 5 min, any excess was blotted off. All preparations were stained with 2% sodium phosphotungstate.

Sucrose was eliminated from density-gradient fractions by either placing the grids on a wet sponge and adding several drops of the preparation per grid, or applying two or three drops of the preparation to the grid, allowing it to air-dry, then washing with distilled water from a Pasteur pipette.

Particle size measurements were made on electron micrographs and compared to micrographs of diffraction grating replicas of 2160 lines/mm.

Serology

An Ouchterlony agar double diffusion test formula adapted from methods developed at Braunschweig, West Germany (R.H. Converse, personal communication) was used. TRV antiserum No. PV AS 75 from the American Type Culture Collection (ATCC) was used. After reactions were complete, 0.05 M solutions of D L-3,4-dihydroxyphenylalanine (DOPA) (Madhosingh and Wood, 1971) in 0.1 M phosphate buffer pH 7.2, was applied. Crude sap from tobacco plants with TRV (PV-73-FD from ATCC), normal serum (at same dilutions as antiserum) and antihealthy serum prepared by R.H. Converse, were used as standard and controls, respectively.

RESULTS

Symptoms

Symptoms induced by the TRV isolate from potato fields are shown in Figure 2 and described in Table 4. Systemic symptoms developed on N. tabacum, and P. lunatus (Figure 2). Local symptoms developed within 72 hrs in N. tabacum and systemic symptoms developed within 6 days after inoculation. When tobacco plants were inoculated with high concentrations of TRV, severe stem mottling occurred as soon as 8 to 9 days after inoculation. The original inoculum was maintained by serial transfer on plants of N. tabacum for more than 1 year without any visible changes on infectivity and/or symptoms under controlled environment.

Primary symptoms on P. lunatus appeared 6 days after inoculation, while systemic symptoms took 18 to 30 days to develop. Some of the inoculated plants never showed systemic symptoms. Symptoms in C. quinoa appeared three days after inoculation and in C. sativus two to four days after inoculation. Other plant species such as Mentha piperita (peppermint) were inoculated but they did not show symptoms and virus could not be recovered from them.

Purification and Electron Microscopy

Yield: All purification methods yielded abundant virus particles that were infective and serologically active (Table 5). Purification method A was attempted twice and provided similar virus yields. Method B, which included a sucrose rate-zonal density

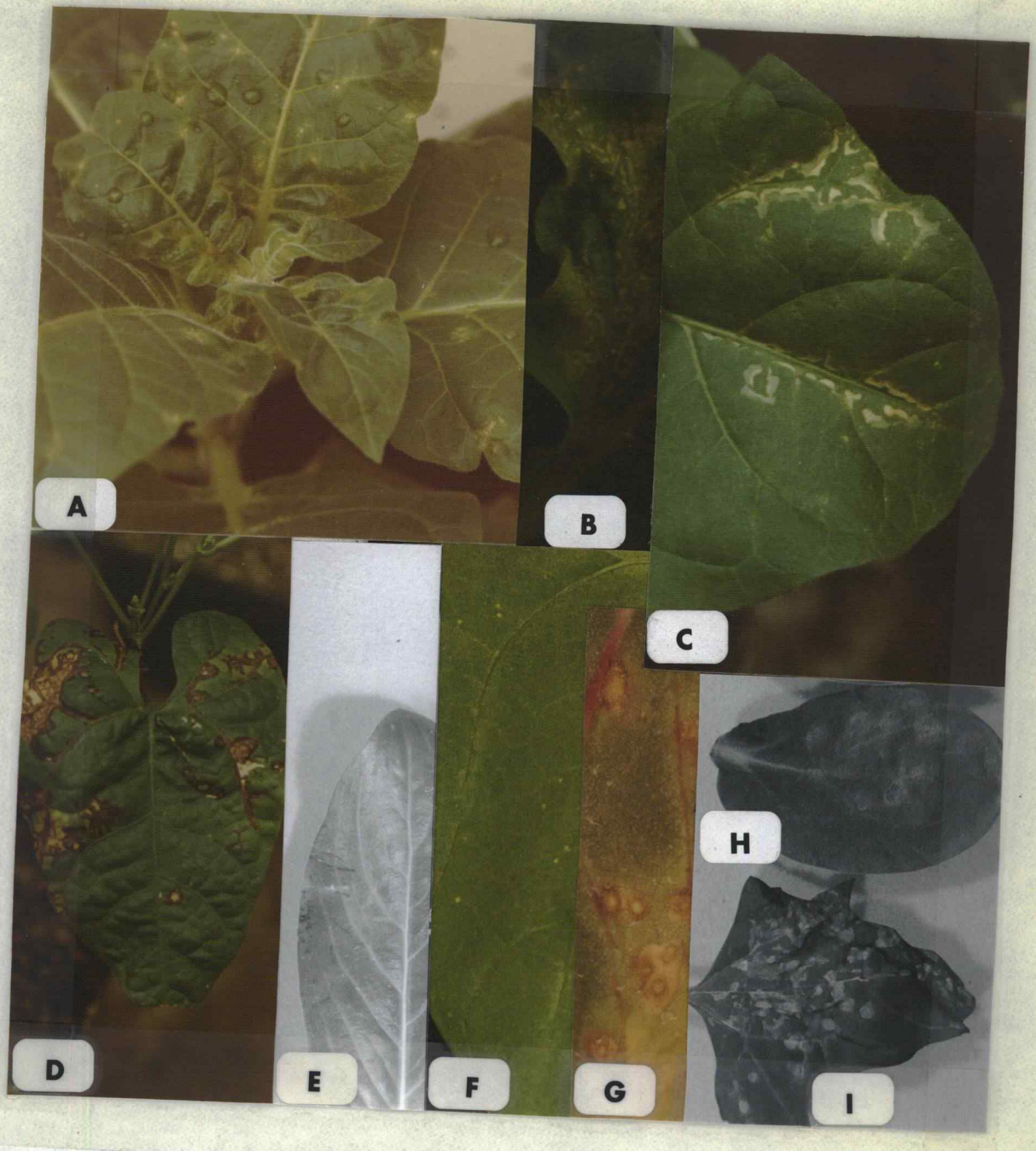


Figure 2. Symptoms on plants inoculated with a tobacco rattle virus isolate from Oregon potato fields. (A, B, C) Nicotiana tabacum cv. 'Samsun NN'. (D) Phaseolus lunatus 'Henderson's Bush'. (E) Vinca rosea 'Purity'. (F) Phaseolus vulgaris 'Bountiful'. (G) Gomphrena globosa. (H) Cucumis sativus. (I) Chenopodium quinoa

gradient, yielded less virus, as was expected. However, optical density (OD) readings produced two distinct peaks related to the two lengths characteristic of TRV (Figure 3).

Table 4. Description of symptoms on plants inoculated with a strain of tobacco rattle virus obtained from Solanum sarrachoides plants collected in Oregon potato fields.

Test plants	symptoms
Chenopodium quinoa	Chlorotic circles and triangular spots, generally chlorotic and coalescing.
Cucumis sativus	Diffuse, chlorotic rings and spots.
Gomphrena globosa	Red rings with chlorotic to brownish center. Often diffuse.
Nicotiana tabacum 'Samsun NN'	Initial chlorotic arcs, further browning, vein necrosis and leaf distortion. Stem mottle and splitting.
Phaseolus lunatus 'Henderson's Bush'	Dark red purple arcs with chlorotic centers, rings and spots on inoculated leaves. Systemic invasion of upper leaves, mostly causing solid red specks and occasionally circles.
Phaseolus vulgaris 'Bountiful'	Small dark lesion on young plants less than 10 days old, browner or lighther on older.
Vinca rosea 'Purity'	Black streaks.

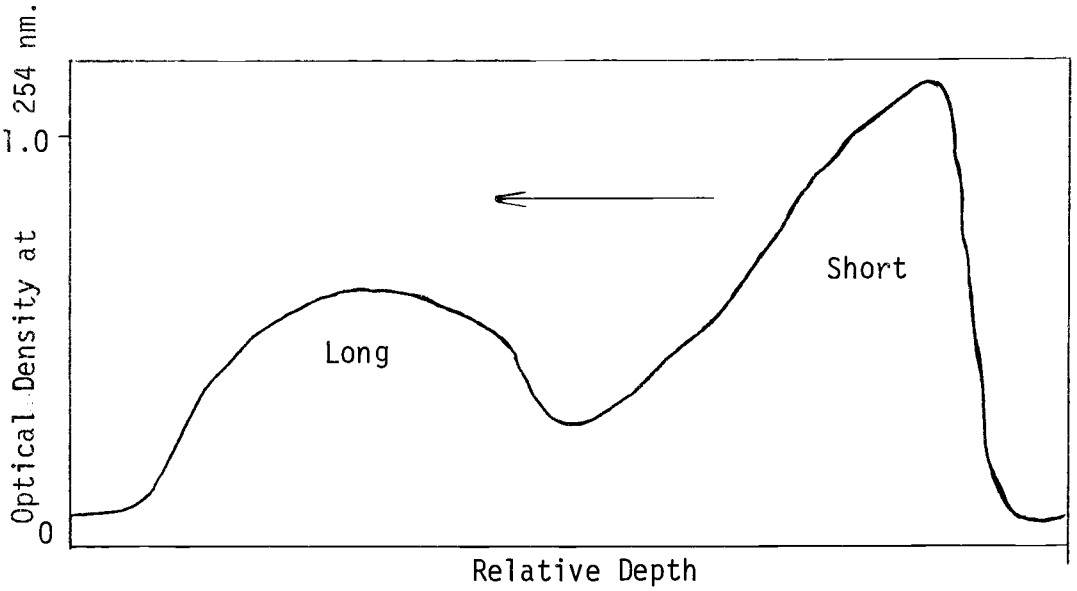


Figure 3. Absorbancy profile of density gradient centrifugation of tobacco rattle virus from Oregon potato fields. Arrow indicates direction of sedimentation.

Table 5. Yield and purity of tobacco rattle virus preparations obtained through different purification procedures.

Method	Yield in mg/kg	Purity as 260/280 ratios (nm). Correction for light scattering
A	13.0	1.11
A	15.7	1.18
B	8.5[1/]	1.12
C	56.0[2/]	1.52
D	-- [3/]	1.36
E	--	1.18
F	--	1.17
Supern. after E, F		1.52-1.60

1/ As estimated from area under absorbancy profile.
2/ High value due to impurities as revealed by high 260-280 ratio, reaction w/antihealthy serum, and large absorbancy readings in the supernatants when E and F followed.
3/ Not determined, only reduction estimated by the reading given by the supernatants.

No clear separation of long and short TRV particles was obtained with only one density gradient such as indicated by Harrison and Nixon (1959). Although method C provided a high absorbance reading, this can be attributed to excess of host material, probably ribosomal RNA. Methods D, E, and F, which included treatments subsequent to method C, caused reduction in absorbance. Much of the lost absorbance remained in the supernatant (Figure 4).

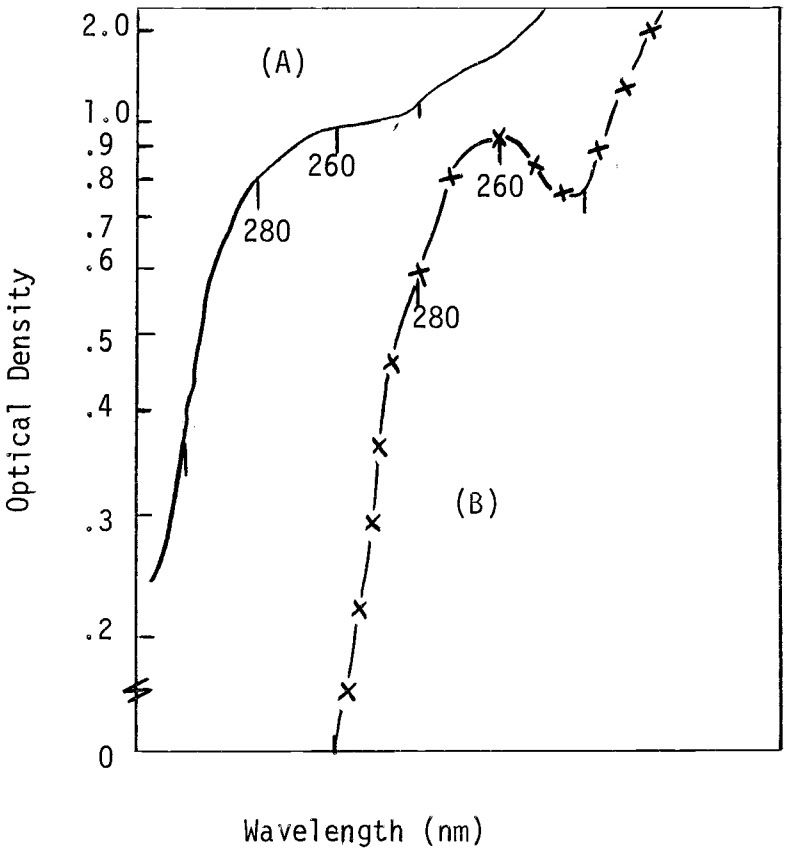


Figure 4. Ultraviolet spectra of supernatants from tobacco rattle virus preparations. A, obtained after sucrose-PEG gradient and PEG precipitation (method F). B, after precipitation with PEG only (method E).

Purity: The 260-280 ratios for method A were close to those reported in the literature (Lister and Bracker, 1969) (Table 5, Figure 5). The high value obtained by method C (Table 5) (56.0 mg/kg) was correlated to a high 260-280 ratio and indicated excessive host material. This also was indicated by the absorption spectra of supernatant fluids obtained after sucrose-PEG gradient and PEG precipitation (method F), and after precipitation with PEG only (method E) (Table 5, Figure 4). When fractions of these supernatant liquids were bioassayed on P. vulgaris L. cv. 'Bountiful', the infectivity was about one-tenth of that obtained from the resuspended pellets.

The addition of the sucrose-PEG gradient (method D) did not reduce the 260-280 ratio (Figure 5-4). Precipitation with PEG alone or added to the sucrose-PEG gradient (methods F and E, respectively) reduced the 260-280 ratio to 1.18 and 1.17 (Figure 5-1).

Particle Size: Purification methods and preparation for electron microscopy produced breakage of the particles which made particle size determination difficult.

Purification methods had some influence on particle breakage (Figures 6-9, 13-14). Methods C, D, E, and F slightly reduced particle breakage, compared to A and B. When unfixed preparations (stained with PTA) from methods A and F were compared, method F (Figure 8) provided a larger number of particles within the modal lengths than method A (Figures 7, 13-14). For method A, 7.8% of

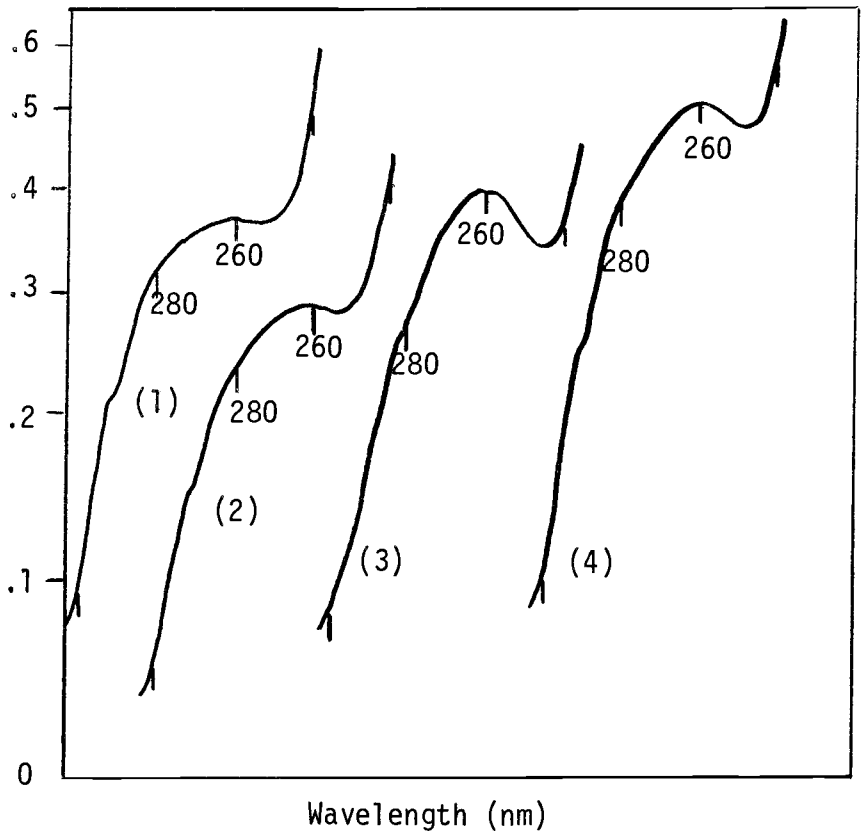
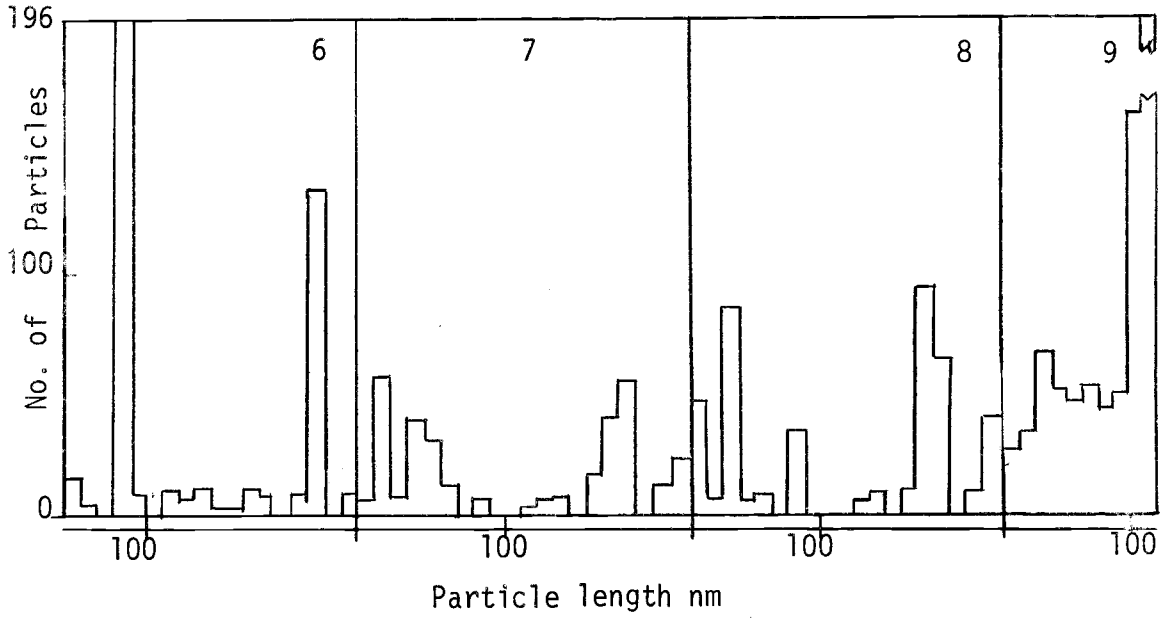


Figure 5. Comparison of ultraviolet spectra of tobacco rattle virus preparations obtained by (1) method F, (2) method A, (3) method C, and (4) method D. Lines for methods A and F show the smallest differences between measurements of optical density at 260 and 280 nm.

the total number of particles measured fell within the modal length for long particles, as compared to 15% from method F. For the short particles, comparable values were 1.5 and 7.5%, respectively.

However, the most relevant factor in reducing particle breakage was the fixation with glutaraldehyde (Figures 6, 11-12). When preparations obtained from methods C-G were fixed with glutaraldehyde, particle breakage was substantially reduced.



Figures 6-9. Histogram of 10 nm categories of particle length measurements made from enlargements of electron micrographs of tobacco rattle virus. (6) virus prepared by method F, fixed; (7) virus prepared by method A, not fixed; (8) virus prepared by method F, not fixed; (9) partial histogram from Harrison and Woods (1966) (modified).

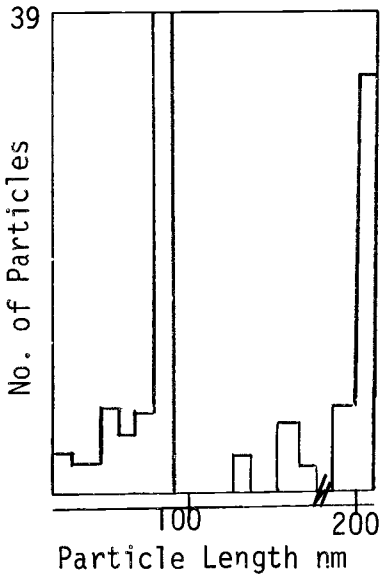
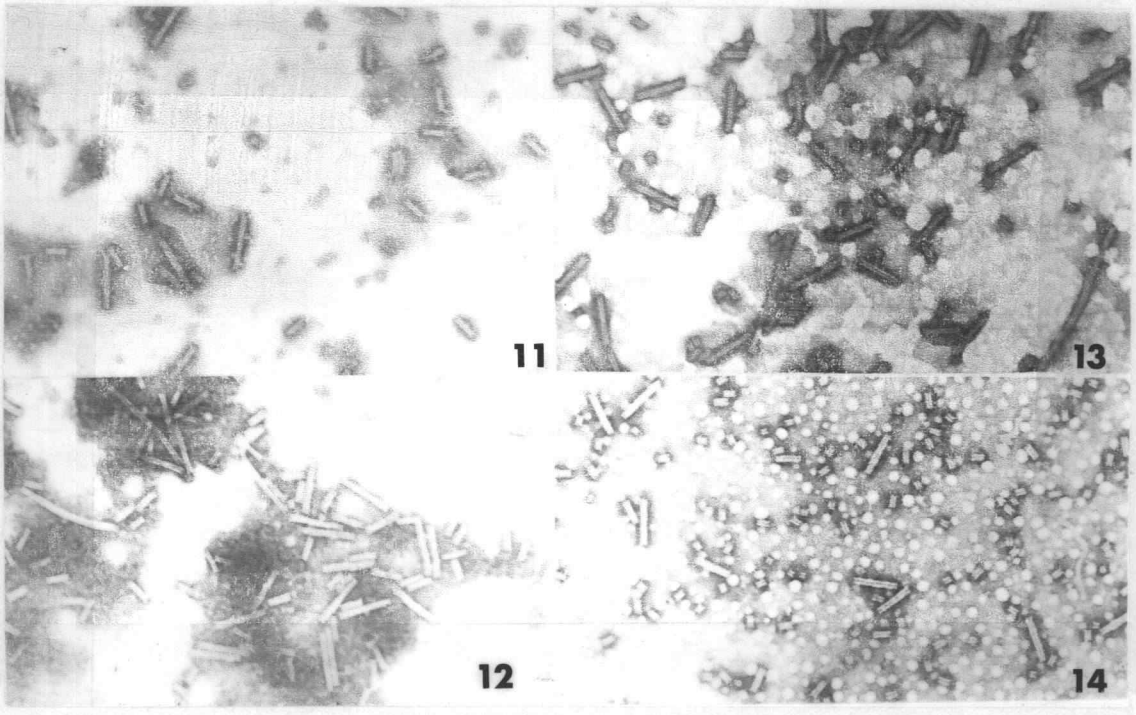


Figure 10. Histogram of 10 nm categories of particle length measurements made from enlargements of electron micrographs of tobacco rattle virus. The virus source was clarified sap after sucrose-PEG density gradient centrifugation.

Particle size determinations of fixed preparations stained with PTA were made on more than 400 particles. Measurements carried out to the nearest 2.7 nm and arranged on 10 nm intervals provided two well defined modal lengths, 206.7 and 85.4 nm for long and short particles, respectively. The width of the particles was estimated as approximately 26 nm by measuring across adjacent particles.

Modal lengths and proportions of particles obtained after clarified sap was layered on a sucrose-PEG gradient were similar to those obtained by the more elaborate methods just described (Figure 10).



Figures 11-14. Electron micrographs of negatively stained tobacco rattle virus preparations from method F fixed with glutaraldehyde; (13) method A not fixed; and (14) method C not fixed, extracted with borate buffer. Magnification 36284 X.

Serology

The serological identification test was carried out using both crude sap of tobacco plants directly inoculated from S. sarrachoides Serdt. and the purified virus preparation. Crude sap end point ranged from 1:8 to 1:64 dilution of the antiserum (titer 512), and 1:128 when testing the purified preparation at an approximate concentration of 0.1 mg/ml. The inocula always were compared to the ATCC (PV-73) culture as a standard and to juice from healthy plants. Normal serum also was used. No reaction with sap from healthy tobacco was detected with titer as low as 8 and normal serum at a titer of 16 showed no reaction to any inocula. Antihealthy serum did not react against antigens except for the supernatants previously mentioned, and antigen from method C.

DISCUSSION

Although recovered from a different potato-growing area in Oregon and a different host than the ATCC strain of TRV, the strain isolated from Oregon potato fields was remarkably similar in host range, symptoms, and serological reactions.

Purification methods provided varied estimates of yield and purity. The standard procedure (method A) using chloroform-butanol provided adequate and consistent yields and 260/280 ratios similar to those reported by other authors (Semancik, 1966; Lister, 1966; Lister and Bracker, 1969). Lister and Bracker reported a yield of 20 to 40 mg/kg when extraction was done from N. clevelandii processed 10 to 14 days after inoculation. Addition of sucrose rate-zonal density gradient reduced yield about 45% (method B). The chloroform procedure (method C) provided a high OD reading due to excessive amount of host material. This was revealed by large OD remaining in the supernatants when subsequent steps were carried out, and excessively high 260/280 ratios. Addition of sucrose-PEG gradient did not change the ratios (method D), obtained in method C. Precipitation with PEG following methods D or C (methods E and F, respectively) resulted in virus preparations with 260/280 ratios of 1.17 and 1.18 but reduced yields.

Although slightly more TRV particle breakage was obtained from the standard procedure (method A) compared to method F, glutaraldehyde fixation was most important in preventing breakage as was found by Hampton et al. (1974). About 84% of all particles

measured fell within the modal lengths, as compared to 47% shown by other authors (Harrison and Nixon, 1959).

Particle size determinations of preparations fixed with glutaraldehyde and stained with PTA provided well identifiable particle lengths, 206.7 and 85.4 nm (for 10 nm intervals), a 2.42 length ratio. The width of the particles was about 26 nm. The long to short ratio (2.42) and the size of short particles (85.4 nm) were within the values found in the literature; 1.79 to 3.21 (Allen, 1963; Lister, 1968) for ratios and 57 to 106 nm (Lister, 1968; Allen, 1963) for short particle size. These values were for the Oregon isolate. The long particles, however, were longer than previously reported.

The concentration of particles within the two modal lengths seemed to be the result of preventing particle breakage. Silberschmidt *et al.* (1971) demonstrated breaking points on TRV particles and indicated that milder handling of the virus resulted in less damage as well as in a larger proportion of long particles. Our results showed that there were 71% as many long particles as short particles, higher than commonly shown (Harrison and Nixon, 1959 and Lister and Bracker, 1969).

We believe that the fixation protected the particles and enhanced the accuracy of TRV particle-length determinations. Advantages of modifying the standard purification method to avoid particle breakage were negligible when compared to the benefits of using glutaraldehyde as a fixative.

Particle breakage seemed to be mainly an artifact of electron microscope preparatory methods. This phenomenon therefore would not have affected U.V. absorbancy characteristics or correlated infectivity measurements.

STUDY OF AN INTERACTION BETWEEN TOBACCO RATTLE VIRUS (TRV) AND POTATO VIRUS X (PVX)

INTRODUCTION

Recovery of tobacco rattle virus (TRV) from infected potato tubers is frequently difficult, and erratic incidence of the disease has complicated attempts to detect it (Van Hoof, 1964). In addition, TRV usually does not occur in above-ground portions of field-grown potato plants.

Although potato seed stocks are carefully tested, plants from inspected stocks are often found to be infected with another virus, potato virus X (PVX). Even virus-tested stock programs have not insured freedom from PVX.

Data presented previously in this thesis have demonstrated double infections of wild hosts with PVX and TRV. In those studies, detection of TRV by electron microscopy was complicated by the presence of PVX. Tobacco plants doubly infected with TRV and PVX showed variable symptoms which were not easy to differentiate from each other. TRV detection and transmission from potato plants also may be difficult because of natural TRV variability, but may be further impaired by the presence of PVX. PVX may also directly affect TRV transmission, detection, and symptoms.

Related viruses generally interact strongly. However, as Loebenstein (1972) indicated, most studies on depressive effects have been based on symptom expression rather than on concentration, so interference between unrelated viruses could be more common than

expected if quantitative data were available. A striking interaction between unrelated viruses was reported by Bawden and Kassanis (1945) where potato virus Y was depressed by severe etch. PVX has been shown to interact in different ways with other viruses (Rochow and Ross, 1955; Close, 1964; Goodman and Ross, 1974, a, b, c). However, despite their occurrence together in potatoes, an interaction between TRV and PVX has not been previously reported.

Therefore, we decided to study the effect of their interaction on TRV detection on plants of Nicotiana tabacum L. cv. 'Samsun NN'. The study was divided into two phases: (1) preliminary work, aimed at improving the sensitivity of the bioassay by (a) determination of the effect of age of Phaseolus vulgaris L. cv. 'Bountiful' plants on the sensitivity of the bioassay, and (b) development of a standard dilution curve of pure virus suspensions to be used in estimating virus concentrations; and (2) quantification of the interaction between TRV and PVX by (a) determining whether TRV concentration was influenced by sequence or time of entry of TRV or PVX, (b) studying the effect of initial TRV concentration on the interaction, and (c) studying the kinetics of the TRV-PVX interaction during various periods of incubation.

MATERIALS AND METHODS

General

Virus isolates studied were strains of TRV from wild hosts of Oregon potato fields and the PV-54 from the American Type Culture Collection for TRV and PVX, respectively. The viruses were maintained by serial transfer every 10 days on plants of N. tabacum L. cv. 'Samsun NN' which were kept in separate growth chambers providing 14 hrs light (7560 lux cool, white lamps) at 19 C, favorable to both viruses (Close, 1964, and Harrison, 1970).

Tobacco plants were grown in the greenhouse at approximately 21 C during the day and 18 C at night. Plants in the four- to five-leaf stage were selected for uniformity and moved to growth chambers 48 hrs before inoculation. Celite was added to plant extracts prior to mechanical inoculation.

For interaction studies, inoculum was applied on lower leaves of tobacco plants or the whole plant by 10 strokes/leaf with a pipe cleaner. Virus-containing sap was diluted 1:2 with 0.05 M phosphate buffer, pH 7.0. When TRV and PVX were inoculated to the same plants, equal quantities of sap from plants infected separately with each virus were mixed. Several leaves from tobacco plants singly or doubly inoculated were harvested. Unless otherwise indicated, extraction was made from inoculated leaves. Leaf tissue was harvested with a corkborer. An equal number of discs were removed from midribs as well as interveinal tissue from the same area of similar leaves for all treatments. Discs were weighed and weights equalized.

Infectivity was assayed by inoculating P. vulgaris L. cv. 'Bountiful' using the half-leaf method. Because minor variations in procedure caused major variation in resulting local lesion counts, the method we used is described in some detail. A notched piece of cardboard was used to support the leaf being inoculated. The petiole rested in the notch and the leaf blade lay flat on the cardboard. The tip of the leaf was held against the cardboard and the leaf was rubbed three times from tip to base with a pipe cleaner. Although assay results could be read at about 15 hrs after inoculation, readings were taken at 24 hrs. Infectivity of extracts from singly (TRV=A) and doubly (TRV + PVX = B) inoculated tobacco plants were compared on opposite half-leaves of 'Bountiful' beans, or by inoculating each inoculum A or B against a standard (S). When inoculation with TRV (A) and TRV + PVX (B) was carried out on opposite halves of 'Bountiful' beans, the data (number of lesions) were compared using a t-test for related samples and the results were expressed as the average of the A/B ratios. When inoculation of TRV and TRV + PVX was carried on opposite halves of 'Bountiful' beans against the standard, ratios R_A and R_B were obtained, respectively. They were compared using a t-test for unrelated samples and the results expressed as average ratios R_A, R_B, and as ratios R_R obtained by dividing average R_A/average R_B (=A/B). The standard (S) consisted of extracts from lower leaves of tobacco plants showing typical TRV symptoms 10 days after inoculation. When this standard was used, concentrations could be estimated by referring to the regression equation (described later) that related concentration to infectivity.

RESULTS

Plant Age

'Bountiful' beans 11, 14, and 17 days old, were inoculated with 1:6 and 1:12 dilutions of frozen sap on opposite half-leaves. In another experiment, plants 9, 11, 13, and 18 days old were inoculated with 1:1.5 dilution of crude sap from freshly inoculated tobacco plants. Within each age plants were selected with the same size of shoot measured from the insertion of the primary leaves to the apex of the first trifoliate leaf.

'Bountiful' bean plants were more sensitive at 11 days after seeding than at other ages (Table 6), regardless of the concentration of the inocula. For subsequent tests, TRV infectivity was assayed on 11-day old plants with a shoot of about 1 cm long measured as previously indicated.

Table 6. Response of Phaseolus vulgaris L. cv. 'Bountiful' of different ages to inoculation with different concentrations of tobacco rattle virus (TRV). Exp. 1 frozen sap; Exp. 2 fresh sap.

Age when inoculated[1/]	Average number of lesions/half leaf		
	Exp. 1 1:6 dilut.	Exp. 1 1:12 dilut.	Exp. 2 1:1.5 dilut.
9	-- [3/]	--	255 b
11	43.0 a [2/]	29.6 a	407 a
13	--	--	99 c
14	3.6 b	2.1 b	--
17	2.9 b	2.1 b	--
18	--	--	13 d

[1/] Days after seeding
[2/] Treatment averages within a column followed by the same letter
[3/] are not different at the 1 percent level of probability.
Not tested (--)

Dilution Curve

The relation of lesion numbers on inoculated leaves to concentration of purified TRV was determined to indicate the range over which infectivity was proportional to virus concentration. By knowing this range, the approximate dilutions of extracts from singly and doubly infected plants used to evaluate infectivity could be estimated. The curve obtained was used to provide an estimation of the virus concentration. TRV was purified following a procedure to be detailed elsewhere (Locatelli et al. unpublished data). Virus suspensions of TRV at concentrations ranging from 6.5×10^{-7} to 3.3×10^{-4} g/ml were prepared based on an extinction coefficient ($E^{0.1\%}_{260\ nm}$) of 3.0 (Harrison, 1970) expressed against a standard (S).

Plotting virus concentration ($OD_{260\ nm}$) against previously described infectivity ratios (R_A) resulted in an exponential curve. R_A was proportional to OD up to OD = 0.125 and R_A reached a plateau at OD = 0.5. Therefore, in order to estimate virus concentration (OD) in interaction studies, the region of six lower points of the curve (Figure15) was chosen. Transformation of the data to Log ($R \times 10^3$) and Log ($OD \times 10^3$) provided an almost perfect linear relationship ($r = 0.97$) whose coordinates are described by the equation.

Log ($R \times 10^3$) = 1.3962 + 1.0296 Log ($OD \times 10^3$) (I). Concentrations of TRV in g $\times 10^{-5}$/ml were estimated by transforming R values obtained in subsequent experiments to Log ($R \times 10^3$) and substituting them in equation I and using the published extinction coefficient for TRV.

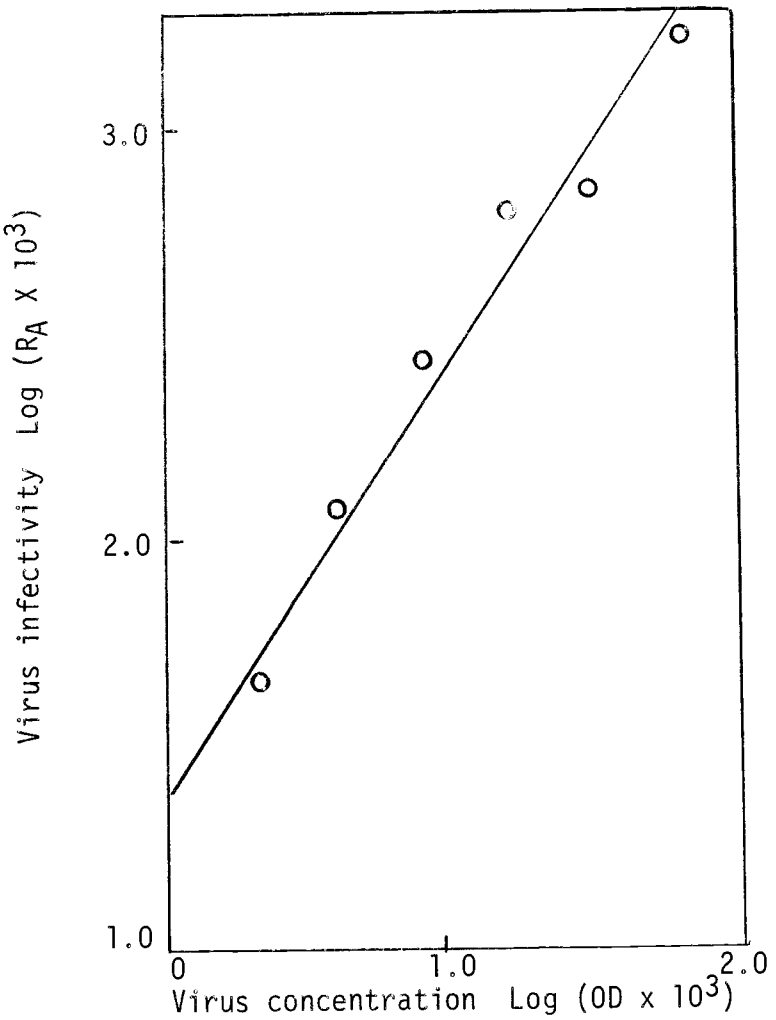


Figure 15. Regression line of the relationship between tobacco rattle virus infectivity ratios and OD transformed to Log (R_A x 10^3) and Log (OD x 10^3), respectively.

Effect of Sequence of Inoculation on the Interaction Between TRV and PVX

Simultaneous inoculations of TRV and PVX were carried out several times and extraction was accomplished at different intervals. Also, PVX was inoculated after TRV, and vice versa, at different intervals. Inoculation with the challenging virus was carried out

on either previously inoculated leaves or on leaves systemically invaded with the challenged inoculum in two different experiments. For inoculation on the same leaves, one group of tobacco plants was simultaneously inoculated with TRV and PVX, two were inoculated with TRV, three others with PVX, and four rubbed with buffer. TRV was inoculated on plants infected with PVX or rubbed with buffer at 48, 96, and 144 hrs. PVX was inoculated on plants previously infected with TRV and on a group of plants rubbed with buffer at 144 hrs after the first inoculation. On systemically invaded leaves, challenge inoculation with TRV or PVX was carried out 240 hrs after the first inoculation. Single inoculation was performed with TRV or PVX on buffer-rubbed plants.

Infectivity of TRV was reduced when extracted from tobacco leaves simultaneously inoculated with PVX and TRV. Values for A and B and R_A and R_B produced the following ratios: A/B = 1.52 and 1.65 for 3- and 14-day incubation periods, respectively, and R_R = 2.47 for a 10-day incubation period. The A and R_A values were always higher than comparable B and R_B values. The A/B ratios for the different incubation periods were similar. Average R_A ratios and average of R_B ratios obtained after 10 days of incubation gave approximate concentrations of 5.18×10^{-5} and 2.15×10^{-5} g/ml of TRV, respectively.

When extraction was made from systemically invaded leaves, the resulting number of lesions was too low to be statistically reliable. However, plants inoculated with TRV alone gave an average of 9 lesions per 12 half-leaves compared to 1.5 lesions induced by

juice from doubly inoculated plants, when the lower inoculated leaves were removed. The comparable average number of lesions for doubly inoculated plants with lower leaves not removed was 0.3.

Sequentially inoculating TRV on leaves already invaded with PVX produced A/B ratios very similar to those produced by simultaneous inoculation (Table 7). Extracts from singly inoculated leaves consistently showed more infectivity than extracts from comparable doubly inoculated plants. The interaction, as indicated by the ratios, was only slightly affected by the period of incubation with PVX. On the other hand, the interaction seemed to be slightly more affected by the order in which the two viruses were introduced, regardless of whether challenging inoculation was made on the same leaves or on systemically invaded leaves. In plants inoculated with TRV, 144 and 240 hrs before inoculation with PVX, TRV depression was higher than when PVX preceeded TRV, as indicated by the A/B ratios obtained (2.04, 2.65, Table 7).

Effect of TRV Dilution on the Interaction Between TRV and PVX

PVX inoculum was maintained at a constant level and TRV was serially diluted by mixing crude sap with buffer. Dilution varied from undiluted to 1:80. Low dilutions (1:4 to 1:6) all induced the same severity of symptoms at about the same time in singly inoculated tobacco plants. Higher dilutions (1:20 to 1:80) produced marked depression on TRV infectivity. The infectivity on bean half-leaves was significantly higher for singly than for doubly inoculated plants within each dilution. The A/B ratios increased

from undiluted to 1:40 dilution of TRV against a constant concentration of PVX inoculum indicating greater depression of TRV at greater dilutions. Average A/B ratios were 1.55, 2.01, 2.25, and 2.15 for undiluted, 1:20, 1:40, and 1:80 dilutions of TRV, respectively.

Table 7. Effect of time and sequence of entry of TRV and PVX on Nicotiana tabacum cv. 'Samsun NN'. Average ratios of number of lesions caused by TRV from extracts of singly inoculated tobacco leaves (A) to those from extract from comparable doubly infected (B) with TRV and PVX.

Challenging virus	Residual virus	Hours after inoculation with challenging virus	Ave ratios A/B [1/]	
TRV	PVX	0	1.65	
TRV	PVX	48	1.70	
TRV	PVX	96	1.52	[2/]
TRV	PVX	144	1.60	
PVX	TRV	144	2.04	
TRV	PVX	240	2.34	
PVX	TRV	240	2.65	[3/]

[1/] The average A values were significantly higher than B values at the 1 percent probability level using a t-test for related samples.

[2/] Inoculation on same leaves, upper leaves removed.

[3/] Inoculation on systemically-invaded leaves.

For subsequent tests, a dilution of 1:40 of TRV in 0.05 M phosphate buffer, pH 7.0 was adopted.

Kinetics of the Interaction TRV-PVX on Simultaneously Inoculated Leaves

Six groups of tobacco plants were inoculated at successive 24-hr intervals. Treatments applied to each group were: (a) TRV alone, (b) TRV + PVX, and (c) PVX alone. Upper leaves were removed 24 hrs prior to each inoculation. Inoculum from TRV-infected and PVX-infected tobacco plants was obtained prior to the first inoculation, and was partially clarified at 10.3 g for 10 min. Mixtures of TRV and PVX were prepared several minutes prior to inoculation. TRV inoculum was diluted 1:20 with buffer to be mixed with PVX and 1:40 to be inoculated alone. Extraction for infectivity assays against the standard (S) were done simultaneously for all groups 24 hrs after the last inoculation was completed.

There was a depression of TRV infectivity in doubly inoculated plants which varied with the period of incubation. The depression was statistically detectable by comparing R_A and R_B ratios with the exception of the 120 hrs incubation.

Both TRV and TRV + PVX infectivity measured as R_A, R_B, or TRV concentration increased (Figure 16) sharply between 24 and 48 hrs of incubation. At 48 hrs, TRV concentration was 5.3 times the concentration at 24 hrs. This indicated that virus remaining on the leaf surface had a negligible effect on infectivity. The infectivity curves in Figure 16 were nearly parallel. Concentration of TRV in extract from singly inoculated plants ranged from 1.06×10^{-6} g/ml at 24 hrs of incubation to 2.36×10^{-5} g/ml at 144 hrs and from 3.98×10^{-7} to 1.13×10^{-5} g/ml for extract from doubly inoculated

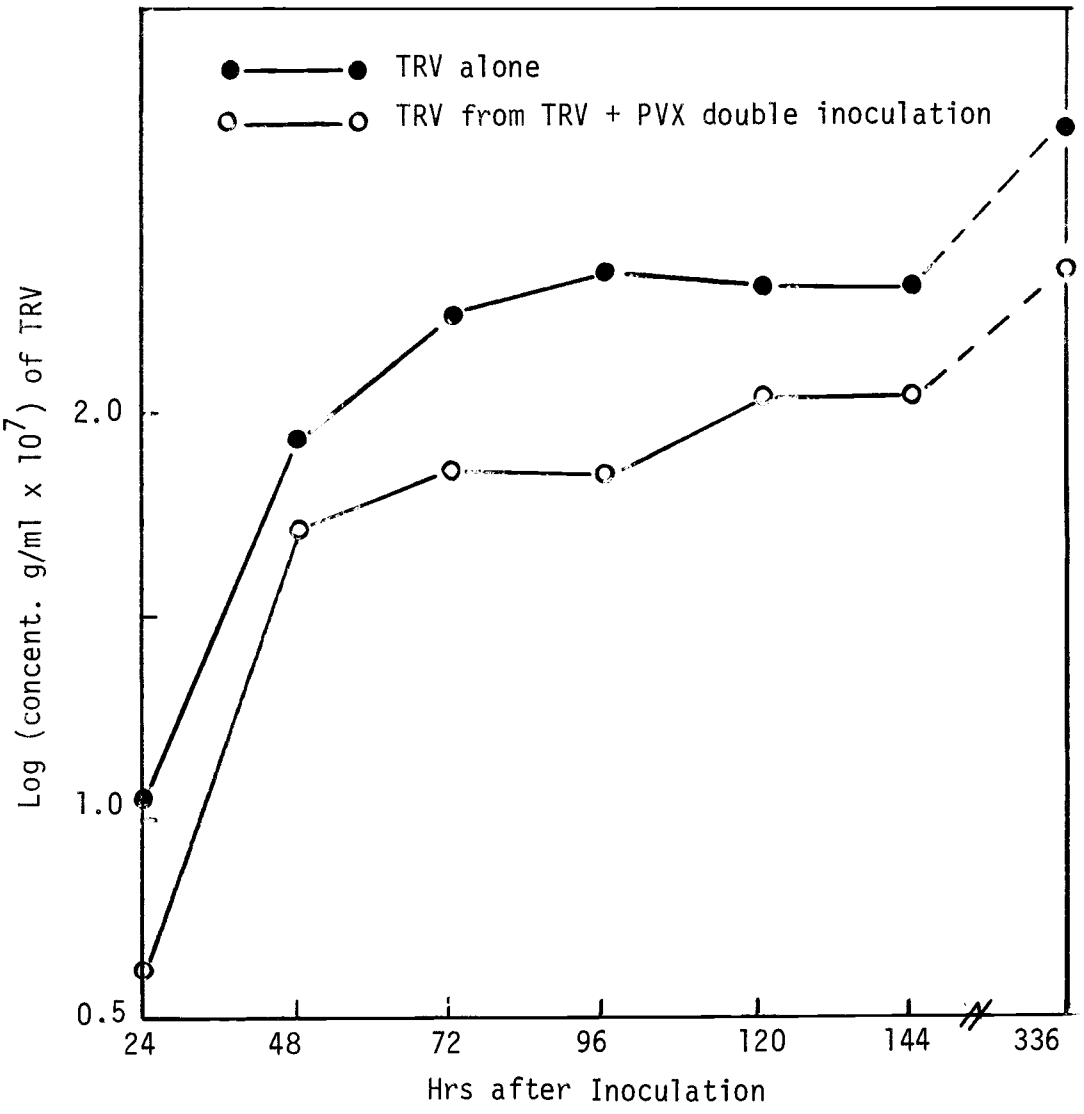


Figure 16. Time course of infection of TRV alone and of TRV in the presence of PVX in tobacco.

plants. There were large differences in R_R ratios (R_A/R_B). At 24 hrs, extract from singly inoculated plants was 2.7 times more infective than that from doubly inoculated plants. This is attributed to the fact that at low lesion levels the ratios were easily changed by the occurrence of a few lesions. Very few lesions were obtained from 24 hrs of incubation, and although extracts from singly inoculated plants gave significantly larger numbers, they were not reliable requiring extrapolation.

Additional studies indicated that infectivity of TRV alone in 'Samsun NN' tobacco increased up to about 15 days after inoculation. Infectivity of extracts from plants singly inoculated with TRV extracted 336 hrs after inoculation was substantially higher (3.98×10^{-5} g/ml of TRV) than that obtained 96 hrs following inoculation (2.36×10^{-5}). However, the interaction did not vary accordingly. After 48 hrs of incubation, TRV alone was 1.9 times more infective than TRV + PVX, and increased to 2.4 at 72 hrs. The ratios showed that infectivity from singly inoculated tobacco plants was 3.0 times as much as the infectivity of comparable extracts from doubly inoculated plants after 96 hrs of incubation. The ratio reached the maximum at 96 hrs, then dropped to 2.1 and 1.9 at 144 and 336 hrs, respectively. These data are interpreted to indicate that the depression of TRV induced by PVX remained relatively constant after reaching a maximum level a few days following inoculation.

Effect of the Interaction TRV-PVX on Symptom Expression and Appearance

Symptoms on tobacco plants singly inoculated with TRV or PVX were evident 72 hrs after inoculation. However, in many instances, TRV related symptoms did not appear at all. TRV symptoms which developed in inoculated leaves a few hrs after inoculation consisted of typical arcs and chlorotic flecks (Figure 17-B, C). Often vein necrosis developed later, resulting in leaf distortion. PVX incited typical mottling, and chlorotic and precisely delimited target spots (Figure 17-D).

The most distinctive symptoms, however, were provided by simultaneous inoculation of TRV and PVX (Figure 17-E). Frequently, symptoms appeared sooner on doubly than on singly inoculated leaves. This was observed in almost all experiments, regardless of whether the whole plant or only the lower leaves were inoculated. Symptoms in doubly inoculated plants were characterized by numerous sunken lesions where the epidermis was sunken. PVX symptoms in doubly inoculated leaves were altered. Target spots were not so obvious and there were less than those in singly inoculated plants. Varying the sequence and time of inoculation did not seem to drastically affect the previously described symptomatology, except that symptoms were less consistent than on simultaneous inoculation. In one instance, plants inoculated with TRV did not show any symptoms; however, when these plants were challenged with PVX 144 hrs later, sunken lesions developed after four days.

High dilution of TRV produced marked decrease in severity of

symptoms in singly inoculated tobacco plants. Doubly inoculated plants showed much more severity of symptoms than plants inoculated singly with either TRV or PVX (Figure 18-A, C). As dilution of TRV was increased, the symptoms in doubly inoculated plants were more severe than symptoms on plants inoculated only with TRV. The damage induced by the 1:40 dilution of TRV in mixed inoculation with PVX was comparable to that induced by undiluted TRV in singly inoculated plants.

The variability of TRV on symptoms appearance was commonly observed throughout all experiments. Despite this fact, infectivity of extracts from singly inoculated plants was considerably higher than in those from doubly invaded plants.

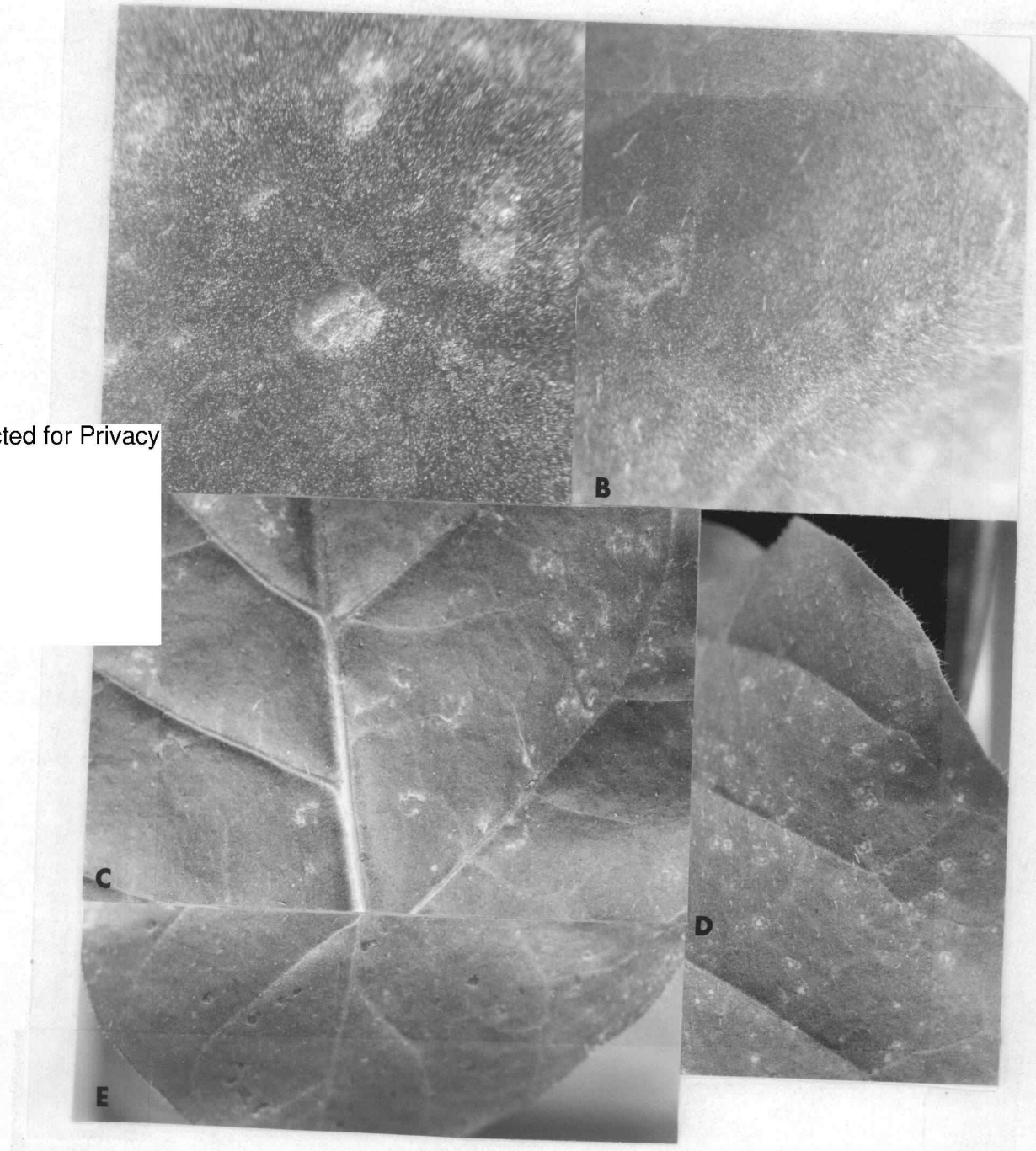


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Figure 17. Symptoms caused in Nicotiana tabacum L. cv. 'Samsun NN' by single and double inoculation with tobacco rattle virus (TRV) and potato virus X (PVX). (A) TRV + PVX, close-up of sunken lesion typical of double inoculation; (B) close-up of arc caused by TRV alone; (C) TRV lesions; (D) PVX lesions; (E) TRV + PVX sunken lesions.

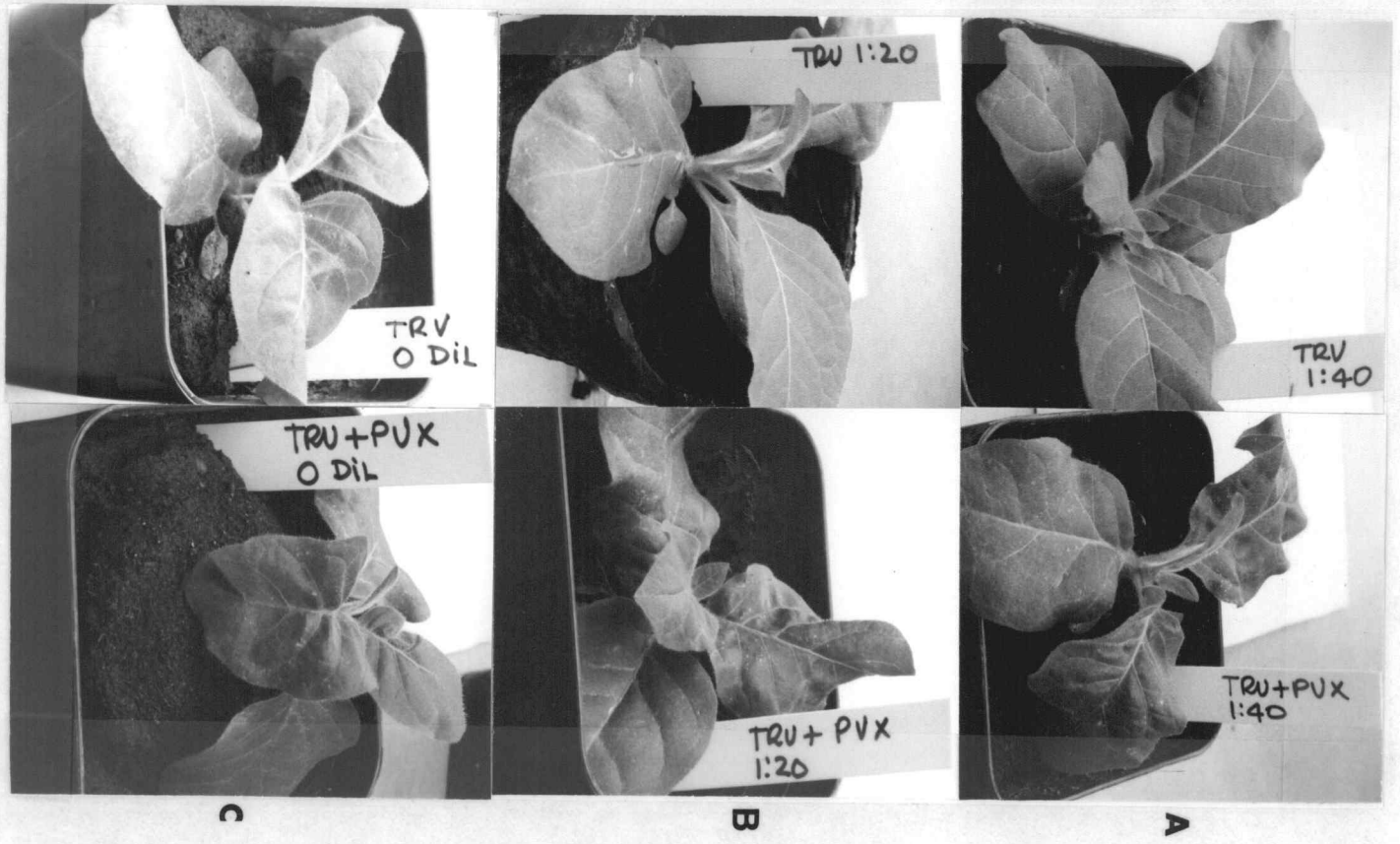

Figure 18. Effect of TRV dilution on singly and doubly inoculated plants of Nicotiana tabacum L. cv. 'Samsun NN'. (A-C) Comparison of singly (TRV) and doubly (TRV + PVX) inoculated plants at decreasing dilutions of TRV.

DISCUSSION

Improved bioassay and inoculation techniques, as well as selection of the proper dilutions of different extracts, permitted work at concentrations where infectivity was proportional to virus concentration. Estimated concentrations ranged from 10^{-7} to 10^{-5} g/ml of TRV, within the range suggested by Loring (1937) to detect differences in concentration of tobacco mosaic virus when bioassayed on P. vulgaris.

Experimental design permitted the detection of a depression of TRV infectivity caused by PVX on tobacco plants. This depression was consistent throughout all experiments. Although inoculation on systemically infected leaves caused the ratios TRV/TRV + PVX (A/B) to be high, they were not larger than the maximum ratio obtained by simultaneous inoculation. Studies on systemically invaded leaves were impaired by the variability of TRV which prevented the selection of leaves with inoculum capable of providing a statistically reliable number of lesions. The interaction also seemed to be independent of TRV and PVX multiplicative cycles in tobacco plants. Assays made beyond the sixth day indicated that TRV concentration continued to increase, and Stouffer and Ross (1961) showed that PVX concentration increased up to the eighteenth day. However, there were no perceptible changes in the TRV-PVX interaction after 96 hrs of incubation.

The drastic changes in symptom expression and time of symptom appearance also suggested an active interaction between TRV and PVX

on doubly inoculated tobacco plants. The appearance of sunken lesions was diagnostic of the interaction and was triggered in different ways. Normally, they appeared with simultaneous inoculation; however, in one instance they also showed up when PVX was inoculated on TRV invaded leaves.

The effect on symptoms cannot be interpreted as additive or synergistic because of the inherent TRV variability, especially in singly inoculated tobacco plants. In many instances, TRV symptoms could not be associated with infectivity since symptomless tobacco plants singly inoculated with TRV diluted 1:20 or more could provide inoculum of relatively high infectivity. On the other hand, comparable tobacco plants inoculated with TRV diluted 1:20 or more while PVX concentration was held constant, showed marked symptoms. This included severe stunting but the leaf extracts were less infective. Possibly this could be considered as a synergistic effect when compared to symptomless tobacco plants singly inoculated with TRV. However, it most likely indicates that there is a threshold level of TRV concentration in tobacco plants which must be surpassed to produce symptoms when only TRV is in tobacco.

The system we established in tobacco plants and evaluated in 'Bountiful' beans might help in the interpretation of some of the problems encountered on TRV detection in potatoes. Although TRV concentrations in naturally infected potatoes seemed to be much lower than the TRV concentration levels we have used, the results obtained may predict what happens at these levels. Several tests have shown that a very small number of lesions are produced on

'Bountiful' beans inoculated with sap from TRV-infected tubers. This low number of lesions is commonly associated with conspicuous symptoms commonly attributed to TRV. Our data, however, suggest the possibility that these symptoms were caused by the presence of PVX and TRV instead of TRV alone in the tubers. Consequently, a combination of (a) increased symptoms in tubers, probably caused by TRV + PVX, (b) symptomless plants, affected by low levels of TRV, and (c) depression of TRV triggered by PVX, could explain some of the problems encountered when screening tubers for the presence of TRV. It is valid to assume that if TRV concentration on naturally infected potato plants and tubers is considerably lower than the ones we worked with, the depressive effect of PVX on TRV could be even higher than that which we have reported here. Consequently, more sensitive bioassays should be devised for further studies on the effect of the interaction of TRV and PVX in potatoes.

The fate of PVX has not been investigated in the present work and we do not know whether TRV depression is paralleled by PVX enhancement.

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APPENDIX

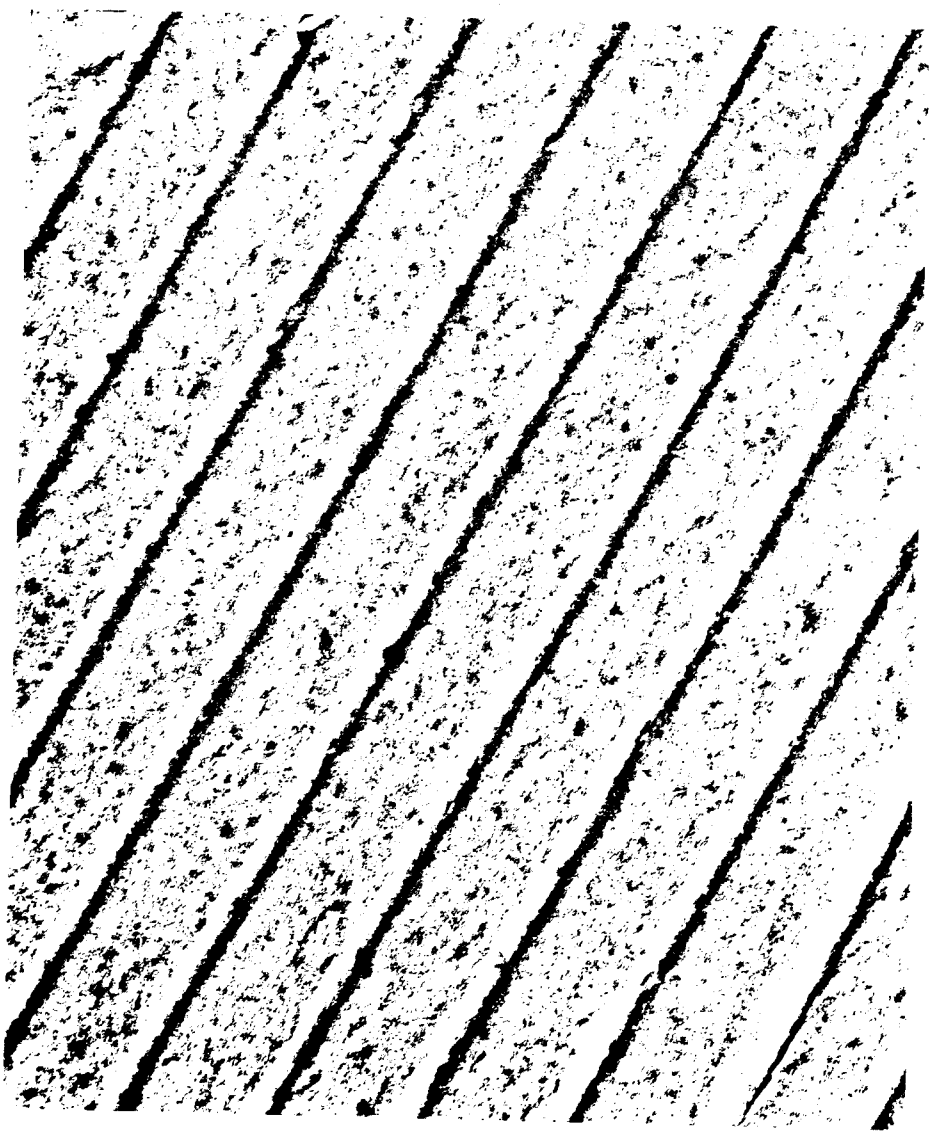

Figure 1. Micrograph of diffraction grating replica of 2160 lines/mm. Used to measure TRV particle size.

Table 1. Data for histogram from photo enlargement of electron micrographs 14127-8. Method F not fixed with glutaraldehyde. Tobacco rattle virus.

Class interval nm	Number of particles
20-30	48
30-40	6
40-50	87
50-60	8
60-70	9
70-80	0
80-90	34
90-100	0
100-110	0
110-120	2
120-130	6
130-140	7
140-150	0
150-160	11
160-170	93
170-180	64
180-190	0
190-200	11
200-210	70
	456

Table 2. Data for histogram from photographic enlargement of electron micrographs 13226-7. Method A not fixed with glutaraldehyde. Tobacco rattle virus.

Class interval nm	Number of particles
10-20	7
20-30	58
30-40	4
40-50	41
50-60	30
60-70	13
70-80	0
80-90	5
90-100	1
100-110	0
110-120	2
120-130	4
130-140	6
140-150	0
150-160	17
160-170	39
170-180	56
180-190	0
190-200	12
200-210	25
	320

Table 3. Data for histogram from electron micrograph enlargements of plates 14196,97. Method F fixed with glutaraldehyde. Tobacco rattle virus.

Class interval nm	Number of particles
30-40	0
40-50	11
50-60	15
60-70	3
70-80	0
80-90	196
90-100	4
100-110	0
110-120	4
120-130	3
130-140	4
140-150	1
150-160	0
160-170	5
170-180	4
180-190	0
190-200	6
200-210	139
210-220	0
220-230	3
230-240	0
240-250	2
250-260	0
	400

Table 4. Data for histogram of clarified sap + SUC-PEG obtained from enlargements of electron micrographs. Method G fixed with glutaraldehyde. Tobacco rattle virus.

Class interval nm	Number of particles
20-30	3
30-40	2
40-50	2
50-60	7
60-70	5
70-80	0
80-90	39
90-100	0
100-110	0
110-120	0
120-130	0
130-140	3
140-150	0
150-160	0
160-170	6
170-180	2
180-190	0
190-200	7
200-210	34
	110

Table 5. Plant age. Time of inoculation of Phaseolus vulgaris L. cv. 'Bountiful'. Local lesions obtained by inoculation with fresh sap from TRV infected tobacco diluted 1:6 and 1:12.

Age when inoculated	Dilution	Observations L. lesion/half leaf 'Bountiful' beans										
		I	II	III	IV	V	VI	VII	VIII	IX	X	Ave.
17	1:6	3	3	3	5	6	3	3	2	1	0	2.9
	1:12	3	2	1	2	3	1	3	2	3	1	2.1
14	1:6	3	2	3	5	3	3	3	2	4	8	3.6
	1:12	0	0	2	1	1	1	2	2	3	9	2.1
11	1:6	39	30	15	24	39	31	50	110	40	32	43.0
	1:12	18	40	6	26	26	26	32	18	60	40	29.2

1/ Days after seeding.

ANOVA 1:6 values				
Source	SS	DF	MS	F
Total	17,001.5	29		
Treatment	10,536.2	2	5268.1	22.0**
Error	6,465.3	27	239.45	

$LSD_{.05}$ = 14.2

ANOVA 1:12 values				
Source	SS	DF	MS	F
Total	6973	29		
Treatment	4896	2	2448	31.8**
Error	2077	27	76.9	

$LSD_{.05}$ = 8.05

**Significant at the 1% probability level

Table 6. Plant age. Time of inoculation of Phaseolus vulgaris L. cv. 'Bountiful'. Local lesions obtained by inoculation with fresh sap from TRV infected tobacco diluted 1:1.5.

Age when inoculated[1/]	Observations L. lesion/half leaf in 'Bountiful' beans						
	I	II	III	IV	V	VI	Ave
9	200	239	286	270	251	285	255
11	391	289	529	434	418	384	407
13	85	68	99	84	136	126	99
18	20	16	18	5	6	12	13

[1/] Days after seeding

ANOVA

Source	DF	SS	MS	F
Total	23	585760		
Treat.	3	545417	181805	90.0**
Error	20	40343	2018	

$LSD_{.01\%} = 74.0$

**Significant at the 1% probability level.

Table 7. Data for dilution curve of pure TRV suspensions. Ratios of local lesions (R_A) obtained by inoculating 'Bountiful' beans with each TRV suspension against a standard 1:8 dilution of TRV infected sap extracted 10 days after inoculation of tobacco plants.

$OD_{260_{nm}}$	Observations R_A[1/]								
	I	II	III	IV	V	VI	VII	VIII	Ave.
1.000	2.428	1.984	2.063	2.143	2.499	4.054	3.000	2.058	2.529
0.500	2.130	2.028	3.130	2.635	2.883	2.975	1.978	3.273	2.630
0.250	1.751	1.886	1.966	2.248	2.673	2.978	2.020	2.480	2.250
0.125	1.662	1.921	2.394	2.012	2.330	2.676	1.520	2.842	2.181
0.063	1.021	1.650	1.460	1.286	1.396	1.489	1.263	1.507	1.384
0.032	0.773	0.865	1.066	0.875	1.094	1.190	0.817	1.100	0.972
0.016	0.302	0.364	0.512	0.700	0.534	0.647	0.400	0.620	0.510
0.008	0.163	0.200	0.289	0.235	0.263	0.326	0.192	0.300	0.246
0.004	0.083	0.089	0.093	0.126	0.128	0.131	0.114	0.102	0.108
0.002	0.023	0.044	0.063	0.027	0.060	0.042	0.025	0.061	0.043

[1/] Local lesion TRV/local lesion standard in 'Bountiful' beans.

Table 8. Data for dilution curve of pure TRV suspensions. Ratios of local lesions (R_A) obtained by inoculating 'Bountiful' beans with each TRV suspension against a standard 1:8 dilution of TRV infected sap extracted 10 days after inoculation of tobacco plants.

$OD_{260_{nm}}$	Observations R_A[1/]								
	I	II	III	IV	V	VI	VII	VIII	Ave.
0.063	1.021	1.650	1.460	1.286	1.396	1.489	1.263	1.507	1.384
0.032	0.773	0.865	1.066	0.875	1.094	1.190	0.817	1.100	0.972
0.016	0.302	0.364	0.512	0.700	0.534	0.647	0.400	0.620	0.510
0.008	0.163	0.200	0.289	0.235	0.263	0.326	0.192	0.300	0.246
0.004	0.083	0.089	0.093	0.126	0.128	0.131	0.114	0.102	0.108
0.002	0.061	0.023	0.044	0.063	0.027	0.060	0.042	0.025	0.043

[1/] R_A = local lesion TRV suspension/local lesion standard in 'Bountiful' beans.

Table 9. Data for dilution curve of pure TRV suspensions. Logarithmic transformation [Log (1000 x R_A)] of ratios of local lesions obtained by inoculating 'Bountiful' beans with each TRV suspension against a standard 1:8 dilution of TRV infected sap extracted 10 days after inoculation of tobacco plants.

$OD_{260_{nm}}$	Log (1000 R_A)[1/]								
	I	II	III	IV	V	VI	VII	VIII	Ave.
0.063	3.009	3.217	3.164	3.109	3.145	3.173	3.101	3.178	3.137
0.032	2.888	2.937	3.028	2.942	3.039	3.076	2.912	3.041	2.983
0.016	2.480	2.561	2.709	2.845	2.728	2.811	2.602	2.792	2.691
0.008	2.212	2.301	2.461	2.371	2.420	2.513	2.283	2.477	2.380
0.004	1.919	1.949	1.968	2.100	2.107	2.117	2.057	2.009	2.028
0.002	1.362	1.643	1.799	1.431	1.778	1.623	1.398	1.785	1.602

[1/] R_A = local lesion TRV/local lesion standard in 'Bountiful' beans.

Table 10. Effect of simultaneous inoculation on TRV-PVX interaction in tobacco plants. Local lesions obtained by inoculating 'Bountiful' beans with extracts from singly (A) and doubly (B) inoculated tobacco plants. Extracted 3 days after inoculation and diluted 1:6 at extraction.

	Observations L. lesion/half leaf 'Bountiful' beans												
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Ave
A	69	98	82	125	120	189	223	260	301	349	202	285	191.0
B	125	50	42	99	93	97	139	169	189	234	168	149	129.5
A/B	.55	1.96	1.95	1.26	1.29	1.95	1.60	1.54	1.59	1.49	1.20	1.91	1.52
Diff.	-56	42	40	26	27	92	84	91	112	115	34	136	61.5
5.55**													

1/ Significant at the 1% probability level using a paired t-test

Table 11. Effect of simultaneous inoculation on TRV-PVX interaction in tobacco plants. Ratios of local lesions obtained by inoculating 'Bountiful' beans with extracts from singly (R_A) and doubly (R_B) inoculated tobacco plants against a standard TRV preparation. Extracted 10 days after inoculation and diluted 1:10 at extraction.

	Observations R_A, R_B[1/]													Ratio R_R (Ave R_A/Ave R_B
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Ave.	
R_A	0.45	0.33	0.54	0.46	0.31	0.38	0.57	0.52	0.29	0.37	0.31	0.52	0.42	2.47
R_B	0.08	0.16	0.09	0.18	0.16	0.12	0.16	0.36	0.20	0.16	0.25	0.10	0.17	

t = 6.77**

[1/] R_A = local lesion TRV/local lesion standard in 'Bountiful' beans.
R_B = local lesion TRV = PVX/local lesion standard in 'Bountiful' beans.

**Significant at the 1% probability level using an unpaired t-test.

Table 12. Effect of simultaneous inoculation on TRV-PVX interaction in tobacco plants. Local lesions obtained by inoculating 'Bountiful' beans with extracts from singly (A) and doubly (B) inoculated tobacco plants. Extracted 14 days after inoculation and diluted 1:6 at extraction.

	Observations L. lesion/half leaf 'Bountiful' beans								
	I	II	III	IV	V	VI	VII	VIII	Ave.
A	291	442	424	356	482	273	195	289	356
B	159	312	326	241	296	254	86	157	228
A/B	1.83	1.41	1.30	1.48	1.63	1.46	2.27	1.84	1.65
Diff.	132	130	98	115	186	118	109	132	127.5

t = 13.6**

**Significant at the 1% probability level using a paired t-test.

Table 13. Effect of time and sequence on TRV and PVX entry on the TRV-PVX interaction in tobacco plants. Local lesions obtained by inoculating 'Bountiful' beans with extracts from singly (A) and doubly (B) inoculated tobacco plants. Extracted 10 days after inoculation and diluted 1:10 at extraction.

	Observations L. lesion/half leaf 'Bountiful' beans									
	I	II	III	IV	V	VI	VII	VIII	IX	Ave
A48	402	365	169	125	504	277	508	150	426	325
B48[1/]	243	200	99	86	293	182	327	75	228	193
A/B	1.65	1.82	1.70	1.46	1.72	1.52	1.55	2.01	1.87	1.70
t = 6.27**										

[1/] A48 = TRV inoculated 48 hrs after PVX was inoculated on other plants.
B48 = TRV inoculated 48 hrs after PVX was inoculated on the same leaves.

**Significant at the 1% probability using a paired t-test.

Table 14. Effect of time and sequence of TRV and PVX entry on the TRV-PVX interaction in tobacco plants. Local lesions obtained by inoculating 'Bountiful' beans with extracts from singly (A) and doubly (B) inoculated tobacco plants. Extracted 10 days after inoculation and diluted 1:10 at extraction.

	Observations L. lesion/half leaf 'Bountiful' beans									
	I	II	III	IV	V	VI	VII	VIII	IX	Ave
A96	573	324	321	478	308	552	637	194	57	382
B96[1/]	384	250	204	287	301	395	403	115	29	263
A/B	1.49	1.30	1.57	1.67	1.02	1.40	1.58	1.69	1.97	1.52
t = 4.55**										

[1/] A96 = TRV inoculated 96 hrs after PVX was inoculated on other plants.
B96 = TRV inoculated 96 hrs after PVX was inoculated on the same leaves.

**Significant at the 1% probability level using a paired t-test.

Table 15. Effect of time and sequence of TRV and PVX entry on the TRV-PVX interaction in tobacco plants. Local lesions obtained by inoculating 'Bountiful' beans with extracts from singly (A) and doubly (B) inoculated tobacco plants. Extracted 10 days after inoculation and diluted 1:10 at extraction.

	Observations L. lesion/half leaf 'Bountiful' beans									
	I	II	III	IV	V	VI	VII	VIII	IX	Ave
A144	253	241	231	210	333	281	273	303	265	265
B144[1/]	216	153	144	145	222	180	156	160	135	169
A/B	1.17	1.58	1.60	1.45	1.50	1.49	1.75	1.89	1.95	1.60
Diff.	37	88	87	65	111	93	117	143	130	97
t = 8.86**										

[1/] A144 = TRV inoculated alone 144 hrs after PVX was inoculated on other plants.
B144 = TRV inoculated 144 hrs after PVX was inoculated on the same leaves.

**Significant at the 1% probability level using a paired t-test.

Table 16. Time and sequence of TRV and PVX entry on the TRV-PVX interaction in tobacco. PVX inoculated 144 hrs after TRV on same leaves. Local lesions obtained by inoculating 'Bountiful' beans with extract from singly (A) and doubly (B) inoculated tobacco plants. Extracted 10 days after inoculation and diluted 1:4 when extracted.

	Observations L. lesion/half leaf 'Bountiful' beans												
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Ave.
A	146	108	153	107	90	198	225	76	125	77	80	195	132
B	101	63	60	54	92	79	107	63	35	37	32	103	68
A/B	1.44	1.71	2.55	1.98	0.98	2.51	2.10	1.20	3.67	2.08	2.5	1.89	2.04
Diff.	45	45	93	53	-2	119	107	13	90	40	48	92	61.9

t = 5.38**

**Significant at the 1% probability level using a paired t-test.

Table 17. Effect of time and sequence of TRV and PVX entry on the TRN-PVX interaction in tobacco TRV inoculated on PVX systemically invaded leaves 240 hrs after PVX inoculation. Local lesions obtained by inoculating 'Bountiful' beans with extracts from singly (A) and doubly (B) inoculated tobacco plants. Extracted 6 days after inoculation and diluted 1:2 at extraction.

	Observations L. lesion/half leaf 'Bountiful' beans												
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Ave.
A	125	76	98	177	49	187	201	115	49	120	60	100	113
B	66	32	45	91	49	62	36	45	20	130	20	85	57
A/B	1.89	2.38	2.18	1.94	1.0	3.01	5.58	2.55	2.45	0.92	3.0	1.18	2.34
Diff.	59	44	53	86	0	125	165	70	20	-10	40	15	57.1

t = 4.08**

**Significant at the 1% probability level using a paired t-test.

Table 18. Effect of time and sequence of TRV and PVX entry on the TRV-PVX interation in tobacco. PVX inoculated on TRV systemically invaded leaves 240 hrs after TRV inoculation. Extracted 6 days after inoculation and diluted 1:2 at extraction.

	Observations L. lesion/half leaf in 'Bountiful' beans												
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Ave.
A	312	239	445	296	326	298	179	117	329	398	317	251	292
B	83	112	123	107	106	98	70	136	121	147	108	143	112
A/B	3.75	2.13	3.62	2.76	3.07	3.04	2.55	0.86	2.72	2.70	2.94	1.75	2.65
Diff.	229	127	322	189	220	200	109	-19	208	251	209	108	180

t = 7.8**

**Significant at the 1% probability level using a paired t-test.

Table 19. Effect of TRV dilution on TRV-PVX interaction in tobacco plants. Local lesions obtained by inoculating 'Bountiful' beans singly (A) and doubly (B) inoculated tobacco plants.

TRV dilution		Observations L. lesion/half leaf 'Bountiful' beans								
		I	II	III	IV	V	VI	VII	VIII	Ave.
0	A	364	290	202	438	157	526	350	200	315
	B	220	145	140	369	120	309	176	166	206
	A/B	1.61	2.00	1.44	1.19	1.31	1.70	1.99	1.20	1.55
Diff.		134	145	62	69	37	217	172	34	109

t = 4.53**

**Significant at the 1% probability level using a paired t-test.

Table 20. Effect of TRV dilution on TRV-PVX interaction in tobacco plants. Local lesions obtained by inoculating 'Bountiful' beans with extracts from singly (A) and doubly (B) inoculated tobacco plants. TRV was diluted while PVX was held constant. Extracted 10 days after inoculation and diluted 1:10 at extraction.

TRV dilution		Observations L. lesion/half leaf 'Bountiful' beans								
		I	II	III	IV	V	VI	VII	VIII	Ave.
1:20	A	257	398	286	297	198	312	127	495	296
	B	155	221	145	112	99	141	70	244	148
	A/B	1.66	1.80	1.97	2.65	2.00	2.20	1.80	2.03	2.01
Diff.		102	177	141	185	99	171	57	251	148

t = 6.84**

**Significant at the 1% probability level using a paired t-test.

Table 21. Effect of TRV dilution on TRV-PVX interaction in tobacco plants. Local lesions obtained by inoculating 'Bountiful' beans with extracts from singly (A) and doubly (B) inoculated tobacco plants. TRV was diluted while PVX was held constant. Extracted 10 days after inoculation and diluted 1:10 at extraction.

TRV dilution		Observations L. lesion/half leaf 'Bountiful' beans								
		I	II	III	IV	V	VI	VII	VIII	Ave.
1:40	A	218	163	161	200	182	236	180	196	192
	B	76	110	110	78	68	82	120	85	91
	A/B	2.87	1.48	1.46	2.55	2.94	2.87	1.50	2.30	2.25
Diff.		142	53	51	122	114	154	60	111	101

t = 6.99**

**Significant at the 1% probability level using a paired t-test.

Table 22. Effect of TRV dilution on TRV-PVX interaction in tobacco plants. Local lesions obtained by inoculating 'Bountiful' beans with extracts from singly (A) and doubly (B) inoculated tobacco plants. TRV was diluted while PVX was held constant. Extracted 10 days after inoculation and diluted 1:10 at extraction.

TRV dilution		Observations L. lesion/half leaf 'Bountiful' beans								
		I	II	III	IV	V	VI	VII	VIII	Ave.
1:80	A	110	126	160	75	48	90	111	45	96
	B	38	41	128	26	24	50	63	29	50
	A/B	2.89	3.07	1.25	2.88	2.00	1.80	1.76	1.55	2.15
Diff.		72	85	32	49	24	40	96	16	46

t = 5.53**

**Significant at the 1% probability level using a paired t-test.

Table 23. Kinetics of the interaction TRV-PVX in tobacco plants. Ratios of local lesions obtained by inoculating 'Bountiful' beans with extracts from singly (R_A) and doubly (R_B) inoculated tobacco plants against a standard TRV preparation.

Extraction at hrs after inoc.		Inocula	Observations R_A, R_B [1/] I	II	III	IV	V	VI	VII	VIII	Ave.	Ratio R_R (Ave R_A/Ave R_B)
24	R_A	TRV	.109	.078	.048	.082	.039	.087	.065	.036	.071	2.73
	R_B	PVX + TRV	.012	.021	.038	.030	.038	.012	.027	.029	.026	t = 3.2**
48	R_A	TRV	.39	.49	.45	.28	.52	.30	.36	.26	.38	1.90
	R_B	TRV + PVX	.14	.15	.17	.18	.21	.21	.25	.26	.20	t = 4.71**
72	R_A	TRV	.70	.4	.50	.91	.80	.4	.95	.98	.705	2.47
	R_B	TRV + PVX	.20	.32	.27	.19	.24	.21	.25	.6	.285	t = 4.27**
96	R_A	TRV	.95	.90	.83	.33	.93	1.03	1.0	.81	.85	3.04
	R_B	TRV + PVX	.22	.25	.41	.26	.30	.34	.26	.20	.28	t = 5.57**
120	R_A	TRV	.38	1.20	1.14	.45	.40	.50	1.11	1.30	.81	1.8
	R_B	TRV + PVX	.86	.45	.28	.23	.20	.28	.71	.60	.45	t = 2.13 NS
144	R_A	TRV	.99	1.10	.85	1.24	.86	1.28	.99	.5	.98	2.13
	R_B	TRV + PVX	.38	.48	.40	.67	.61	.40	.40	.36	.46	t = 5.35**

*Significantly different at the 5% level of probability using an unpaired t-test.
**Significantly different at the 1% level of probability using an unpaired t-test.
NS - not significant.
1/ R_A = local lesion TRV/local lesion standard in 'Bountiful' beans.
R_B = local lesion TRV + PVX/local lesion standard in 'Bountiful' beans.

Table 24. Kinetics of the interaction TRV-PVX in simultaneously inoculated tobacco leaves. Ratios obtained by extracting at different intervals after inoculation.

	Hours after inoculation. Ratios R_A, R_B and dilutions at extraction.											
	24	Diltn.	48	Diltn.	72	Diltn.	96	Diltn.	120	Diltn.	144	Diltn.
TRV alone (R_A)[1/]	.071	1:145	.38	1:2	.705	1:2	.85	1:2	.81	1:2	.98	1:2
TRV + PVX (R_B)[1/]	.026	1:145	.20	1:2	.285	1:2	.28	1:2	.45	1:2	.46	1:2
R_R[2/] ratio	2.73		1.90		2.47		3.04		1.80		2.13	

[1/] R_A = lesion TRV/lesion standard, R_B = lesion TRV + PVX/lesion standard in 'Bountiful' beans.

[2/] R_A/R_B

Table 25. Kinetics of the interaction TRV-PVX in simultaneously inoculated tobacco leaves. Estimated concentrations calculated by substituting average of ratios R_A[1/] and R_B on regression equation. Extraction from tobacco at different intervals after inoculation.

	Hours after inoculation					
	24	48	72	96	120	144
TRV alone (conc. g/ml)	1.06×10^{-6}[2/]	9.40×10^{-6}	1.71×10^{-5}	2.06×10^{-5}	1.96×10^{-5}	2.36×10^{-5}
TRV + PVX (conc. g/ml)	3.98×10^{-7}[2/]	5.04×10^{-6}	7.11×10^{-6}	6.99×10^{-6}	1.11×10^{-5}	1.13×10^{-5}
Log (conc. g/ml x 10^7) TRV	1.025	1.973	2.233	2.314	2.292	2.373
Log (conc. g/ml x 10^7) TRV + PVX	0.600	1.702	1.852	1.844	2.045	2.053

[1/] R_A = local lesion TRV/local lesion standard in 'Bountiful' beans

R_B = local lesion TRV + PVX/local lesion standard in 'Bountiful' beans.

[2/] Extrapolation.

Table 26. Effect of simultaneous inoculation on TRV-PVX interaction in tobacco plants. Ratios of local lesions obtained by inoculating 'Bountiful' beans with extracts from singly (R_A) and doubly (R_B) inoculated tobacco plants against a standard TRV preparation. Extracted 336 hrs after inoculation and diluted 1:10 at extraction. TRV diluted 1:40 at inoculation of tobacco plants.

	Observations R_A, R_B [1/]													Ratio R_R (Ave R_A/Ave R_B)
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Ave	
R_A	0.22	0.29	0.32	0.39	0.40	0.36	0.33	0.23	0.21	0.40	0.43	0.26	0.320	1.9
R_B	0.17	0.15	0.14	0.15	0.14	0.18	0.17	0.26	0.13	0.14	0.18	0.21	0.168	

TRV Concentration: R_A 0.32 = 3.98×10^{-5} g/ml and TRV, R_B 0.168 = 2.13×10^{-5} g/ml of TRV

t = 6.13**

**Significant at the 1% probability level using an unpaired t-test

[1/] R_A = local lesion TRV/local lesion standard in 'Bountiful' beans.
R_B = local lesion TRV + PVX/local lesion standard in 'Bountiful' beans.