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Patterns of disease manifestation in tomato seedlings singly or doubly infected with *Potato* X *potexvirus* and *Tobacco Mosaic tobamovirus*

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Abstract

Plants of a common tomato (*Lycopersicon esculentum* Mill.) cultivar i.e. Fukuju # 2, were raised under greenhouse conditions at different times in Japan. Both the initial and long-term symptom responses to single and mixed infections with potato X potexvirus (PVX) and the L (wild strain) of tobacco mosaic tobamovirus (TMV) were monitored. Symptoms expression, both in rapidity as well as in severity varied among the treatments and were influenced, not only by virus strains but also by the inoculation regime. The reduction in sizes of parenchyma and collenchyma cells from the stem, plant height, number of leaves, stem girth and yield of fresh and dry shoot and root as well as fresh fruit that were recorded in all infected plants, showed strong correlation with observed symptom severity which in turn was found to be a function of the concentration, as measured by enzyme linked immunosorbent assay (ELISA) of the accumulated viral particles in the host during the acute stage of infection. Simultaneous mixed inoculation of PVX and TMV-L, which induced the most severe symptoms also led to relatively more growth reduction than sequential mixed inoculation with either virus, 4 days before the other, and single infections with PVX or TMV. Significantly delayed flowering and a reduction of at least 80 % in fresh shoot weight and fruit yield were recorded for all mixed infected plants regardless of sequence of infection.

Key words: ELISA, virus strains, symptom severity, simultaneous and sequential inoculations.

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INTRODUCTION

Generally, virus infection of plants often cause visible symptoms such as various forms of mosaic and distortions with consequent reductions in growth and crop yield. Such severe responses have from long being reported for many crops (Goodman and Ross, 1974; James, 1974; Hampton, 1975; Lastra and Uzcategi, 1975; Whenham et al., 1985). Although reduction in plant size is the most general symptom induced by virus infection, according to Matthews (1991) there is probably some slight general stunting of growth even with 'masked' or 'latent' infections where the systemically infected plant shows no obvious sign of disease.

Understanding the mechanisms that control the effects observed in infected plants has however always been a daunting task. Although Fraser et al. (1986) used multiple regression analysis to attempt an understanding of how virus-induced effects are controlled especially the quantitative relationships between severity of pathogenic symptoms and inhibition of host growth in tobacco and tomato under infection by TMV, the mechanism by which these effects are controlled remain to be perfectly understood.

Besides, mixed infections with two unrelated viruses, which are common in field plants, especially in tropical areas, often produce a more severe disease than that caused by either virus alone. For instance, tobacco on infection with potato virus X (PVX) and potato virus Y normally develop a more severe disease than that induced by either virus alone (Vance, 1991).

It is worthy of note though that not all mixed infections involving viruses result in more severity of disease. As observed long time ago by McKinney (1929), tobacco plants inoculated with a mild mosaic strain of tobacco mosaic virus failed to develop additional symptoms when challenge-inoculated with a yellow mosaic strain. This phenomenon referred to as cross protection, however, is usually found only between virus strains, which are serologically related and adapted to the same host (Marrou and Migliori, 1971).

In view of the economic importance of the tomato plant the world over, research aimed at further understanding the mechanisms of its disease problems, among which viral –induced ones are prominent is justified. The overall aim of this study therefore, was to determine the extent of superiority of mixed over single infections with respect to induction of severe disease symptoms, and the growth and yield responses of the host to different infection levels. The study of the infection sequences was to help evaluate its influence on development of the synergistic effects and the possibility of its manipulation as an option in disease management.

MATERIALS AND METHODS

Propagation of plants and inoculation with viruses

Cultivar Fukuju # 2, tomato seedlings were raised under greenhouse conditions throughout the year in all the experiments. Temperature during the summer months was a maximum of 32 ^{o}C during the day and 20 ^{o}C at night. Natural daylight was the regime for all experiments. Sandy-loam soil or Levington compost, steam-sterilized at 121^{o}C for 30 min and supplemented with N P K fertilizer at the rate of 2:2:1 g per liter pot, and some vermiculite at seeding or transplanting was used in all cases. Plants were watered adequately daily to avoid water stress in all experiments.

The O strain of PVX, and the L strain of TMV that were used for the different experiments were multiplied in *Nicotiana tabacum* cv xanthi and purified separately according to standard procedures before use. The primary leaves i.e. the first 2 true leaves from the base of plants at the 5 to 6 true-leaf stage were inoculated by rubbing with a suspension of 0.2 mg of virus per ml of either phosphate buffer, pH 7.0, or sterile deionized water. Control plants were mock inoculated with buffer or sterile de-ionized water. Leaves were dusted lightly with carborundum prior to inoculations and washed with running water immediately after inoculations that followed pre-determined treatment designs in which the viruses were inoculated singly and in various

combinations. Simultaneous mixed inoculations were carried out by mixing equal volume of inoculum of both viruses and then gently rubbing as usual on same leaf position as for single inoculations. All plants were kept on platforms in the greenhouse following completely randomized design (CRD) pattern to enable subsequent relevant statistical analysis of variance.

Disease, plant growth and yield assessment

Plants were monitored daily to record visible changes such as time of first appearance and type of symptoms and days to flowering among others. Weekly records of plant height and number of leaves, and stem girth were also taken. Leaf samples for ELISA and SDS-PAGE (to confirm success and level of infection) were collected at various times postinoculation and kept frozen when necessary at -40 ^{o} C until assayed. At 7 weeks post inoculation, shoots of some plants were cut from the base with a scalpel and weighed while the roots were also removed from the soil, carefully rid of attached soil particles and then weighed. Both shoots and roots were then wrapped individually in papers properly labeled and oven-dried to constant weight at 80 ^{o}C over 24 h after which the dry weight was taken. Fruits derived from the first round of flowering were harvested and weighed at first sign of ripening from at least four plants. Analysis of variance was carried out and for comparison between treatments Tukey–Kramer's HSD test was used, with probability at 5 % level of significance.

Stem cell length measurement

The internode between the 8th and 9th leaves of both healthy and infected plants shoot was excised at harvest after weighing but before oven-drying the shoot. They were kept in cellophane bags to prevent early dehydration. At the laboratory, random segments about 1 cm long, of each of the treatments were later mounted in turn on a DSK microslicer (Dosaka EM co. Ltd., Japan) and longitudinal thin sections, 10 µm in thickness were cut. Pieces were temporarily mounted on slides and viewed with a Nikon Phase contrast microscope (Nippon kogaku, Tokyo, Japan) equipped with ocular micro calibrator. Lengths of at least 20 of each of parenchyma and collenchyma cells from the cortex, for each sample, were measured and the mean cell length estimated.

SDS-PAGE to evaluate host and viral proteins accumulation.

Protein fractionation was carried out by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the methods described by Sambrook et al. (1989). Known quantities (0.1 g - 0.3 g) of leaf samples collected from infected and healthy plants were macerated, using precooled mortars and pestles, in homogenizing buffer (0.05 M Tris-HCl, pH 6.8 containing 1% PMSF or 1% 2-Mercaptoethanol) at a ratio of 1 g: 10 ml buffer. An equal volume of 2x SDS gel loading buffer (Sambrook et al., 1989), was then added and mixed thoroughly before boiling for 8 min at 100 ^{o} C to denature the protein. Samples were loaded at 20 µl or 30 µl per lane on 12.5 % standard 1.6-mm diameter gels and resolved at 30 mA until the dye head reached the bottom of the gel. Staining of the gel was carried out with Coomassie brilliant blue 25G and destaining was done with 3 changes of destaining solution i.e. ratio 9: 1 of Methanol / Water (50/50): Glacial Acetic, over 4 h.

Enzyme Linked Immunosorbent Assay (ELISA) procedure.

ELISA for confirmation of infection as well as virus concentration, in both the preliminary and substantive experiments of this study was carried out according to the indirect method described by Koenig (1981). Leaf samples were ground for 1 min using pre-cooled mortar and pestle in freshly prepared 0.02 M sodium carbonate (Na_2CO_3) in the ratio of 1 g of tissue: 10 ml buffer. The homogenate was centrifuged for 10 min at 10,000 rpm and the supernatant was removed carefully and was then diluted in two-fold steps in the homogenizing buffer from 1: 100 to 1: 12,800 for all treatment samples.

Sample preparations, as described above, were coated directly unto Corning microtiter plates and incubated at room temperature for 1

h. Washing and blocking with Tris- buffered Saline (TBS-T) (50 mM Tris-HCl, pH 7.6; 0.15 M NaCl, 0.05% NaN3, 0.05% Tween 20) was done 4 times at three min interval and antibody against relevant viruses were added accordingly at 5 µg/ml final concentration in TBS-T. Goat antirabbit IgG- alkaline phosphatase conjugate (Biosource International, Camarillo Ca. USA) was used as the second antibody at 1:2,000 dilutions. Color was developed with p- nitro phenyl phosphate at 1 mg/ml of 10 % diethanolamine, pH 9.8. Absorbance was measured using a 405 nm filter of micro plate photometer (Corona Electric Tokyo, Japan). The concentration of each virus in the samples was estimated from a standard curve established using purified virus preparations that had been passed through sucrose gradients and concentration measured spectrophotometrically (Hitachi U-1100 spectrophotometer).

RESULTS

Effect of treatments on symptom expression

All virus-inoculated plants were successfully infected as confirmed by ELISA and SDS-PAGE analyses. As shown by the results of the SDS- PAGE analysis in plate 1, the virus-specific coat protein bands in lanes containing samples from infected plants are of varying intensities. Infected plants also manifested systemic symptoms, which however, showed variations in the mode and time postinoculation of appearance and the nature of symptoms.

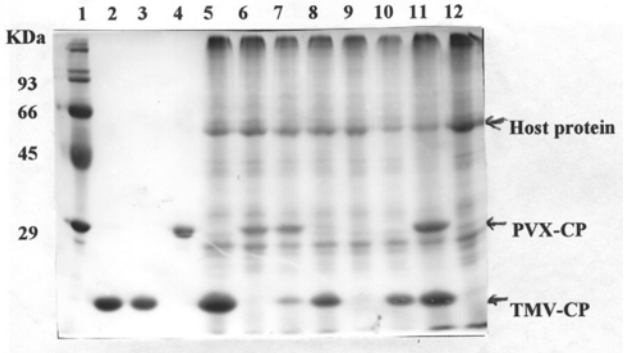

Plate 1: SDS-PAGE analysis of virus specific and host proteins accumulated in the systemically infected upper leaf 5 of cultivar Fukuju # 2 tomato, 7 days after single or mixed inoculation with PVX and TMV.

Lane 1= Pre-stained protein marker, lanes 2, 3, and 4= Purified TMV-L, TMV-L11A and PVX respectively (used as markers). Lane 5= TMV-L alone, lane 6= PVX alone, lane 7= PVX before TMV-L, lane 8= TMV-L before PVX, lane 9= PVX+ TMV-L (in leaf 3, used as an infected control), lane 10= TMV-L11A, lane 11= PVX+TMV-L, lane 12= Healthy control.

Plants inoculated with PVX alone expressed chlorotic mottling in addition to becoming subsequently stunted compared to the healthy control. First appearance of symptoms, as yellowish spots, was as early as 5 days postinoculation (dpi) in plants inoculated in late autumn and winter. This period is characterized by dramatic changes in environmental conditions, which become colder, with shorter daylight and with weaker light intensity. It was as late as 14 dpi in experiments carried out during hotter periods of late spring and summer. In both cases, although almost fully expanded upper leaf positions number 4 or 5, i.e. from the stem base, were the first targets more severe symptoms were from leaf no. 5 and above which were rapidly expanding at that time. Symptoms persisted in subsequent new leaves. Plate 2 A shows cv Fukuju #2 tomato plants at 7 dpi following inoculation with PVX alone.

Plants infected with TMV-L alone manifested severe mosaic, which appeared as early as 5 dpi regardless of the season in the youngest leaves. It became more prominent at 9 dpi and thereafter on subsequent new leaves. The lower, fully expanded leaves at inoculation, including the inoculated ones, remained symptomless. Plate 2 B shows TMV-L – infected tomato plant at 7 dpi.

Plants simultaneously inoculated with PVX and the TMV-L manifested characteristic distortions and necrosis on the uppermost leaf as well as on the stem area close to the distorted leaves. The first indication was as early as 4 dpi becoming fully apparent at 7 dpi and by the end of two weeks led to death in weaker plants. Surviving plants were seriously stunted. In treatments where one of the viruses was inoculated 4 days before the other, the characteristic double infection syndrome was milder than that under simultaneous inoculations. Although necrotic spots combined with rugose manifested as late as 14 dpi it became more severe thereafter leading to stunting but not death. Plate 2 C shows the symptom response at 7 dpi, in cv. Fukuju no. 2

tomato plants to simultaneous mixed infections with PVX and TMV-L. The mock-inoculated plants, which served as control, remained healthy (Plate 2 D).

Effects on plant growth

Both healthy and infected plants increased in height with time postinoculation. The final height of plants under all infection treatments i.e., whether infected singly or doubly, was significantly lower than that of the healthy plants (96.3 cm). Although both singly infected plants were significantly taller than plants simultaneously inoculated with TMV-L and PVX, only plants with TMV-L alone were also significantly taller than those with PVX sequentially inoculated before TMV (i.e. Pb4P). Although doubly infected plants mostly did not differ significantly from one another, simultaneous mixed inoculation apparently elicited relatively more damaging response than the other sequences of mixed infection.

Plate 2: Systemic disease symptoms response in tomato 7days after single and mixed infection with PVX and TMV.
A) PVX alone; B) TMV-L alone; C) PVX+ TMV-L (simultaneous infection); D) Healthy plant.

With regards to the effects on stem cell length, healthy plants had significantly longer stem parenchyma and collenchyma cells than the infected ones. The control was an average of 134 µm and 216 µm (Table 1). The trend was as for plant height with singly infected plants having longer cells than mixed infected ones at the region of the stem examined. Sequence of

Table 1: Some growth parameters in healthy and diseased tomato plants under single and mixed infection with TMV and PVX

Treatment combinations	Height at harvest (cm)	Number of leaves at harvest	Final stem diameter (mm)	Stem cell length (µm)		parenchyma/ collenchyma ratio
				parenchyma	collenchyma	
PVX alone	64.7 bc	17.8 a	7.4 b	120.8 bc	173.3 bc	0.70 a
TMV-L alone	70.3 b	16.3 b	6.9 bc	124.3 b	184 b	0.68 a
PVX plus **TMV-L**	51.9 d	13.8 c	6.3 c	106.0 d	158 d	0.67 a
PVX before **TMV-L**	57.7 cd	16.0 b	6.6 bc	114 c	166 cd	0.69 a
TMV-L before **PVX**	62.8 bc	15.5 b	6.7 bc	119.5 bc	171 c	0.70 a
Healthy control	96.3 a	18.8 a	10.3 a	134.3 a	214.5 a	0.63 b

Figures followed by the same letter in a column are not significantly different at P=0.05, Tukey-Kramer's HSD test.
Each value is a mean of 4 plants.

inoculation appeared to have effect on cell elongation response to mixed infection as plants inoculated with TMV before PVX had relatively longer parenchyma and collenchyma cells than those inoculated with PVX before TMV and significantly different from those with simultaneous inoculation.

Considering the lengths of the parenchyma cells in relation to those of the collenchyma, it is shown that healthy plants have significantly lower parenchyma: collenchyma length ratio (0.63) compared to the infected ones (0.67-0.70), which did not differ significantly among one another (Table 1). Higher ratios indicate that the viral infections generally suppressed the extension of the stem cortical collenchyma more than it did that of the parenchyma in the infected plants.

A general increase in the number of leaves per plant with time was recorded, in all treatments, up to 6 weeks post-inoculation regardless of the season of growth. As shown in Table 3, comparison of the number of living leaves at harvest among treatments showed the control plants with 19 leaves, and those with PVX alone (18 leaves) not being significantly different. Simultaneously infected plants had significantly the least number (14 leaves) (Table 2).

The effect on stem girth followed the same trend as the number of leaves. Healthy control plants had significantly thicker stems than infected plants at harvest (Table 2). Singly infected plants did not differ significantly between each other, while those with TMV-L alone had significantly the same stem girth as all other viral-inoculated plants. Stem diameter ranged between 6.3 mm (in simultaneously mixed infected plants) and 10.3 mm in the control.

Linear regression analysis showed that plant height had negative correlation with virus levels both PVX and TMV (Figs 1 and 2).

Table 2: Shoot and root yield in healthy and diseased tomato plants under single and mixed infection with PVX and TMV

Treatment	Shoot weight (g)		Root weight (g)		% Dry matter* in		% loss in wt of shoot**		% loss in root wt**	
combinations	**Fresh**	**Dry**	**Fresh**	**Dry**	**Shoot**	**Root**	**Fresh**	**Dry**	**Fresh**	**Dry**
PVX alone	53.1 b	7.9 b	7.5 c	1.2 c	14.9 b	15.3 c	33.9	44.3	48.1	61
TMV-L alone	53.0 b	8.1 b	10.1b	1.7 b	15.3 b	17.0 b	34.1	42.9	30.1	41.7
PVX plus **TMV-L**	41.5 d	4.4 d	5.2 d	0.6 e	10.7 d	11.4 e	48.4	68.8	64.3	80.0
PVX before **TMV-L**	43.9 cd	4.9 d	5.6 d	0.7 de	11.2 d	13.2 d	45.4	65.4	62.1	75.6
TMV-L before **PVX**	47.9 c	6.3 c	6.6 c	1.0 cd	13.1 c	14.4 cd	40.5	55.5	54.2	67.5
Healthy control	80.4 a	14.2 a	14.5 a	3.0 a	17.7 a	20.4 a	0	0	0	0

Figures followed by the same letter in a column are not significantly different at P= 0.05, Tukey-Kramer's HSD test.
Each value is a mean of 4 plants.
** Comparison among treatments was based on log-transformed data. The control was the benchmark.*
***Absolute difference between the values of the control and the respective infection treatment expressed as a % of the control.*

Effects on yield components

Samples for measurement of fresh weight of tops at harvest were taken for all treatments. As with most growth parameters, the healthy plants had significantly higher fresh and dry matter weight than infected plants (Table 2). Comparison of the sequences of mixed infection showed plants that were simultaneously infected with PVX and TMV-L and PVX before TMV had apparently the lowest weights. The percent dry matter compositions of the shoots as well as the percent loss in weight, based on the control values, are shown in Table 2. These values followed the trend of the absolute values of fresh and dry weights. As high as 48 % loss was recorded for fresh weight while ca. 70 % loss was recorded on dry weight basis for PVX +TMV-L treatment, indicating a higher loss of dry matter.

As with shoot weight, on absolute value and percentage basis, the control had significantly higher fresh and dry root weight than the other treatments at harvest. As usual, plants with PVX and TMV combined had the smallest weights followed by the singly infected ones among which those with TMV- L alone had the highest fresh, dry and percent dry weight (Table 2). The percentage dry matter ranged between 11.4 and 20.4. Figures 1 and 2 show that shoot weight negatively correlated with increase in the level of PVX and TMV in the plants

The average number of days post-inoculation to flower appearance was recorded. Plants of the healthy control and singly infected treatments were the earliest to bloom and were not significantly different from one another. The Mixed infected plants, that bloomed at all, had significant delay of several days (Table 3). Apart from this very long delay, rate of flower abortion was observed to be high and indeed, many of the doubly infected plants did not flower at all before the termination of the experiments.

The average number of edible fruits as at first harvest was 3 in healthy plants. This was

not significantly different from those of singly infected plants, which had an average of 2 each.

Table 3: Fruit yield in healthy and diseased tomato plants under single and mixed infection with PVX and TMV

Treatment combinations	Flowering (dpi)	Yield of edible fruits per plant at 1st harvest*			% loss of fruit yield per plant**		
		Mean no.	Total wt. (g)	Mean wt. (g)	mean no.	total wt.	mean wt.
PVX alone	34.3d	1.8a	247.5b	142.9ab	35.7	44.1	11.5
TMV-L alone	35.8cd	1.8a	241.3b	138.5b	35.7	45.5	14.2
PVX plus **TMV-L**	40.5a	0.3b	17.8c	71.0c	89.3	96	56
PVX before **TMV-L**	38.3ab	0.3b	20.5c	82.0c	89.3	95.4	49.2
TMV-L before **PVX**	38.0bc	0.5b	41.3c	82.5c	82.1	90.7	48.9
Healthy control	33.8d	2.8a	442.5a	161.4a	0	0	0

Means followed by the same letter in a column are not significantly different using Tukey-Kramer's HSD test (P= 0.05)

**Edible fruit (ripe tomato of any size) derived from the first round of flowering.*

***Absolute difference between the values of the control and the respective infection treatment expressed as a % of the control.*

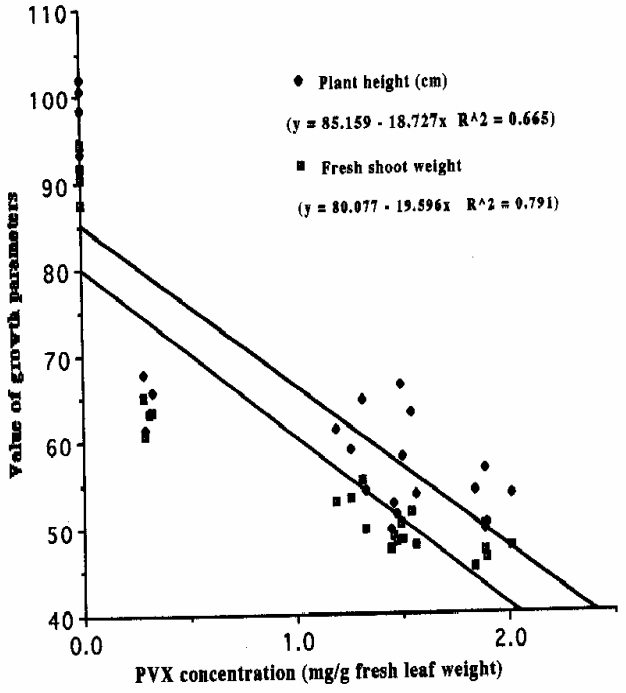

Fig 1: Relationship between some growth components at harvest and PVX concentration measured during the acute stage of disease in tomato under single or mixed infection with PVX and TMV.

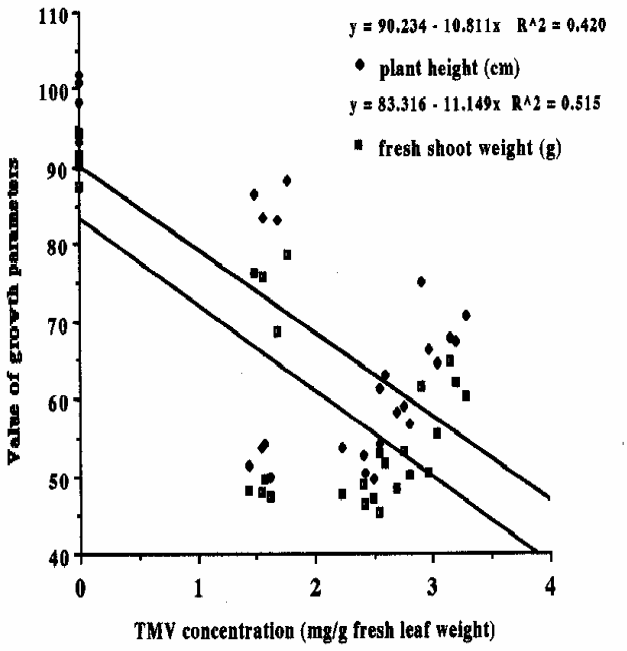

Fig 2: Relationship between some growth components at harvest and TMV concentration measured during the acute stage of disease in tomato under single or mixed infection with PVX and TMV.

This group, however, significantly differed from those with PVX plus TMV in various combinations, which had a mean value of 0.5 fruits per plant (Table 3).

As also shown on Table 3, although the mean total fruit weight per plant was significantly

higher in the control than in other groups, the average weight of a fruit was essentially not

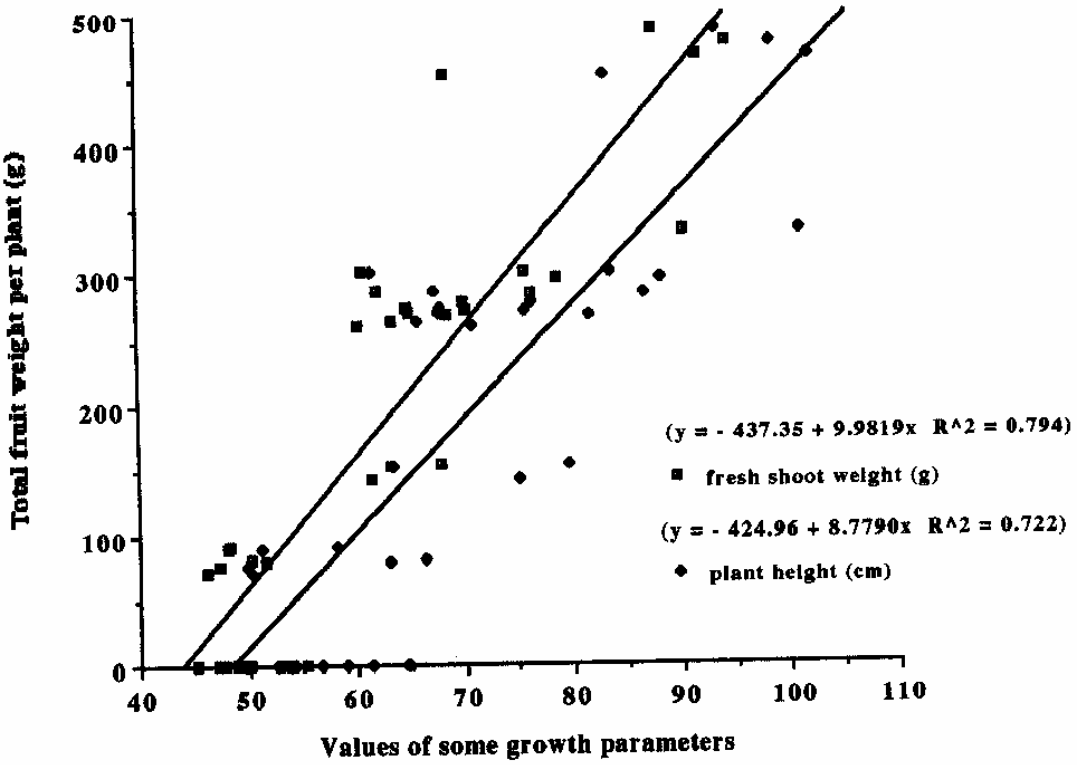

Fig 3: Relationship between total edible fruit weight at first harvest with plant height and fresh shoot weight.

different for those of the PVX only-infected plants. While the control was 161 g, the PVX alone treatment was 142.9 g. Mixed infected plants had the significantly lowest weights (about 70 g/fruit). Fig 3 shows that fruit yield is a function of the tomato growth level with positive correlation (r = 0.85)

DISCUSSION

The results of the various experiments carried out in this study reveal different levels of susceptibility in tomato to infection by PVX and TMV strain inoculated singly or in combination with one another under the prevailing conditions of the experiments. Plants that were either simultaneously or sequentially doubly infected had significantly higher severity of disease and consequently more reduced growth and yield compared to the healthy and singly infected plants.

The severity of disease is normally influenced by factors that may be both external and/or internal to the plant milieu Matthews (1991). Mixed infection with viruses, and the attendant synergistic effects were factors identified to have highly influenced disease development and the growth and yield responses of plants in this study. Reduction in plant size is said to be the most general symptom induced by virus infection. According to Matthews (1991), there is probably some slight general stunting of growth even with masked or latent infections where the systemically infected plant show no obvious sign of disease. For example, Matthew (1949) showed through carefully designed experiments, a reduction of between 7 % and 15 % in tuber yield in potato under infection with mild strains of PVX.

At inoculation, all plants were of uniform height of about 8 cm with 4-5 leaves each (Data not shown). As at first harvest 7 weeks later, however, the tallest was the control (96.3 cm and ca. 19 leaves) and the shortest, PVX + TMV-L treatment, was 52 cm with about 14

leaves. Mixed infection with PVX and TMV generally caused more reduction in height and number of leaves than single infection. The initial distortions and necrosis of the apical part (an indicator of severity) that characterized mixed infection may be partially responsible for keeping plant growth in check. Poor differentiation of the meristem resulting under such condition as well as the reduced cell elongation capability could have contributed to the overall inhibition of stem extension and decreased number of leaves witnessed in this study. In fact, linear regression analysis shows a positive correlation (r = 0.96) between stem collenchyma cell length and plant height.

Hampton (1975) observed that reductions in yield could be due either to fewer fruits or smaller fruits per plant. In this study, plants singly infected with PVX apparently but not significantly, produced fewer numbers of fruits than the control. The yield loss, based on mean weight of a fruit and total fruit weight per plant, however, was 11.5 % and 44.1 % respectively. The doubly infected plants were invariably worst affected regardless of the sequence of infection; having at least 90 % loss based on the total fruit weight per plant indicating that the effect of the interacting viruses on the plant was additive.

Analysis of the SDS-PAGE results in plate 1 shows the signal of PVX, especially under simultaneous mixed infection, appearing bolder than that of PVX alone in the same leaf position. This is an indication of PVX concentration under mixed infection being enhanced by its interaction with TMV. Furthermore, Figures 1 and 2 show that the concentrations of the viruses during the acute stage of disease, as measured by ELISA, had considerable negative correlations ($r > -0.81$ for PVX, and $r > -0.65$ for TMV) with the growth of plants, while Figure 3 shows a positive correlation ($r > 0.85$) between growth and fruit yield. Fraser et al (1986), using multiple regression analysis, found that TMV concentration alone could account significantly for growth reduction in tobacco but that was not sufficient in tomato. However, severity of disease was found to correlate well with growth reduction in both tobacco and tomato. Under mixed infection in this study, the concentration of PVX appeared to contribute more to the severity of disease.

Although field results might vary to some extent, in view of the fact that conditions that favor the establishment of a mixed infection in tomato can be easily met in field environment of the tropics, considerations of the potential economic loss to the grower makes this situation worth studying and preventing. As observed by Crute and Pink (1996), cultivar –specific resistance controlled by a resistance gene has proven to be the best long term means of protection. It also remained the best hope for curtailing the 'streak' disease in tomato that had been studied here should a natural outbreak occur.

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