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Seedling Age at Inoculation and Infection Sequence Affect Disease and Growth Responses in Tomato Mixed Infected with Potato Virus X and Tomato Mosaic Virus

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ABSTRACT

Simultaneously mixed inoculation of container grown seedlings of tomato, *Lycopersicon esculentum* Mill, cv GCR 236 (+/+) at the nursery age of 14 days after germination was studied. Inoculation of 0.1 mg mL^{-1} each of potato virus X (PVX, genus *Potexvirus*) and tomato mosaic virus (ToMV, genus *Tobamovirus*) induced an acute symptomatic response and eventual death of over 90% of the plant population 15 days after inoculation. On the other hand, 28 days old plants inoculated either simultaneously or sequentially, manifested only a synergistic disease response but no deaths were recorded. Sequentially mixed infected plants manifested significantly less severe synergistic disease symptoms than simultaneously infected ones. Double antibody sandwich (DAS)-ELISA of samples collected from systemically infected leaves revealed a higher concentration of PVX antigen in simultaneously mixed infected plants than in sequentially infected ones during the early but not the chronic stage of infection. Western blot analysis showed patterns of accumulation of PVX and ToMV coat proteins that was consistent with the ELISA results. Generally, mixed infected plants had poorer plant growth and fruit yield than singly infected and healthy ones. The results indicate that cultural control strategy can be adopted in viral disease management; keeping young seedlings free of infection source for as long as possible especially when using non-resistant host cultivars so as to take advantage of disease tolerance conferred by age.

Key Words: Sequential infection; Synergistic disease; Cultural control strategy

INTRODUCTION

Generally, viral infection often causes visible symptoms such as various forms of mosaic and distortions in plants with consequent reductions in crop growth and yield. While reduction in plant size is the most general symptom induced by virus infection, there is probably some stunting of growth even with 'masked' or 'latent' infections, where the systemically infected plant shows no obvious sign of disease (Matthews, 1991). In nature, higher plants are commonly co-infected with multiple viruses and a number of disease syndromes are caused by interaction of two independent viruses. The accumulation dynamics of the interacting viruses in such mixed infection often change drastically (Otsuki &Takebe, 1979). Many such synergistic diseases involve a member of the potyvirus group of plant viruses and the other virus of the pair may be any of a broad range of un-related viruses. RNA viruses such as potato virus X (PVX) (Vance, 1991) and cowpea mosaic virus (Anjos *et al.,* 1992) among others have been reported earlier.

The best characterized of the plant viral synergisms, no doubt, is the interaction between PVX and the potyvirus-potato virus Y (PVY) in tobacco (Goodman & Ross, 1974a, b; Vance, 1991). However, several other potyvirus-associated synergistic diseases have also been examined in detail. In each, a dramatic increase in host symptoms has been observed in doubly infected plants (Pruss *et al*., 1977). The increase in symptoms is normally correlated with an increase or decrease in the level of potyvirus (Stouffer & Ross, 1961; Calvert & Ghabrial, 1983; Poolpol & Inouye, 1986; Goldberg & Brakke, 1987; Vance, 1991; Scheets, 1998; Kim *et al.*, 2004).

Variations in host system and strains of virus involved, among other factors are known to influence the accumulation dynamics of the interacting viruses and the subsequent disease severity in mixed infections. In the mixed infection involving PVX and PVY in tobacco, PVX was reported as being enhanced, while the concentration of PVY remained un-changed (Vance, 1991). In a study involving PVX and some strains of ToMV in the TMV-susceptible cultivar Fukuju No. 2 (a common Japanese tomato cultivar), the concentration of PVX at the acute stage of infection, was found to increase between 4 and 6 folds in the systemically infected leaves of simultaneously mixed infected plants, compared to that in plants singly infected

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with PVX (Balogun *et al*., 2002). The concentration of L strain of ToMV (that caused the higher enhancement of PVX) was reported to be reduced by about 25%, while under the same condition the concentration of the attenuated ToMV strain (i.e., L11A), remained unchanged. In the ToMV-non susceptible cultivar GCR 237 (Tm^{-1}), PVX concentration in a mixed infection with ToMV, failed to increase significantly higher than the level in plants singly infected with PVX (Balogun *et al*., 2005) and there was no synergistic disease symptom. These reports indicated that the accumulation of ToMV and indeed its enhancing effect on PVX concentration was not only virus strain -dependent but also host cultivar- dependent.

Understanding the mechanisms that control the effects observed in virus infected plants is a difficult task. Nevertheless, 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, especially with a view to finding relatively easy to adopt disease management strategies, continues to be justified. The hypotheses of this study were: (i) the accumulation of PVX and ToMV antigens in simultaneously infected tomato plants was not significantly different from that in sequentially infected ones; (ii) the accumulation of PVX and ToMV antigens in younger seedlings was not significantly different from that in older seedlings, (iii) the eventual disease, growth and yield responses of infected tomato was not significantly different in young and older seedlings inoculated with PVX and ToMV. In view of the above hypotheses, objectives were to determine the manipulation of seedling age at inoculation and the sequence of infection of PVX and ToMV as a cultural control strategy against the synergistic disease induced by mixed infection.

MATERIALS AND METHODS

Greenhouse experiments. The experiments were carried out in a greenhouse of the Tokyo University of Agriculture and Technology, Japan. Cultivar GCR 236 (+/+) (a ToMV-susceptible, European cultivar) plants were raised under conditions in which maximum temperature was 30°C (day time), while the minimum (at night) was 22°C. The medium of growth was sandy loam soil, which was steam-sterilized at 121°C for 30 min before potting in 1.5 L oven-sterilized clay pots. NPK fertilizer was supplemented at planting (5 g pot^{-1}) and the soil surface was covered with vermiculite. The arrangement of the pots was completely randomized with six replicates (Balogun *et al*., 2002 & 05).

Inoculation of the transplanting age seedlings (at the 5 - 6 true leaf stage, 28 days after germination) was by rubbing the two carborundum-dusted primary leaves i.e., leaves 1 and 2 from the stem base, with 0.2 mg mL^{-1} (in phosphate buffer pH 7.2) of any one virus alone or with a mixture of equal quantities of PVX and ToMV-L. For sequential mixed inoculation, the same quantities of the viruses as used in single inoculations were inoculated on the same plants but at four days interval. Inoculation on nursery stage seedlings (i.e., at the 2 - 3 true leaf stage, 14 days after germination) was on the cotyledonary leaves. Mock-inoculated plants served as control in both cases. The inoculated leaves were washed with water after every inoculation regime. Plants were watered as and when necessary to avoid water stress during the growth period.

Determination of viral antigenic concentration by ELISA. Leaf samples from both healthy and infected plants were ground for one min in 0.02 M sodium-phosphate buffer, pH 7.2, with pre-cooled mortar and pestle in the ratio of 1 g of tissue: 10 mL buffer. ELISA, for estimation of viral antigen concentration in samples, was carried out according to the direct double antibody sandwich (DAS) method of CLARK and ADAMS (1977). The concentration of each virus in the samples was estimated from a standard curve established for each set of assay with sucrose density gradient-purified virus preparations of each of the viruses. Viruses were diluted in buffer and for comparison purposes, dilutions were also made in crude extracts from healthy plants (Balogun *et al*., 2005).

Evaluation of viral coat protein accumulation by Western blot analysis. Leaf samples from infected and healthy plants were macerated with precooled mortars and pestles in homogenizing buffer (0.05 M Tris-HCL, pH 6.8 containing 1% 2-Mercaptoethanol) at a ratio of 1 g: 10 mL buffer and proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) essentially as described by SAMBROOK *et al*. (1989). The fractionated proteins were electro-transferred to hybond-C extra nitrocellulose membrane (Amersham Life Science, Japan) and assayed by western blot analysis as earlier described (Balogun *et al*., 2002 & 05).

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 were also taken. Leaf samples for ELISA, SDS-PAGE and Western blot analysis were collected at various times postinoculation and kept frozen at -40°C when necessary until assayed. Measurement of fresh and dry shoot, root and fruit weights were as described earlier (Balogun *et al*., 2002). Analysis of variance was carried out and for comparison between treatments means, the new Duncan's multiple range tests were used, with probability at 5% level of significance.

RESULTS

Symptoms. Single infection with PVX alone on the seedling at both the 2 - 3 leaf and 5 - 6 leaf stages of growth elicited a generally mild chlorotic mottling of the systemically infected leaves and subsequent general stunting of the whole plant with increasing age of infection. ToMV-L alone elicited severe mosaic, whereas simultaneous double

Table I. Comparative viral antigenic concentrations at different leaf positions of cv. GCR 236 tomato seedlings, as measured by ELISA at different times following single or simultaneous mixed inoculation of the cotyledons at 14 days after germination

Virus Assayed	Treatments	Inoculated cotyledon, at 5 dpi		Uppermost leaf (third true	leaf), at 7 dpi
	PVX alone	1.29±0.04		0.42±0.08b	
PVX	PVX plus ToMV-L	1.49±0.03 NS	(1.1)	1.85±0.06a	(4.4)
	ToMV-L alone	1.80±0.08a		2.72±0.15	
ToMV	PVX plus ToMV-L	1.65±0.07b	(0.91)	2.24±0.11 NS	(0.82)

*Values (mg g^{-1} fresh leaf weight) are means ± standard deviation of 4 plants independently assayed. Different superscripts, in the same column, following the mean values for any one virus type indicates significant differences at P=0.05.
N.S= Not significant.
Leaf position denotes location from the stem base upwards. The primary leaves are numbered 1 and 2.
Values in parentheses are ratios of individual virus concentration in doubly: singly infected plants for individual virus.

Table II. Comparative PVX antigenic concentration at different leaf positions of cv GCR 236 tomato plants, as measured by ELISA, at different times after inoculation with PVX and ToMV-L

Treatments	Concentration of PVX (mg/g fresh leaf weight) at different leaf positions Assayed							
	Leaf 1, at 7 dpi		Leaf 5, at 7 dpi		Leaf 21 dpi		Leaf 12, at 28 dpi	
PVX alone	1.37±0.08a*		0.39±0.04c*		0.43±0.03c		0.58±0.04b	
PVX plus ToMV-L	1.44±0.08a	(1.05)	1.70±0.08a	(4.36)	1.57±0.04a	(3.65)	1.28±0.05a	(2.21)
PVX before ToMV-L	1.51±0.03a	(1.10)	0.87±0.01b	(2.23)	1.35±0.09ab	(3.14)	0.96±0.05a	(1.66)
ToMV-L before PVX	0.64±001b	(0.47)	0.47±0.03c	(1.21)	1.16±0.02b	(2.70)	1.01±0.06a	(1.74)

*Values (mg g^{-1} fresh leaf weight) are means ± standard deviation of 4 plants independently assayed.
Leaf position denotes the location of leaf from the stem base upwards. The inoculated primary leaves are numbered 1 and 2.
Values in parentheses are ratios of PVX concentrations in doubly: singly infected plants for the respective treatment.
Means followed by the same letter (s) in a column, are not significantly different at P=0.05 using the New Duncan' s Multiple Range Test.

Table III. Comparative ToMV antigenic concentration at different leaf positions of cv GCR 236 tomato plants, as measured by ELISA, at different times after inoculation with PVX and ToMV-L

Treatments	Concentration of ToMV (mg g^{-1} fresh leaf weight) at different leaf positions Assayed							
	Leaf 1, at 7 dpi		Leaf 5, at 7 dpi		Leaf 21 dpi		Leaf 12, at 28 dpi	
ToMV-L alone	3.26 a ± 0.04a		2.89±0.08a*		2.24±0.09a		1.39±0.04ab	
PVX plus ToMV-L	3.21 a ± 0.04a	(0.98)	2.22±0.04b	(0.77)	2.16±0.11ab	(0.96)	1.28±0.02bc	(0.92)
PVX before ToMV-L	2.01 b ± 0.05b	(0.62)	1.18±0.06c	(0.41)	1.98±0.04b	(1.06)	1.22±0.06c	(0.88)
ToMV-L before PVX	3.32 a ± 0.09a	(1.01)	2.69±0.13a	(0.93)	2.18±0.10a	(0.97)	1.53±0.08a	(1.10)

*Values (mg g^{-1} fresh leaf weight) are means ± standard deviation of 4 plants independently assayed.
Leaf position denotes the location of leaf from the stem base upwards. The primary leaves are numbered 1 and 2.
Values in parentheses are ratios of ToMV-L concentrations in doubly: singly infected plants. Means followed by the same letter (s) in a column, are not significantly different at P=0.05 using the New Duncan's Multiple Range Test.

infection with PVX and the ToMV-L on nursery age seedlings (2 - 3 true leaf stage) resulted in death of other 90% of plants as at two weeks after inoculation (Plate 1A). However, simultaneous double infection in transplanting age seedlings (5 to 6 true-leaf stage) resulted only in a severe 'streak' synergistic disease but no death was recorded. Such plants became severely stunted over time with chlorotic and distorted leaves, in addition to varying degrees of top necrosis (Plate 1B). In contrast, sequential inoculation with the viruses not only led to relatively late-manifesting synergistic disease, the severity was milder than that induced by simultaneous double infection (Plate 1C). Mock-inoculated plants remained healthy and manifested no symptoms regardless of the age at inoculation (Plate 1A).

Comparative concentration of viral antigens in infected plants. The concentration of PVX antigen in the inoculated cotyledons and systemically infected uppermost leaf of tomato seedlings at the 2 - 3 true-leaf stage is shown in Table I. The inoculated leaf contained about the same amount of the virus, single or mixed infection not withstanding; the double to single ratio being 1.1. In the systemically mixed infected leaf, however, at 7 dpi the level was about 4.4 times that in the singly infected plant and the difference was significant ($P < 0.05$). The trend of accumulation of PVX in plants inoculated at the 5 - 6 true-leaf stage followed that recorded in plants inoculated at the earlier stage. Inoculated leaves supported no enhancement, while varying degrees of enhancement of PVX antigenic concentration were recorded in the systemically infected leaves (Table II). Moreover, plants that were mixed-infected had higher levels of PVX than singly infected ones. Simultaneously mixed infected had the highest level of PVX, followed by those inoculated with PVX before ToMV-L. Plants sequentially infected with ToMV-L before PVX invariably had the lowest levels. The difference in level was significant ($P \leq 0.05$) during the early stage of disease but non significant ($P > 0.05$) with increase in age of infection.

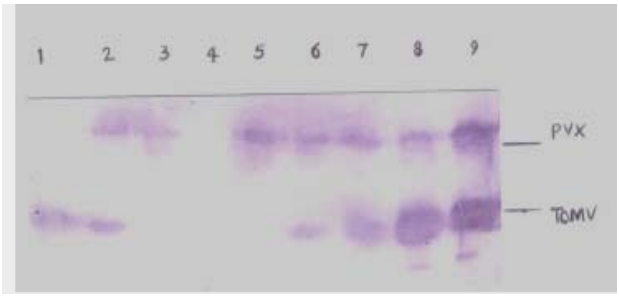
The level of ToMV-L in the inoculated cotyledons in plants inoculated at the 2 - 3 true-leaf stage did not differ

Plate 1. Symptoms manifested at different times in cultivar GCR 236 (+/+) tomato plants following mock, or mixed inoculation with PVX and ToMV-L at various stages of growth

From left: Dead seedlings (simultaneously doubly inoculated with PVX plus ToMV-L); and healthy ones (Mock-inoculated with buffer only) at 15 dpi. Cotyledons of tomato seedlings at the 2-3 leaf stage (2 wks after germination) were inoculated. (B) Plants at 28 days after simultaneous inoculation with PVX +ToMVL at the 5-6 true leaf stage (4 wks after germination). (C) Plants at 28 days after the initial inoculation of TMV 4 days before PVX (sequential inoculation) at the 5-6 true leaf stage (4 wks after germination).

Plate 2. Comparative Western blot analysis of viral coat proteins accumulated in the inoculated primary leaf and systemically infected upper leaf no 5 of cv GCR 236 tomato plants 7 days after initial single or mixed infection at 28 days after germination with PVX and ToMV-L

Lane 1= Purified ToMV-L virion (Marker); 2= Simultaneous PVX plus ToMV-L (in the inoculated primary leaf). Lane 3= Purified PVX virion (Marker); 4= Mock inoculated (healthy primary leaf). Lane 5= PVX alone (in leaf 5); 6= PVX before ToMV-L (in inoculated primary leaf); 7= PVX before ToMV-L (in leaf 5); 8= ToMV-L before PVX (in leaf 5); and 9= PVX plus ToMV-L simultaneously (in leaf 5). Bands were detected by using a mixture of antisera against PVX and ToMV-L.



significantly in singly and doubly inoculated plants. The upper leaf, however, showed different levels with singly infected plants having generally higher concentration than doubly infected ones (Table I). Plants inoculated at six-true leaf stage and assayed at different times after inoculation were not different from those inoculated at the earlier growth stage (Table III). The inoculated primary leaf, assayed at 7 dpi, contained the highest level in both the singly and mixed infected plants and with the exception of plants inoculated with PVX before ToMV, the concentration was significantly the same for all treatments. In the systemically infected upper leaf No. 5 at 7 dpi, the concentration (2.89 mg) in singly infected plants was higher than that in treatments PVX + ToMV-L (2.22 mg) and PVX before ToMV-L (1.18 mg) but similar to those infected with ToMV-L before PVX (2.69 mg). There was a general decrease in contents over time in the upper leaves but at 21 dpi in leaf 9 and 28 dpi in leaf no.12, plants sequentially infected with ToMV-L before PVX had highest concentration of the mixed infection treatments (Table III).

Analysis of accumulated viral coat proteins. The result of western blot analysis of SDS-PAGE-fractionated coat proteins (CP) accumulated in the systemically infected fifth leaf of singly and mixed infected plants at 7 dpi followed the trend of results obtained by ELISA (Plate 2). There was a considerably higher accumulation of PVX-CP under simultaneous mixed infections than in other infection regimes. Although the CP bands for the ToMV-L appeared generally bold regardless of the mixed infection type, it is considerably boldest in sample in which ToMV was inoculated before PVX.

Plant growth and yield responses. As a result of the plant death by the end of the second week of inoculation of all the plants simultaneously mixed infected at the 2 - 3 leaf stage, growth and yield data recorded was limited to plants, which did not die under infection after inoculation i.e., at the 5 - 6 leaf stage. The data were recorded for various growth and yield attributes (Table IV). Healthy plants had significantly better values than infected ones, while singly infected plants had better values than mixed infected ones. Of the mixed infected plants, those with sequential inoculation of ToMV-L before PVX generally had better values than those with PVX before ToMV or PVX plus ToMV. The highest yield loss (90.9 & 92.4%) in number of fruits per plant (90.9%) and mean weight of a fruit (92.4%) were recorded in simultaneously mixed infected plants. These values for the treatment - ToMV before PVX, was relatively lower (69.7 & 55.0%, respectively).

DISCUSSION

Results in this study revealed that age of plant at inoculation had little or no effect on the initial accumulation of the antigens of PVX and ToMV, especially in the early stage of infection. Both viruses accumulated to high levels and in similar proportions, regardless of the age of seedling at inoculation, in the inoculated and systemically infected leaves (Table I &II). Moreover, insignificant enhancement of PVX was observed in the inoculated leaves, while systemically infected leaves supported significantly higher

Table IV. Comparative values of some growth parameters in healthy and diseased cv. GCR 236 tomato plants under single and mixed infections with PVX and ToMV-L

Treatment Combination	Height at 7 wpi (cm)	No. of leaves	Shoot weight (g)		Root weight (g)		% loss in fresh wt of shoot**
			Fresh	Dry	Fresh	Dry	
PVX alone	80.3c	22.5b	64.8b	12.1b	10.5c	1.61c	32.4b
ToMV-L alone	91.0b	20.1c	61.3c	10.8c	13.0b	2.15b	36.0c
PVX plus ToMV-L	61.0e	14.0f	49.5e	7.4e	6.3d	1.01d	48.3e
PVX before ToMV-L	67.3de	17.0e	51.0e	8.1e	7.0d	1.14d	46.8e
ToMV-L before PVX	70.8d	18.8d	55.6d	9.7d	9.8c	1.69c	42.0d
Healthy control	123.8a	25.0a	95.8a	18.8a	16.8a	3.75a	0a

Means followed by the same letter in a column are not significantly different at P=0.05 using the New Duncan' s Multiple Range Test.
**Absolute difference between the values of the control and the respective infection treatment expressed as a percentage of the control. Each value is a mean of 4 replicates.

Table V. Comparative values of some yield parameters in healthy and diseased cv. GCR 236 tomato plants under single and mixed infection with PVX and ToMV-L and mixed infections with PVX and ToMV-L

		Yield of Edible Fruits at 1st Harvest*			% loss in fruit yield**	
Treatment Combinations	No. of days to flowering (dpi)	No. per plant	Total fresh weight (g)	Mean wt. of a Fruit (g)	Based on No. of Fruits	Based on Total wt of fruit
PVX alone	45.0d	2.3b	130.5b	59.2b	30.3b	15.4b
ToMV-L alone	45.5d	2.5b	132.5b	53.8b	24.2b	23.1c
PVX plus ToMV-L	60.8a	0.3c	5.3c	5.3d	90.9d	92.4f
PVX before ToMV-L	56.3b	0.5c	13.5c	13.5d	84.8d	80.7e
ToMV-L before PVX	50.8c	1.0c	31.5c	31.5c	69.7c	55d
Healthy control	44.5d	3.3a	230.6a	70a	0a	0a

Means followed by the same letter in a column are not significantly different at P=0.05 using the New Duncan's Multiple Range Test.
*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 percentage of the control. Each value is a mean of four replicates. dpi: days post inoculation.

PVX growth in both age categories. It can be inferred therefore that age of plant at inoculation does not significantly affect the accumulation of the viruses in the host plant.

The consequential influence of seedling age at inoculation on disease response was however, clearly demonstrated in this study. Seedlings that were simultaneously mixed infected at the nursery age of 14 days after germination (2 - 3 true-leaf stage) manifested extremely severe synergistic symptoms and then died off as evident at 2 weeks after inoculation. Older plants (28-days old) infected under similar conditions survived despite high virus accumulation and the subsequent high disease severity. In a related study of the effect of mixed infection of some viruses including cowpea mottle virus (CMeV), cowpea aphid borne- mosaic virus (CABMV) and southern bean mosaic viruses (SBMV) on cowpea, Taiwo and Akinjogunla (2006) reported that the age of plant at time of infection significantly influenced the assessed growth and yield parameters. Early inoculation at 10 days after germination led to significant reductions in the parameters compared to the values recorded for plants inoculated at 28 days after germination and the healthy control.

Matthews (1991) reported that the severity of disease is normally influenced by factors that may be both external and/or internal to the plant milieu. The morphological and physiological maturity associated with age was obviously lacking in seedlings at the earlier growth stage and may have facilitated, at least in part, the relatively early death of such plants. This showed that while age may not be a deterrent to virus accumulation it surely was influential in mitigating the disease response. Balogun *et al.* (2005) have earlier reported varying viral accumulation dynamics and disease response in different cultivars of tomato under simultaneous mixed infection with PVX and ToMV.

Further consideration of the results in this study revealed apparently lower concentrations and lesser extent of PVX enhancement in sequentially mixed infected plants compared to the levels under simultaneous mixed infection especially during the early stages of infection (Table II). These revealed that sequence of mixed infection does have significant influence on viral accumulation. As noticeable in Plate 1B and C, although both plants were mixed inoculated at the transplanting age of 4 weeks after germination, there are apparent discrepancies in disease severity as at 28 days after initial inoculation. This observation mirrored the apparent differential intensities, as seen in Lanes 8 and 9 of plate 2, of the PVX and ToMV-L coat protein bands, in samples obtained from the same plants at 7 days after initial inoculation.

Plants under sequential mixed infection, especially ToMV-L before PVX a combination that elicited the least PVX antigenic enhancement (Table II), had considerably less severe disease symptoms and consequently relatively better growth and yield (Table IV & V), than plants under simultaneous mixed infection. Furthermore, highest yield losses (90.9 & 92.4% based on number of fruits per plant & mean weight of a fruit, respectively) were also recorded in

simultaneously mixed infected plants whereas treatment - ToMV-L before PVX, had significantly lower values (69.7 & 55.0%, respectively). These data support the hypothesis that sequence of infection has marked effect on the viral accumulation dynamics in mixed infection and the eventual synergistic disease development in tomato pathosystem.

CONCLUSION

The findings of this study have highlighted the potential practical importance, in viral disease management, of the cultural control strategy of keeping young seedlings free of infection as long as possible especially when growing non-resistant host cultivars, in order to take advantage of improved disease tolerance conferred on plants by age. This may be especially advisable for growers in resource -poor domains, where access to improved seeds may be a bit problematic.

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