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Influence of the host cultivar on disease and viral accumulation dynamics in tomato under mixed infection with *Potato virus X* and *Tomato mosaic virus*

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Summary. The primary leaves of seedlings of tomato (*Lycopersicon esculentum* Mill.) cultivar Fukuju No. 2 (a common Japanese cultivar that is susceptible to *Tobacco mosaic virus* (TMV, genus *Tobamovirus*) were inoculated at the five-true leaf stage with the O strain of *Potato virus X* (PVX, genus *Potexvirus*) and with a mixture of that strain plus *Tomato mosaic virus* (ToMV, genus *Tobamovirus*). Inoculation resulted in varying degrees of disease manifestation. During the acute stage of the resulting severe disease (between 5 and 12 days postinoculation), PVX and ToMV levels rose considerably in both the inoculated and the systematically infected leaves. Furthermore, levels of PVX in the systemically infected upper leaves (positions 5 to 7) of plants with a mixed infection were three to six times as high as in plants given the single infection, as determined by direct double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA). In tomato cv. GCR 236 (+/+), symptom manifestation and the accumulation of both PVX and ToMV closely followed the pattern recorded for cv. Fukuju No. 2. In cv. GCR 237 (*Tm-1*) plants, however, only PVX accumulated while ToMV whether inoculated singly or mixed with PVX was detected neither in the inoculated nor in the systemically infected leaves even 14 days after inoculation. In contrast to other cultivars, SDS-PAGE, Western blot and Northern blot hybridization did not reveal any enhancement of the coat protein and genomic RNA of PVX in such systemically infected leaves. Consequently, the characteristic severe symptoms normally associated with mixed infection in TMV-susceptible cultivars were absent.

Key words: *Lycopersicon esculentum*; cv. Fukuju No. 2; cv. GCR 236 (+/+); cv. GCR 237 (*Tm-1*); PVX and ToMV.

Introduction

The elicitation of visible symptoms, such as various forms of mosaic and distortion with consequent reduction in growth and crop yield, has long been associated with virus infection of plants. Such severe responses have also been reported for many crop plants including tomatoes. In Nigeria, with its tropical climate, the most common and important virus diseases of tomato are tomato mosaic virus, tomato bunchy top virus and tomato yellows. Tomato mosaic and tomato yellows virus diseases are the most serious, causing losses from 20 to as much as 90% (Lana and Adegbola, 1977). The scourge of virus disease is however not limited to the tropics.

The task of managing viral diseases has always been a daunting one. It often requires particular cultural practices and the exploitation of host resistance, whose effectiveness hinges on many extraneous factors. Resistance of plants to viruses has usually been divided into two types: 1. total

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resistance, involving all the members of a plant species (immunity); 2. partial (qualitative) resistance, when some members of a species are resistant while some are susceptible. This second type of resistance is often controlled either by a single dominant gene or by a small number of genes (Mansky and Hill, 1993).

In tomato, three genes, *Tm-1, Tm-2,* and *Tm-2²* (Pelham, 1966; 1972) have been reported to confer constitutive resistance to *Tobacco mosaic virus* (TMV, genus *Tobamovirus*). The *Tm-1* gene confers a symptomless reaction, whereas *Tm-2* and *Tm-2²* confer a hypersensitive response. Immunoflorescence microscopy and infectivity assays revealed that resistance conditioned by *Tm-1* is maintained even in the protoplast (Motoyoshi and Oshima, 1979; Watanabe *et al*., 1987).

In nature, mixed viral infections are not uncommon. They occur in both plants and animals, and doubly infected organisms commonly display more severe disease symptoms and higher levels of one or both viruses than the same organism when singly infected (Pruss *et al*., 1997). Bennett (1949) first showed quantitatively that infection by one virus could affect the multiplication of another virus, as in the case of dodder latent mosaic virus which was markedly stimulated in mixed infections with some other viruses. Otsuki and Takebe (1976) showed that pairs of related and unrelated viruses often replicated in the same cells and interacted synergistically or antagonistically. Goodman and Ross (1974) had earlier reported that *Potato virus X* (PVX, genus *Potexvirus*) was enhanced within cells of tobacco when co-infected with *Potato virus Y* (PVY, genus *Potyvirus*, family *Potyviridae*).

The mechanisms involved in interactions between viruses in mixed infections vary with the host and with the viral strains involved. Goldberg and Brakke (1987) reported a 5.4-fold increase in the concentration of *Maize chlorotic mottle virus* (MCMV, genus *Machlomovirus*) in a mixed infection with strain B of *Maize dwarf mosaic virus* (MDMV-B, family *Potyviridae*), but there was no corresponding increase in MDMV-B. Scheets (1998), however, reported that while the MCMV concentrations in corn co-infected with *Wheat streak mosaic virus* (WSMV, genus *Rymovirus*, family *Potyviridae*) increased 3.3- to 11.2- fold compared to the average for plants inoculated with MCMV alone, WSMV concentrations also went up in such mixed infection, becoming at least 2.1 times what it was in plants infected with WSMV alone. Similar increases have been reported in soybean infected with *Soybean mosaic virus* (SMV, genus *Potyvirus*, family *Potyviridae*) plus another non-potyvirus member (Anjos *et al*., 1992). Vance (1991) reported that in co-infections with PVX and PVY, PVX experienced alterations in the replication of its RNA, leading to higher levels of anti-sense RNA.

In view of the different interactions in various pathosystems, this study focused on the influence of the host cultivar in host-viral interactions, and especially on the accumulation dynamics of PVX and TMV inoculated singly or doubly in three different cultivars of tomato.

Materials and methods

Plant propagation and inoculation with viruses

Three cultivars of tomato, Fukuju No. 2 (a common Japanese cultivar), GCR 236(+/+) (TMV-susceptible) and GCR 237 (*Tm-1*) (homozygously resistant to TMV) were grown under normal greenhouse conditions, maximum temperature 32°C by day, minimum 18°C at night. Sandy loam soil was steam-sterilized at 121°C for 30 min and supplemented with NPK fertilizer elements at planting. In the experiments, which were carried out over a two-year period, the arrangement of the pots followed a completely randomized design. Inoculation of the plants at the 5-leaf stage was by rubbing the carborundum-dusted primary leaves (leaves 1 and 2) with 0.2 mg ml^{-1} (in phosphate buffer, pH 7.2) of one virus alone or simultaneously with a mixture of equal quantities of PVX and ToMV. Mock-inoculated plants (with buffer only) served as the control.

Analysis of viral concentrations by enzyme linked immunosorbent assay (ELISA)

Leaf samples from plants with PVX or ToMV alone, or with a mixture of those viruses were ground for 1 min in 0.02 M sodium-phosphate buffer, pH 7.2 with a pre-cooled mortar and pestle in the ratio of 1 g of tissue to 10 ml buffer. The homogenate was centrifuged for 10 min at 10,000 *g* and the supernatant was carefully removed and serially diluted. To estimate virus concentration in

the samples ELISA was carried out essentially according to the double antibody sandwich (DAS) method of Clark and Adams (1977). Initial viral IgG was applied at 5 μg ml^{-1} (final concentration) while antibody-alkaline phosphatase conjugate was applied at 2 μg ml^{-1}, final concentration. Color was developed with *p*-nitrophenylphosphate at 1 mg ml^{-1} concentration in 10% diethanolamine, pH 9.8 and absorbance was read at 405 nm using a microplate photometer (Corona Electric model MTP-22, Corona Electric Co., Ibaraki, Japan). The concentration of each virus in the samples was estimated from a standard curve established for each set of assays with purified virus preparations of each virus.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Protein fractionation and Western blot analysis for the assay of virus-specific protein accumulation in infected plants were carried out according to the standard method of Sambrook *et al.* (1989). Known quantities (0.1–0.3 g) of leaf samples collected from the same positions on infected or healthy plants were macerated with pre-cooled mortars and pestles in homogenizing buffer (0.05 M Tris-HCl, pH 6.8 containing 0.1% 2-mercaptoethanol) at a ratio of 1 g of sample to 10 ml of buffer. Samples were loaded at 10 μl per lane on 15% minigels and resolved at 30 mA until the dye head reached the bottom of the gel.

For immunoblot analysis, proteins were electrotransferred to hybond-C extra nitrocellulose membrane (Amersham Life Science, Tokyo, Japan) at a constant current of 180 mA for 30 min. Antiviral antibodies were used at 5 μg ml^{-1} of blocking buffer. Goat anti-rabbit antibody-alkaline phosphatase conjugate (Biosource International, Camarillo, CA, USA) was used as a second antibody at 1:1,000 dilution. Virus-specific protein bands were visualized in nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution in 15 ml of alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; 5 mM $MgCl_2$) containing 100 μl of NBT solution (50 mg of NBT per ml of 70% dimethyl formamide) and 50 μl BCIP solution per ml of dimethyl formamide). The reaction was terminated as soon as the desired intensity was attained, usually within 5 min with PBS containing 1 mM EDTA (i.e. 200 μl of 0.5 M EDTA, pH.8.0, per 50 ml of PBS).

Isolation and purification of total RNA and Northern blot hybridization

Total RNA was isolated from both mock-inoculated and infected leaf samples according to the method of Logemann *et al.* (1987), which made use of a guanidine buffer (8 M guanidine hydrochloride, 20 mM Mes (4-morpholineethansulfonic acid), 20 mM EDTA, and 50 mM mercaptoethanol at pH 7.0) to homogenize the sample. The homogenate was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and RNA was extracted from the aqueous phase by mixing with precooled 0.7 vol. ethanol and 0.2 vol. of 1 M acetic acid and keeping at -70°C for 1 h. The pelleted RNA was washed first with 3 M sodium acetate, pH 5.2 and then with 70% ethanol. The RNA pellet was dissolved in sterile DEPC-treated water. Aliquots of 10 μl were quantified spectrophotometrically using the Ultrospec 3000 spectrophotometer (Pharmacia/Biotech, Tokyo, Japan) while the remaining RNA was kept at -70°C until used.

For analysis of the abundance of genomic and sub genomic RNA, equal quantities of total RNA (30 μg) of each treatment sample were fractionated on 1.2% formaldehyde-agarose gel and transferred by capillary elution overnight to a hybond nylon membrane in 20×SSC (1×SSC=0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). An RNA probe, complementary to 350 nts of the PVX coat protein gene, inserted into a T7 plasmid vector and labeled in the laboratory with digoxigenin (DIG) (Boehringer, Mannheim, Germany) according to manufacturer's instructions, was hybridized to the total RNA. Prehybridization, hybridization and washing were carried out using the standard procedures (Sambrook *et al.*, 1989; Ausubel *et al.*, 1995). The immobilized PVX RNAs were then immunodetected using anti-DIG alkaline phosphatase conjugate. Color development was with 15 ml NBT/BCIP solution in 2× alkaline phosphatase buffer i.e. double the concentration normally utilized for protein detection.

All numerical data were subjected to analysis of variance (ANOVA). Means were compared with Tukey-Kramer's HSD test at P=0.05 level of significance.

Results

Symptoms

In cultivars Fukuju No. 2 and GCR 236 (+/+), infection with PVX alone elicited a generally mild chlorosis of the systemically infected leaves and subsequent stunting of the whole plant, while infection with ToMV alone elicited a severe mosaic that manifested itself first in the young upper leaves around the 7th day after inoculation. Subsequent leaves also developed mosaic, and were much smaller. In cv. GCR 237 (*Tm-1*), PVX elicited the same response as in the other cultivars but ToMV inoculated alone did not elicit any symptoms.

In cv. Fukuju No. 2 and GCR 236, mixed infection with PVX and ToMV led to severe streak disease that also manifested itself first on the systemically infected uppermost leaves. The plants so infected were severely stunted, with chlorotic and distorted leaves, in addition to varying degrees of necrosis on the leaves and stems. No severe symptoms formed on doubly inoculated cv. GCR 237. Figure 1a shows the absence of symptoms on cv. GCR 237, compared with the symptoms in the other two cultivars (Fig. 1b and c) 7 days after mixed inoculation with PVX and ToMV.

Comparative concentration of viruses in plants with single and double infections

PVX concentration

ELISA revealed that in samples from all three cultivars PVX levels were considerably higher in the primary leaves inoculated either singly or doubly at the 5 to 6-leaf stage. No significant differences (P=0.05) were found between singly or doubly inoculated plants of any cultivar. Seven days after inoculation, the virus concentration in cv. GCR 236 plants was relatively more than, but not significantly different from virus levels in cv. Fukuju No. 2. The virus level in cv. GCR 237 was significantly (2.5 to 3 times) less than the levels recorded with both GCR 236 and Fukuju No. 2 plants (Table 1).

Analysis of the systemically infected upper leaves showed that while doubly infected plants had significantly higher virus levels than singly-infected plants in cv. Fukuju No. 2 and GCR 236, the level in cv. GCR 237 remained the same for both singly and doubly infected plants (Table 2).

Fig. 1. Foliar symptoms in three cultivars of tomato after mixed infection with PVX and ToMV-L, 7 days after inoculation. A, GCR 237 (Tm-1); B, GCR 236 (+/+); C, Fukuju No. 2.

The virus level (1.93 mg) in Fukuju plants with double infection was the highest and differed significantly from the others. The ratios of concentration of doubly infected to that of singly infected plants were 6.03, 4.36 and 0.93 for cultivars Fukuju No. 2, GCR 236 and GCR 237 respectively (Table 2).

When virus levels in the inoculated leaves were compared with those in systemically infected leaves of the same plant for each of the 3 cultivars, those in systemically infected leaves of the singly infected cv. Fukuju No. 2 and GCR 236 were significantly lower, and those in cv. GCR 237 non-significantly lower (Tables 1 and 2). Virus concentration in systemically infected leaves, compared to the levels in the inoculated leaves, were 23 and 21% respectively for Fukuju No. 2 and GCR 236, and 80% for GCR 237.

ToMV concentration.

The mean concentrations of ToMV in inoculated leaves of the 3 tomato cultivars are shown in Table 1. Seven days after inoculation both the singly and doubly infected plants of cv. Fukuju No. 2

Table 1. Virus concentration, as determined by ELISA, in the inoculated leaves of three cultivars of tomato with single and mixed infection with *Potato virus X* and *Tomato mosaic virus*.

Host cultivar	Virus treatment	Concentration (mg g^{-1} fresh leaf weight) in the inoculated leaf at 7 dpi[a]			
		PVX concentration[b, c]		ToMV concentration[b, c]	
Fukuju No. 2	PVX or ToMV alone	1.32 a±0.04		1.92 b±0.14	
	PVX+ToMV	1.40 a±0.10	(1.06)	1.75 b±0.10	(0.91)
GCR 236 (+/+)	PVX or ToMV alone	1.37 a±0.04		3.26 a±0.04	
	PVX+ToMV	1.44 a±0.08	(1.05)	3.21 a±0.04	(0.98)
GCR 237 (*Tm-1*)	PVX or ToMV alone	0.57 b±0.05		0.00 c±0.00	
	PVX+ToMV	0.51 b±0.04	(0.89)	0.00 c±0.00	(0.0)

[a] Figures are means ± the standard deviation of data obtained from 4 plants individually assayed in replicates.
[b] Figures in the same column followed by the same letter are not significantly different at P=0.05 using the Tukey-Kramer HSD test.
[c] Numbers in parenthesis denote the ratio of double to single infections with the viruses.

Table 2. Virus concentration, as determined by ELISA, in the systemically infected leaves of three cultivars of tomato with single and mixed infection with *Potato virus X* and *Tomato mosaic virus*.

Host cultivar	Virus treatment	Concentration (mg g^{-1} fresh leaf weight) in the inoculated leaf at 7 dpi[a]			
		PVX concentration[b, c]		ToMV concentration[b, c]	
Fukuju No. 2	PVX or ToMV alone	0.32 c ±0.03		3.12 a±0.15	
	PVX+ToMV	1.93 a±0.06	(6.03)	2.34 b±0.14	(0.75)
GCR 236 (+/+)	PVX or ToMV alone	0.39 c±0.04		2.89 a±0.08	
	PVX+ToMV	1.70 b±0.07	(4.36)	2.22 b±0.04	(0.77)
GCR 237 (*Tm-1*)	PVX or ToMV alone	0.40 c±0.04		0.00 c±0.00	
	PVX+ToMV	0.43 c±0.05	(0.93)	0.00 c±0.00	(0.0)

[a] See Table 1.
[b] See Table 1.
[c] See Table 1.

and GCR 236 had substantially high virus concentrations in the inoculated leaves. Cultivar GCR 236, however, had significantly higher virus levels than cv. Fukuju No. 2. For any given cultivar, the difference between single and double infection was insignificant; the ratio between double and single infection being 0.91 for cv. Fukuju No. 2, and 0.98 for GCR 236. No ToMV accumulation was recorded in cv. GCR 237 at 7 dpi.

Unlike what occurred with PVX, in the systemically infected leaves ToMV concentrations in doubly infected plants of cv. Fukuju No. 2 and GCR 236, were significantly lower than those in singly infected plants (Table 2). The reduction was about

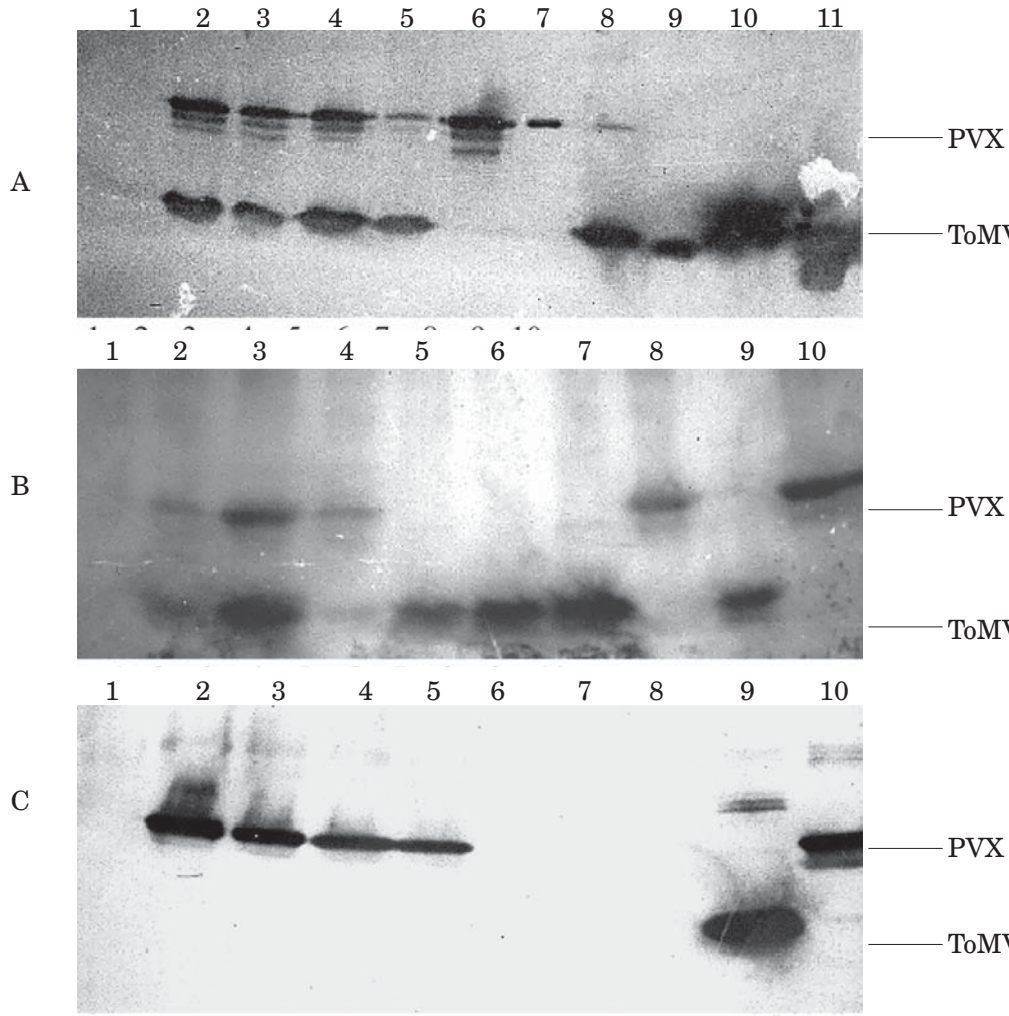

Fig. 2. Western blot analysis of accumulated viral coat proteins in the inoculated leaf of different tomato cultivars under single or mixed infection with PVX and different strains of TMV. (A) Fukuju No. 2 at 5 dpi. Lane 1, control; 2, P+L+ L11A; 3, P+L; 4, P+L11A; and 5, P+OM. Lane 6, PVX (P) alone; 7, purified PVX; 8, L11A alone; 9, OM alone; 10, L alone and 11, purified ToMV-L. (B) GCR 236 at 7 dpi. Lane 1, control; 2, P+ L11A; 3, P+L; and 4, P+OM. Lane 5, OM alone; 6, L11A alone; 7, L alone; 8, P alone; 9, purified TMV-L and 10, purified PVX. (C) GCR 237 at 7 dpi. Lane 1, control; 2, P alone; 3, P+L; 4, P+L11A; 5, P+ OM; 6, L11A alone; 7, OM alone; 8, L alone; 9, purified ToMV-L and 10, purified PVX. All bands were visualized using combinations of polyclonal antibodies raised against the individual viruses in rabbits. The position of the PVX and ToMV bands are as shown.

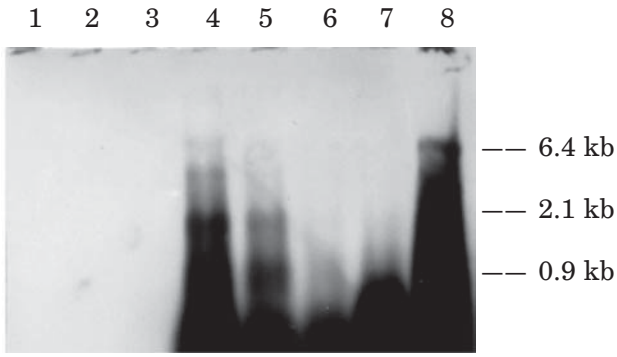

Fig. 3. Northern blot hybridization analysis of the accumulated PVX RNAs in the systemically infected upper leaf No. 5 of three tomato cultivars 7 days after single or mixed inoculation with PVX and ToMV-L.
Lanes 1 and 2 are samples from mock-inoculated (healthy) and ToMV-L alone in cv. Fukuju No. 2 plants while lane 3 is ToMV-L alone in cv. GCR 236. Lanes 4, 5, and 6 are PVX+ToMV-L in cv. Fukuju No. 2, GCR 236, and GCR 237 respectively. Lane 7, PVX alone in GCR 237 and lane 8, 1 μg of purified PVX RNA used as a marker. PVX RNAs were hybridized with a cDNA probe prepared from a 350 nts portion in the coat protein gene of PVX and labeled with Digoxigenin (DIG). The hybridized RNAs were immunodetected using anti DIG-alkaline phosphatase conjugate. The positions of the genomic (6.4 kb) and subgenomic RNAs (2.1 kb and 0.9 kb) determined from preliminary experiments and from standard texts are as indicated.

25% for cv. Fukuju No. 2, and 23% for GCR 236. In cv. GCR 237, ELISA failed to detect ToMV even in the systemically infected upper leaves at 7 dpi (Table 2).

As shown in Tables 1 and 2, there were also differences between cv. Fukuju No. 2 and cv. GCR 236 in the pattern of ToMV increase in the systemically infected leaves as compared with the inoculated leaves of a given plant. Virus levels in systemically infected leaves were higher with both single and double infection in cv. Fukuju No. 2, but were lower in GCR 236. For instance, while the virus level in the inoculated leaf compared to that of systemically infected leaves was 61 and 78%, respectively, for single and double infection in Fukuju No. 2, the level was 111 and 145% in cv. GCR 236. In other words, ToMV accumulation in the upper leaf was more efficient in Fukuju No. 2 than in cv GCR 236.

Analysis of viral proteins accumulated in singly and doubly infected plants

Western blot analysis of SDS-PAGE-fractionated proteins from leaf samples of both inoculated and systemically infected leaves, showed that the pattern of coat protein (CP) accumulation of both ToMV and PVX was similar to that of the virus particles measured by ELISA. Figures 2a and 2b show the uninhibited accumulation of PVX and some ToMV strains in singly and doubly inoculated primary leaves of cv. Fukuju No. 2 (at 5 dpi) and those of cv. GCR 236 (at 7 dpi). In cv. GCR 237, ToMV was not detected in the inoculated leaf nor in leaf 5 by Western blot analysis at 7 dpi, but PVX accumulated to substantial levels in both leaves. Fig. 2c shows Western blot of the CPs accumulated at 7 dpi in inoculated primary leaf No. 1.

Analysis of PVX RNA levels with single and mixed infection

Northern blot hybridization analysis of total RNA samples from leaf position 5 at 7 dpi (acute stage of disease) with a PVX specific probe revealed much higher levels of the PVX subgenomic RNAs in double infected plants of both Fukuju No. 2 and GCR 236. However, the genomic RNA level in GCR 236 was considerably lower than that in cv. Fukuju No. 2 and was undetectable in cv. GCR 237, where only the subgenomic RNAs bands were sparingly visible both in plants with single and in plants with mixed infections (Fig. 3).

Discussion

Enhancement of PVX concentration in systemically infected upper leaves of tomato has been reported (Balogun, 2002; Balogun *et al*., 2002). The experiments in this study, however, revealed that enhancement was also influenced significantly by the characteristics of the tomato host. Tomato genotypes have recognizable, distinguishable morphological and physiological characteristics that impact on the host-pathogen or pathogen-pathogen interactions (Balogun *et al*., 2002), and this was demonstrated in the present study. Tomato cultivar GCR 236 responded to single and mixed infections in a way similar to that of Fukuju No. 2, with both cultivars showing moderate to severe mosaic symptoms in response to single infection. Mixed

infection also elicited a characteristic streak disease, with severe mosaic and necrosis of the upper systemically infected leaves. In this study, however, as shown in Figure 1, the synergistic disease was more severe in cv. Fukuju No. 2 than in cv. GCR 236.

These observed discrepancies also extended to virus accumulation dynamics in the different genotypes. ToMV accumulation in the upper leaves was more efficient in cv. Fukuju No. 2 than in cv. GCR 236. The ToMV level in the systemically infected upper leaf was about 40% higher than in the inoculated leaf of singly infected Fukuju No. 2 plants and 22% higher than in the inoculated leaf of doubly infected Fukuju No. 2. On the other hand, in GCR 236 virus levels were 11% lower in systemically infected leaves than in inoculated leaves with single infection, and 45% lower with double infection. This could have been a result of easier viral phloem movement in the cv. Fukuju No. 2 plants due to genetic factors. It could also account at least in part for the differences in the levels of PVX (a 6-fold increase in cv. Fukuju No. 2 and a 4-fold increase in cv. GCR 236) when infected together with ToMV, hence the differences in the severity of the disease in these hosts. Balogun (2002) using linear regression analysis reported a positive correlation between PVX enhancement and disease severity during the acute stage of disease resulting from PVX and ToMV infection.

In cultivar GCR 237, which has the *Tm-1* gene that confers homozygous resistance to TMV, ToMV accumulated virus particles to undetectable level in the inoculated leaves, and/or to low levels in the systemically infected upper leaves of both singly and doubly inoculated plants (Tables 1 and 2). Consequently, in spite of the PVX concentration remaining high, it was not significantly higher in plants with double infection than in plants with single infection, nor was there a manifestation of the symptoms characteristic of mixed infection.

Results with ELISA, Western blot analysis of the CPs and the Northern blot hybridization of the RNAs all indicated that though PVX is generally enhanced by co-infection with ToMV, the extent of enhancement in tomato depended not only on having a mixed inoculation with ToMV, but also on the ability of the enhancing ToMV strain to accumulate to a certain substantial level in the leaf that is supporting enhancement, and at a time that coincided with active PVX replication. In other words, when there was no substantial ToMV accumulation, PVX enhancement did not occur. Lack of enhancement in turn meant no severe disease manifestation, hence the possibility of good tomato growth and yield.

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