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Patterns in disease progress and the influence of single and multiple viral infections on pepper (*Capsicum annuum* L.) growth

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Abstract The patterns and progress of disease caused by multiple infections of *Cucumber mosaic virus* (CMV), *Pepper mild mottle virus* (PMMoV) and *Pepper mottle virus* (PepMoV) and their effects on growth of pepper plants (*Capsicum annuum* L.) were investigated in this study. Each virus induced distinct symptoms, but more severe symptoms, including reduced growth rates, were observed when pepper plants were simultaneously infected by more than one virus. When CMV was included in multiple viral inoculations, co-inoculations and sequential inoculations, PepMoV and PMMoV symptoms were observed but the symptoms characteristic of CMV were not masked, even though CMV titre did not increase greatly. In multiple viral infections, PepMoV titre and CMV did not increase significantly, but PMMoV titre gradually increased in most cases. Growth rates of pepper plants were greatly reduced during the 30 to 40-day post-inoculation period under both single-infection and multiple-infection conditions, but multiple viral infections of CMV pre-inoculated peppers were affected to a greater extent. A significant reduction in fruit size and fruit number was observed in single and multiple viral inoculations, and fruit malformation rates were high in CMV single-infection and multiple viral infections with CMV.

Keywords *Capsicum annuum* · Disease progress · Multiple virus infection · Pathogenicity · Pepper · Synergism

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Introduction

Over 40 viruses are known to infect peppers, and 10 viruses have been reported for cayenne pepper (*Capsicum annuum* L.) in Korea (Watterson 1993; Kim 2005). The three viruses that cause the greatest economical losses are *Cucumber mosaic virus* (CMV), *Pepper mild mottle virus* (PMMoV) and *Pepper mottle virus* (PepMoV) which belong to the genera *Cucumovirus* and *Tobamovirus* and *Potyvirus*, respectively (Choi and Kwon 1989; Choi et al. 2005). Pepper diseases caused by viruses from 2001 to 2004 in 32 pepper production regions in Korea were caused by

CMV (33.2%), PepMoV (18.5%) and PMMoV (13.9%), and 26.2% of the reported cases were caused by multiple infections of all three of these viruses (Kim 2005). CMV and PepMoV are transmitted by aphids in a nonpersistent manner, whereas PMMoV is transmitted by direct contact. In contrast, viruses other than these comprised less than 5% of the total reported infections.

In this study, the responses of pepper to inoculation with these three viruses, singly or in multiple co-infections established simultaneously and/or sequentially, were investigated. Collected data include effects on fruit yield and vegetative growth, and indicated synergisms between certain viruses.

Materials and methods

Sources of virus inocula and pepper cultivars

The three virus strains used in these experiments were CMV-Mf (Choi et al. 1998), PMMoV-P (Choi et al. 1989) and PepMoV-Vc (Kim 2005). Virus inocula were propagated on young *Nicotiana benthamiana* Domin. plants and the chilli pepper cultivar Nokkwang (Seminis Korea, Seoul).

Virus inoculation combinations and methods to monitor plant growth

Single, multiple co-infection and sequential infection in 17 viral combinations were tested. Both multiple virus co-inoculation and multiple virus sequential inoculation experiments were conducted. Slash (/) symbol indicates a sequential inoculation experiment between primary (pre-inoculated) and challenge inoculation. The plus (+) symbol indicates simultaneous co-inoculation. For the sequential inoculation, challenging virus or viral combinations were inoculated after one week on primary virus pre-inoculated peppers.

Inoculation combinations were: (1) three single inoculations of CMV, PMMoV and PepMoV, (2) five simultaneous multiple co-inoculation combinations: CMV+PMMoV, CMV+PepMoV, PMMoV+PepMoV and CMV+PMMoV+PepMoV, and (3) nine time-difference sequential inoculation combinations; CMV/PMMoV, CMV/PepMoV, CMV/PMMoV+PepMoV, PMMoV/CMV, PMMoV/PepMoV, PMMoV/ CMV+PepMoV, PepMoV/CMV, PepMoV/PMMoV and PepMoV/CMV+PMMoV.

Virus-infected N. benthamiana leaves, either in single virus or as co-inoculation combinations, were ground 10-fold (w/v) in a 0.1 M phosphate buffer solution (pH 7.0) containing 20 mM EDTA and used for inocula. At the time of inoculation, the pepper plants had 4–6 true leaves. The pepper plants were transplanted 10-days post primary inoculation (dpi) and were grown in a greenhouse. Experiments were repleated 2 times with 5 plants per inoculation combination. Each combination of virus-inoculated plants was treated as a block and arranged randomly in the greenhouse. Growth variables for the pepper plants, including plant height, fruit set and other physical characteristics of the fruit were recorded every 10 days following inoculation.

ELISA

Relative virus accumulation, i.e., differences in the relative titre of viruses throughout the pepper growth stages after inoculation was monitored using Enzyme-linked immune-sorbent assay (ELISA). Following virus inoculation, 0.5 g of newly emerged pepper leaves were harvested at 20, 40 and 60 dpi. Pepper leaves were ground with 0.01 M potassium phosphate buffer (pH 7.5) at dilution factor of 1 g/20 mL. The ELISA method used was Double Antibody Sandwich ELISA (DAS-ELISA), and microplates were coated with 125 μl of 1:1,000 diluted antisera of CMV, PMMoV and PepMoV. Antisera to CMV, PMMoV and PepMoV were produced in immunized New Zealand white rabbits (Plant Virus GenBank, Seoul, Korea). Conjugated immunoglobulin concentration was 1:1,000, and reactions were measured with a Model 680 Microplate Reader (Bio-Rad, Hercules, U.S.A.) at 405 nm for 30 min following the addition of a *p*-nitrophenyl phosphate.

Data analysis

Relative virus titre as ELISA measurement and plant height data were transformed to a comparative index to compare changes in virus titres and effects on growth. When data were transformed, the average of accumulated virus amounts from single infections, and also of healthy plant heights, were considered as 100%. The collected yield, average number of fruits per plant, fruit weight and plant height data were analyzed in an analysis of variance (ANOVA) in a complete randomized block design using the statistical software program SAS (SAS Institute, Cary, N.C., U.S.A.), with a Tukey's

Fig. 1 Developed symptoms of pepper (*Capsicum annuum* cv. Nockwang) leaves that were systemically infected with *Cucumber mosaic virus* (CMV; Mf strain) (A), *Pepper mild mottle virus* (PMMoV; P strain) (B) and *Pepper mottle virus* (PepMoV; strain Vc)(C). Typical symptoms of each virus were mosaic and veinal chlorosis (CMV), mild mosaic and upward curling (PMMoV), and mottle and downward curling (PepMoV). Photographs represent top (row a) and side views (row b)

Studentized Range Test used to test for significant differences among means for effects of virus inoculation combinations on pepper growth.

Results and discussion

Symptom development

Symptoms in CMV, PMMoV and PepMoV single-infected pepper plants began at 6- dpi, and distinct symptoms for each virus were more developed at 10 dpi. Distinct CMV-induced symptoms were yellow mosaic near the leaf vein areas, malformation of leaf edges and reduction of leaf sizes (Fig. 1A), as well as mild mosaic symptoms on the entire leaf area and upward curling symptoms by PMMoV (Fig. 1B), and clear mottle symptoms with downward curling symptoms by PepMoV (Fig. 1C). Leaf malformation was marked in CMV-infected peppers, and to a lesser degree in PMMoV and PepMoV-infected plants.

Simultaneous multiple infection of the three viruses induced more severe symptoms than did single infections. In particular, CMV+PMMoV and CMV+

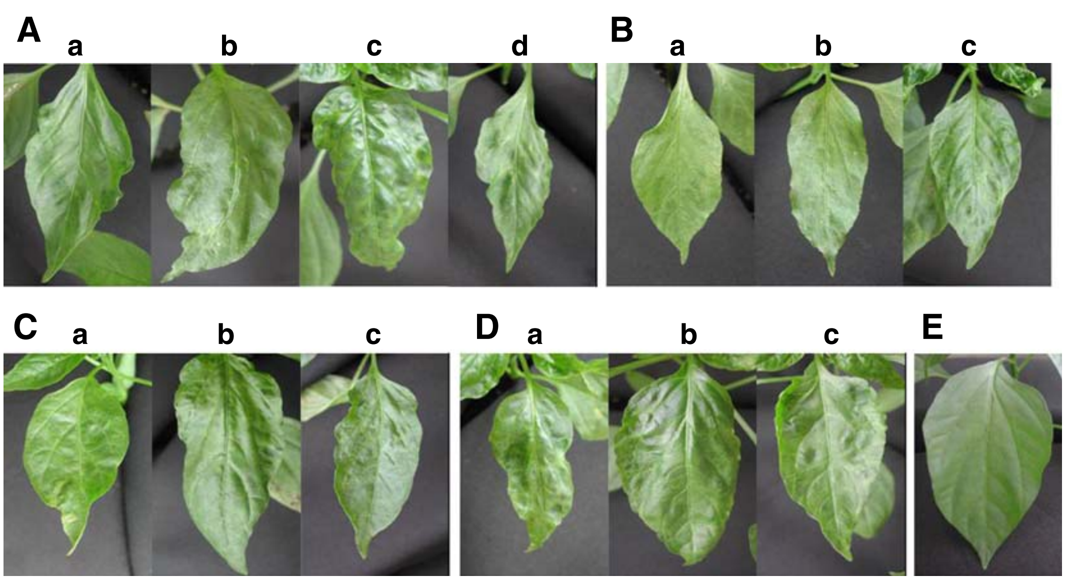

Fig. 2 Symptoms on pepper leaves with multiple combinations of CMV, PMMoV and PepMoV infection. (A) Co-inoculation: CMV+PMMoV(a); CMV + PepMoV(b); PMMoV + PepMoV (c); CMV+PMMoV + PepMoV(d). (B) Sequential inoculation: CMV/PMMoV(a); CMV/PepMoV(b); CMV/PMMoV + PepMoV(c). (C) Sequential inoculations: PMMoV/CMV(a); PMMoV/PepMoV(b); PMMoV/CMV + PepMoV(c). (D) Sequential inoculation: PepMoV/CMV(a); PepMoV/PMMoV(b); PepMoV/CMV + PMMoV(c). (E) Healthy control

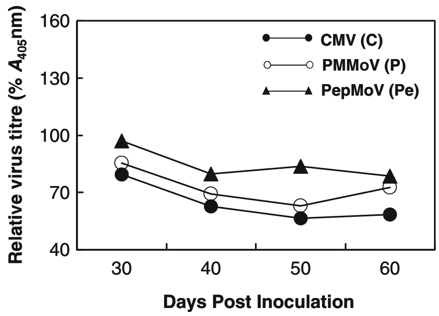

Fig. 3 Relative average virus titre changes in single virus-infected pepper plants compared with 20 dpi as 100%

PMMoV+PepMoV combinations induced pronounced CMV-induced symptoms (Fig. 2A a, d) and PepMoV+PMMoV and CMV+PepMoV induced strong PepMoV-induced mottle symptoms, and leaf distortion was more severe than it was when with a single PepMoV infection (Fig. 2A b, c). When multiple viruses were tested, CMV-induced leaf malformation and PepMoV-induced symptoms were not masked by other viral symptoms.

Symptom development in sequential infections was different from those of multiple virus co-infections. Symptoms of CMV/PMMoV, CMV/PepMoV or CMV/PMMoV+PepMoV-infected peppers were typical of those caused by CMV, for example, they had small, thin leaves (Fig. 2B a, b, c). In contrast, PMMoV/PepMoV or PepMoV/PMMoV-infected peppers showed pronounced pre-inoculated PMMoV or PepMoV symptoms (Fig. 2C b and 2D b), but when CMV was inoculated later, pre-inoculated PepMoV or PMMoV symptoms were observed, but leaf shape was similar to CMV-induced symptoms (Fig. 2C a and 2D a). These results indicate that CMV-induced symptoms were not masked and did not disappear when co-infected or with sequential infections, whether pre-inoculated or inoculated later.

Change of relative titre of viruses

Relative virus titres of single virus-infected plants showed a gradual reduction during the growth period

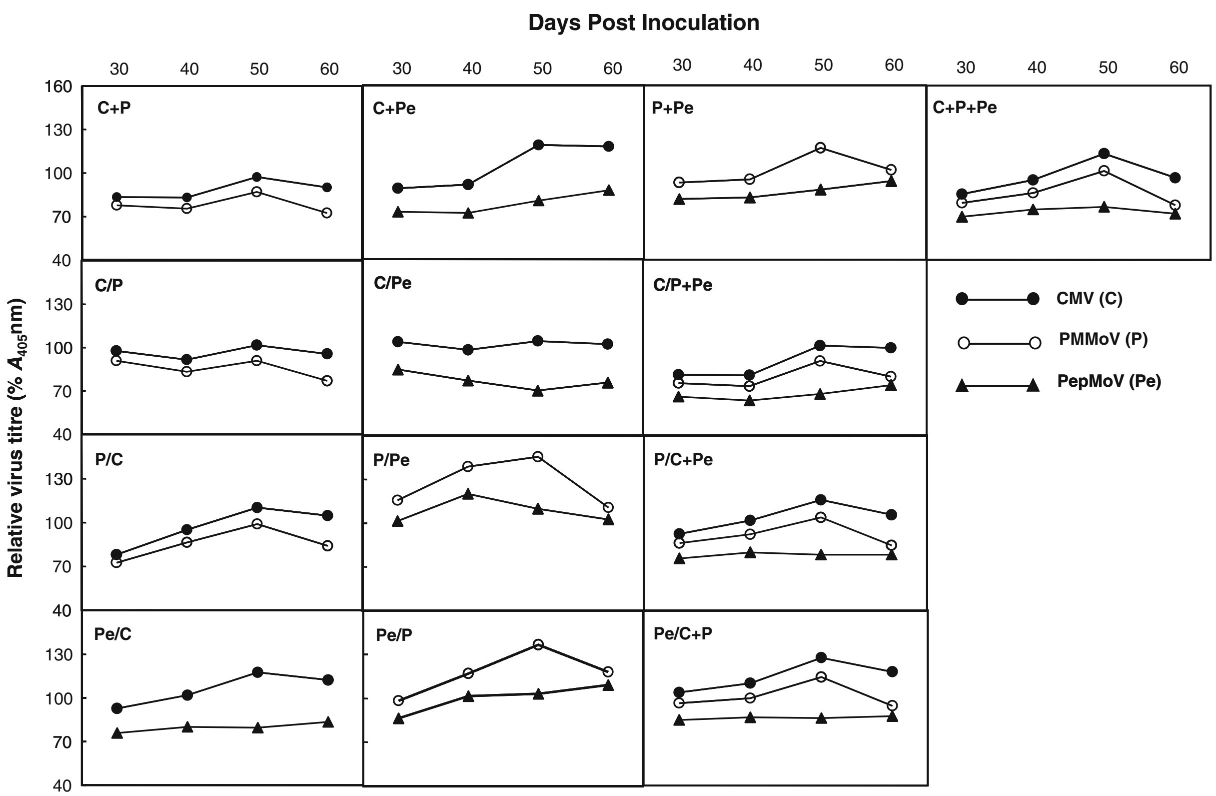

Fig. 4 Average relative virus titre of each virus in multiple virus-infected pepper plants compared with average relative virus titre of single virus-infected pepper plants as 100%. Plus (+) symbol was used for simultaneous co-inoculation and slash (/) symbol was used for sequential inoculation between pre-inoculation and challenging virus inoculation

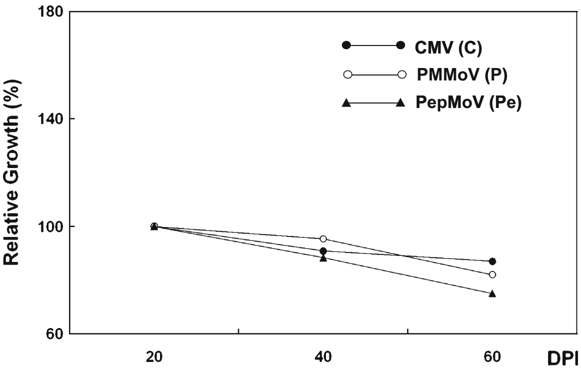

Fig. 5 Relative average growth rate of single virus-infected pepper plants compared with healthy plants as 100%

(Fig.3). This result agreed with a previous report (Siegel et al. 1978) that described an increase in titre after inoculation and a decrease once the titre reached the highest level.

In two virus co-infection experiments, CMV titres did not differ from those of CMV single inoculations during the growth of peppers. PepMoV titres did not differ from PepMoV single inoculation when co-infected with CMV but a slight increase was observed when co-infected with PMMoV. PMMoV titres increased in all co-infection combinations. When the three viruses infected the plants simultaneously, only PMMoV titres were increased (Fig. 4). These results indicate that the titre of PMMoV was not negatively affected by co-inoculated CMV and PepMoV.

In sequential inoculation experiments, titres of PMMoV, PepMoV from CMV/PMMoV or CMV/PepMoV did not differ from the single inoculation of each virus (Fig. 4). These results differed from the co-inoculation results, which showed increased titres of PMMoV and PepMoV. When PMMoV was pre-inoculated, only the titre of PMMoV increased significantly in PMMoV/CMV, PMMoV/ PepMoV and PMMoV/CMV+PepMoV (Fig. 4). These results differed from CMV/PMMoV and CMV/PepMoV results, and it can be concluded that late inoculation with CMV did not have an effect on pre-inoculated virus titres.

PepMoV titre decreased significantly in PMMoV/PepMoV+CMV, and PepMoV/PMMoV+CMV. Interestingly, titres of PMMoV and PepMoV increased in PMMoV+PepMoV, PepMoV + PMMoV and PMMoV/PepMoV, PepMoV/PMMoV (Fig. 4).

These results indicate that PepMoV did not have an effect on the multiplication of other viruses even though plants were pre-inoculated, and there was little

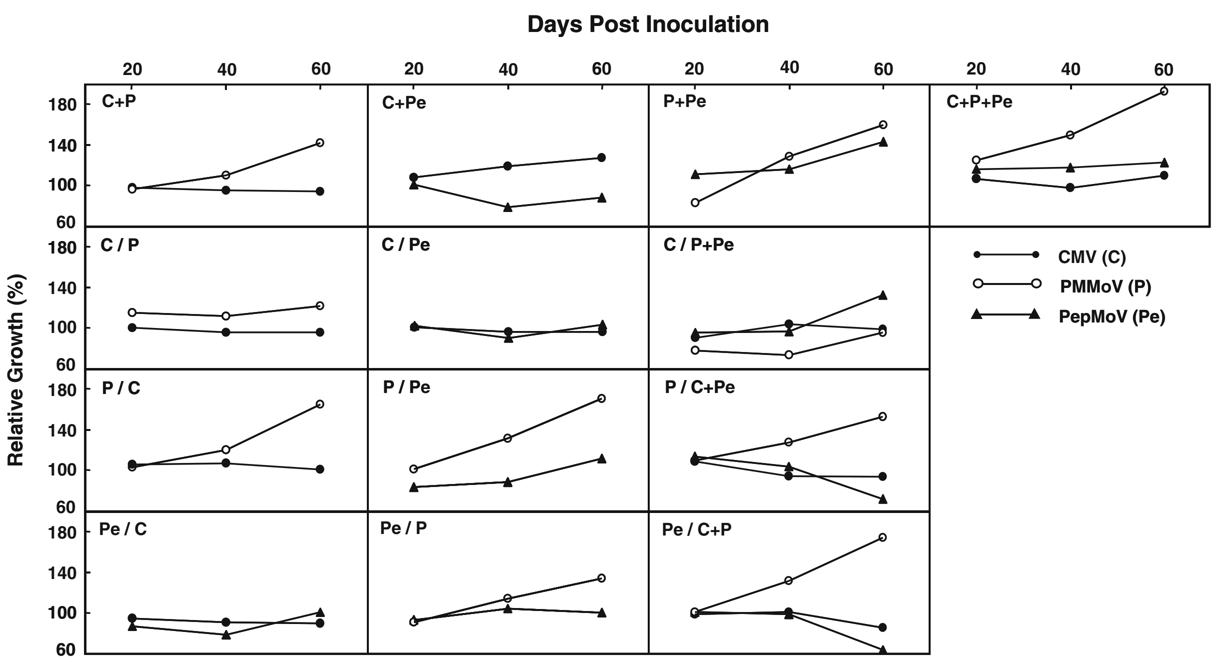

Fig. 6 Relative average growth rate for multiple virus-infected pepper plants compared with single virus-infected plants as 100%. Plus(+) symbol was used for simultaneous co-inoculation and slash(/) symbol was used for sequential inoculation between pre-inoculation and challenging virus inoculation

indication that PMMoV and PepMoV had a negative synergistic effect. Reduction in titre of PepMoV in PMMoV/PepMoV+CMV and PepMoV/PMMoV+CMV might have been caused by CMV.

Generally, changes in potyvirus titres are the result of interactions among viruses (Pio-Ribeiro et al. 1978; Poolpol and Inouye 1986; Sherwood et al. 1986; Sano and Kojima. 1989; Murphy and Kyle 1995). Several cases are reported where the titre of CMV increased when CMV was inoculated with a Potyvirus. When CMV and *Zucchini yellow mosaic virus* (ZYMV) were co-inoculated in cucumber (*Cucumis sativus* L.) and zucchini squash and melon and CMV and *Turnip mosaic virus* (TuMV) in radish (*Raphanus sativus* L.), CMV titres increased and both viruses have been shown to exhibit a synergistic pathological response (Choi et al. 2002; Poolpol and Inouye 1986; Sano and Kojima 1989; Wang et al. 2002). Results from this study confirmed the interactions between CMV and PepMoV. One possible mechanism is that HC-Pro of PepMoV may affect the suppressor function of systemic spread and enhance the pathogenicity of another virus in pepper. The ability of HC-Pro could be due to the silencing of suppressor activity (Kasschau and Carrington 2001; Voinnet 2001). These potyviruses caused an increase in titre of CMV from CMV and PepMoV co-infected pepper plants. In contrast, various co-infection and sequential infection experiments with PMMoV, a tobamovirus, have shown that titres of co-infected virus increased in all combinations after PMMoV infection. A similar phenomenon was observed in tobamovirus studies. When *Brome mosaic virus* (BMV) and *Tobacco mosaic virus* (TMV) were co-inoculated in barley (*Hordeum jubatum* L), BMV

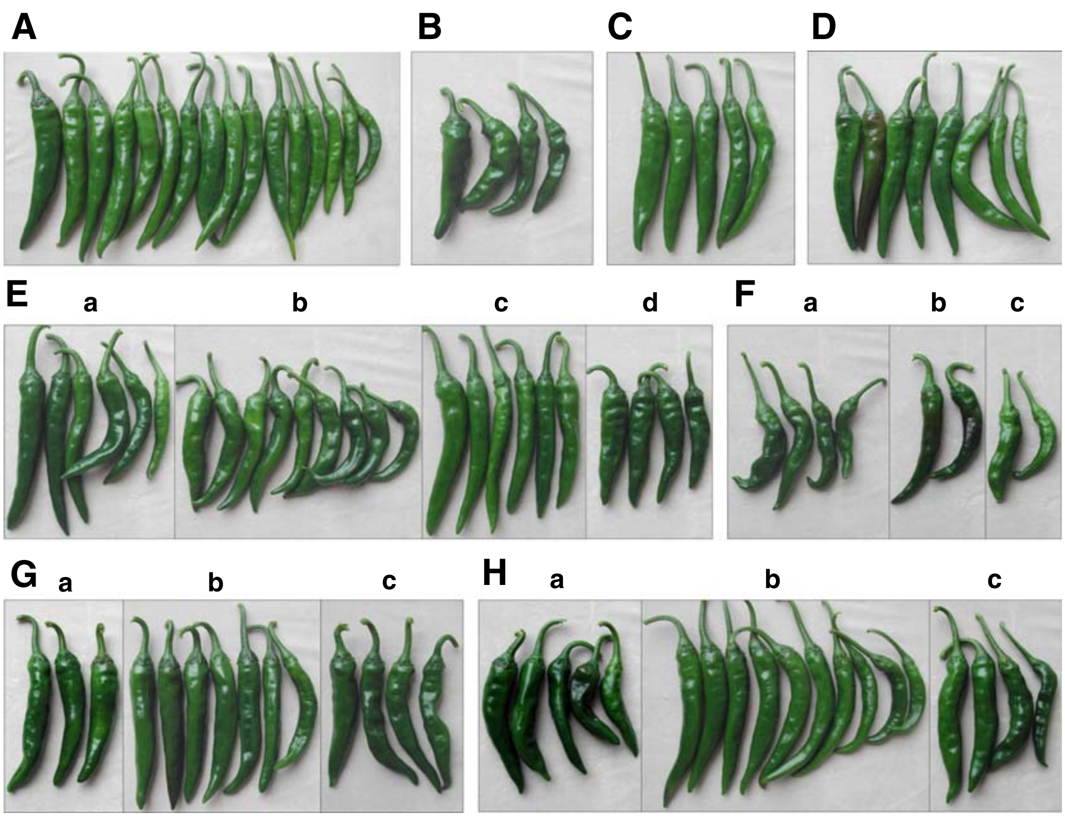

Fig. 7 Fruit morphology and fruit number produced from a single pepper plant infected with multiple combinations of CMV, PMMoV and PepMoV. (A) Healthy control. (B) CMV-infected. (C) PMMoV-infected. (D) PepMoV-infected. (E) Co-inoculations: CMV + PMMoV(a); CMV + PepMoV(b); PMMoV + PepMoV(c); CMV + PMMoV + PepMoV(d). (F) Sequential inoculation: CMV/PMMoV (a); CMV/PepMoV (b); CMV/PMMoV + PepMoV (c). (G) Sequential inoculation: PMMoV/CMV(a); PMMoV/PepMoV(b); PMMoV/CMV + PepMoV(c). (H) Sequential inoculation: PepMoV/CMV(a); PepMoV/PMMoV(b); PepMoV/CMV + PMMoV(c)

acted as a helper virus for systemic TMV infection, which was not caused by TMV alone (Hamilton and Nichols 1977). It is well known that *Potato virus* X (PVX) helps the multiplication of *Potato virus* Y (PVY) in potato (*Solanum tuberosum* L.) (Beemster and Rozendaal 1972). These results indicated that interactions of co-infected viruses in the same plants were case-specific and are affected by interactions among viruses and among viruses and host plants.

Effects on vegetative growth

Growth rates of pepper plants were reduced after 30 dpi. In single virus inoculations, the most severe growth reduction was observed from CMV-infected pepper plants, but PepMoV-infected plants did not show severe growth reductions (Fig. 5). Similar results were observed in multiple virus co-infected plants. When PepMoV was co-infected with other viruses, CMV+PepMoV and PMMoV+PepMoV, severe growth reductions were not observed as in CMV+PMMoV (Fig. 6). These results indicated that PepMoV can positively regulate CMV-induced growth reduction effects in co-infection, and the synergistic effect of PepMoV and PMMoV is not severe.

CMV pre-inoculated plants also showed severe growth reductions in sequential inoculations. In contrast, PMMoV/PepMoV and PepMoV/PMMoV combinations did not induce the severe growth reduction that was observed in CMV, including combinations, and plant heights were similar to those in the control (Fig. 6). These results indicate that pre-inoculated CMV dominated symptom development and growth retardation in pepper plants; and subsequently inoculated PepMoV and PMMoV did not cause fully developed symptoms.

Researchers have reported that multiple virus-infected plants developed more severe symptoms, including leaf shrinkage, than did those with single viral infections (Kuhn and Dawson 1973; Pio-Ribeiro et al. 1978; Calvert and Ghabrial 1983; Poolpol and

Table 1 Tukey's Studentized Range Test of total yield, average fruit number and average fruit weight[a] of pepper plants infected with multiple combinations of CMV, PMMoV and PepMoV

Virus combination[b]	Yield(g)[c]		Avg. no. of fruit [c]		Avg. fruit weight[c]	
Healthy	195.0	a	15.4	a	12.86	abc
CMV	44.6	ghi	4.6	e	9.77	cdef
PMMoV	76.8	de	5.4	de	14.24	ab
PepMoV	125.6	b	8.2	bc	15.32	a
CMV+PMMoV	65.8	ef	5.6	de	11.78	bcd
CMV+PepMoV	83.0	d	7.6	bcd	10.94	bcdef
PMMOV+PepMoV	80.2	d	6.0	cde	13.37	ab
CMV+PMMoV+PepMoV	42.6	ghi	5.2	de	8.19	ef
CMV/PMMoV	33.6	i	3.8	ef	9.01	def
CMV/PepMoV	19.4	j	2.0	f	9.86	cdef
CMV/PMMoV+PepMoV	15.2	j	2.0	f	7.68	f
PMMoV/CMV	49.2	gh	4.2	ef	11.75	bcd
PMMoV/PepMoV	100.8	c	7.2	bcd	14.00	ab
PMMoV/CMV+PepMoV	47.4	gh	4.2	ef	11.45	bcde
PepMoV/CMV	53.6	fg	4.6	e	11.81	bcd
PepMoV/PMMoV	118.2	b	9.6	b	12.28	abcd
PepMoV/CMV+PMMoV	40.4	hi	4.4	ef	9.18	def

[a] Fruits were harvested after 90 days from single and multiple-inoculation combinations of CMV, PMMoV and PepMoV

[b] plus(+) symbol was used for simultaneous co-inoculation, and slash(/) symbol was used for sequential inoculation between pre-inoculation and challenging virus inoculation

[c] For each column, $\alpha=0.05$, Error $df=32$, Critical Value of Studentized Range=5.30

Inouye 1986; Sherwood et al. 1986; Sano and Kojima 1989; Murphy and Kyle 1995). In this experiment, similar severe symptom development, including growth reduction, was also observed in multiple-virus-infected pepper plants, but synergistic effects on symptom development were especially strong in CMV and inoculation combinations.

Effects on fruit yield and morphology

The total number of fruit produced and fruit size were significantly reduced when plants were infected by one or more viruses compared with healthy control plants (Fig. 7, Table 1). Pepper fruits from CMV single inoculation, CMV co-inoculations and CMV-sequential inoculations showed the highest fruit malformation rates from within all inoculation combinations. These results strongly indicate that CMV can directly affect fruit shape, severely limit fruit size and negatively affect fruit number.

In contrast, single PMMoV or PepMoV inoculations and PMMoV+PepMoV, PepMoV/PMMV and PMMV/PepMoV combinations also showed a decrease in fruit number, but fruit shape appeared normal. Interestingly, the treatments appeared to have an effect on fruit weight. For example, average fruit weights of PMMoV or PepMoV-infected plants and PMMoV+PepMoV, PMMoV/PepMoV and PepMoV/PMMoV-infected plants exceeded the average fruit weight of healthy plants. These results show that PMMoV or PepMoV negatively affected fruit number, but did not have an effect on fruit shape or fruit growth following fruit set.

From these experiments, the most severe pepper virus of the three viruses tested was CMV, and damage caused by PMMoV or PepMoV alone was not as severe as reported in other studies. Survey results of pepper yield loss caused by viruses support our experimental results. Average yield losses of pepper from PMMoV were 15–40%, but losses of 50–55% were caused by CMV (Choi and Kwon 1989). Overall, CMV had a strong synergistic effect on symptom development and yield when combined with the other two viruses. These results suggest that CMV-resistant cultivars (Dufour et al. 1989; Caranta et al. 2004) are beneficial even though they do not possess PepMoV or PMMoV resistance, through the prevention of a synergistic effect of CMV.

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