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Ultrastructural Characteristics of Necrosis and Stunt Disease in Red Pepper by the Mixed Infections of *Tobacco mosaic virus*-U1 or *Pepper mild mottle virus* and *Pepper mottle virus*

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In single infection of *Tobacco mosaic virus*-U1 (TMV-U1) or *Pepper mild mottle virus* (PMMoV), mosaic symptoms were produced on the chili pepper cultivars of 'Cheongyang' and 'Wangshilgun'. However, in cultivars of 'Manitta' and 'Bugang', no symptoms were occurred. In single infection of *Pepper mottle virus* (PepMoV), symptoms of mottle and malformation were produced on the tested cultivars of 'Manitta', 'Bugang', 'Cheongyang', and 'Wangshilgun'. In the cultivars of 'Cheongyang' and 'Wangshilgun', synergistic symptoms of stunt and lethal death were induced by mixed infections in the two combinations of TMV-U1+ PepMoV and PMMoV+PepMoV. However, in cultivars of 'Manitta' and 'Bugang', synergistic symptoms were not noted, but mottling which was milder than that of single infection was produced. Cells infected singly with TMV-U1 and PMMoV in the cultivars of 'Cheongyang' and 'Wangshilgun', respectively, had the typical ultrastructures of tobamovirus as the stacked-band structure and multiple spiral aggregate (SA). In the cells and tissues infected with PepMoV on the cultivars of 'Cheongyang', 'Wangshilgun', 'Manitta' and 'Bugang', the potyvirus inclusions of pinwheels, scrolls, lamminated aggregates and amorphous inclusion were observed. In the cells infected mixedly with combinations of TMV-U1+PepMoV and PMMoV+PepMoV, the virus particles and inclusions of the two different viruses were found simultaneously in the same cytoplasm. The amounts of virus particles in mixed infections were more abundant than in single infection. The angled-layer aggregates (ALA) were observed only in the cells infected with both TMV-U1 and PepMoV.

Keywords **:** mixed infection, potyvirus, synergism, tobamovirus, ultrastructure

Virus diseases of crops are very common to occur by mixed infections in the field so that the yield loss becomes more severe. As a typical virus disease caused by mixed infection cowpea stunt disease with *Blackeye cowpea mosaic virus* and *Cucumber mosaic virus* had been reported in southwestern states of the United States (Pio-Ribeiro et al., 1978). In Korea, as a virus diseases caused by mixed infection, oriental cabbage showing necrotic stunt disease with *Turnip mosaic virus* (TuMV) and *Ribgrases mosaic virus* (RMV) was reported in mid eastern alpine area (Cho et al., 1995; Kim et al., 1993). In watermelon, the specific ultrastructures of watermelon necrosis disease caused by mixed infection with *Watermelon mosaic virus* (WMV) and *Cucumber green mottle mosaic virus* (CGMMV) were reported (Cho et al., 2000).

Sometimes mixed infection of the two viruses could make specific arrangement in plant cells. Hexagon is the specific arrangement found in bean dwarf disease that one particle of *Bean yellow mosaic virus* was surrounded by 6 particles of *Cowpea mosaic virus*. Another arrangement octagon is found in cowpea stunt disease that one isometric particle of CMV was surrounded by 8 flexuous rod particles of *Bean common mosaic virus* (Carr and Kim, 1983). Similarly, nonagon in watermelon necrosis disease was that one filamentous particle of WMV was surrounded by 9 rigid rod particles of CGMMV (Cho, 1998). In oriental cabbage cells having new specific ultrastructures by mixed infection with TuMV and RMV, nonagon-like ring (NLR) and spiral aggregates (SA) were formed. The NLR was made by a TuMV surrounded loosely by 9 RMV particles and the SA was formed spirally by fully mixed of the two virus particles. The SA had some NLR in its center, which was observed from cross sectioned SA.

The two virus genus, *Tobamovirus* and *Potyvirus*, are totally different in ecological characteristics, transmission manners, physical properties and morphology of virus particles. Purpose of this study is to understand the relationships between synergism of external symptoms and cytopatic effect when the two different viruses of tobamoviruses for TMV-U1 and PMMoV and potyvirus for PepMoV were mechanically inoculated at the same time on chili pepper.

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Materials and Methods

Viruse sources. *Pepper mottle virus* (PepMoV) was isolated from red pepper (Kim, 2005). Two typical isolate of tobamoviruses, *Tobacco mosaic virus*-U1 (TMV-U1) and *Pepper mild mottle virus* (PMMoV), were obtained from Dr, Choi, G. S. (National Horticultural Research Institute, Rural Development Administration, Korea).

Making of mixedly infected plants. For ultrastructural studies of virus infected cells, diseased plants of peppers were inoculated mechanically. Plants used were 4 cultivars of chili pepper (*Capsicum annum*), 'Manitta', 'Bugang', 'Wangshilgun' and 'Cheongyang', single and mixed infection with TMV-U1 or PMMoV and PepMoV. The same volume of leaves infected with tobamovirus and that infected with PepMoV were macerated with mortar and pestle in a 4 vol. of 0.01 M sodium phosphate buffer (pH 7.0). The combinations of mixed infections were TMV-U1 and PepMoV or PMMoV and PepMoV.

Electron microscopy. Infected tissues at 14 days post inoculation were fixed for 90 minutes at 4°C in 2.5% glutaraldehyde with Millonig's phosphate buffer (pH 7.0). After clearly rinsed with Millonig's phosphate buffer and then post fixation was achieved by 2% osmium tetroxide for 2 hours. The tissues were allowed to soak in the 1.0% uranyl acetate for 2 hours at 4°C after distilled water rinsing. Dehydration was done with 30~100% ethyl alcohol in six steps for 30 minutes each. The dehydrated tissues were embedded in LR white resin and incubated at 60°C for 24 hours. Ultrathin sectioning of 80 nm in thickness was performed in an ultramicrotome (MT-X) with a glass knife. Double staining was conducted with 2% uranyl acetate and 0.5% lead citrate for 15 minutes and 7 minutes, respectively.

Results

Symptom expression of single infections. TMV-U1 produced local necrotic spots on inoculated leaves of 4 cultivars of pepper including 'Manitta' at 5 days post inoculation, and then the inoculated leaves defoliated within 10 days post inoculation (Fig. 1A, Table 1). On the upper leaves, mosaic symptoms were produced on the cultivars of 'Cheongyang' and 'Wangshilgun' (Fig. 1B). However, no symptoms were produced on cultivars of 'Bugang' and 'Manitta'.

PMMoV produced necrotic local lesions on the inoculated leaves of 4 cultivars of pepper including 'Manitta' at 5 days post inoculation, and then the inoculated leaves were dropped at 10 days post inoculation. On the upper leaves, mosaic symptom was produced on the cultivals of 'Cheongyang' (Fig. 1C, Table 1) and 'Wangshilgun'. However, no symptoms were produced on cultivars of 'Bugang' and 'Manitta'.

Fig. 1. The singly infected red pepper with TMV-U1 produced necrotic spot and leaf drop at one week after mechanical inoculation 'Bugang' (A), and mosaic symptoms of 'Wangshilgun' (B). The singly infected red pepper with PMMoV produced mosaic symptoms of 'Cheongyang' (C). The singly infected chilli pepper with PepMoV produced mottle and malformation symptoms of 'Cheongyang' (D). The red pepper infected doubly with TMV-U1 and PepMoV were shown the different plant growth two weeks after inoculation (E). From up to down row, 'Bugang', 'Manitta', 'Cheongyang', 'Wangshilgun'. The red pepper infected doubly with PMMoV and PepMoV were shown the different plant growth two weeks after inoculation (F). From up to down row, 'Bugang', 'Manitta', 'Cheongyang', 'Wangshilgun'.

PepMoV produced symptomless on the inoculated leaves of 4 cultivars of red pepper including 'Manitta'. Severe mottle symptoms on the upper leaves of the those cultivars were produced at 12 days post inoculation, and then malformation symptoms were induced at 18 days post inoculation (Fig. 1D, Table 1).

Symptom expression of mixed infections. Devastating symptoms of necrotic spots along with general leaf necrosis were produced on the inoculated leaves of 4 cultivars by the mixed virions of TMV-U1 and PepMoV (Table 1). Plant death occurred at 15 days post inoculation with mixed virions of TMV-U1 and PepMoV in 7 out of 8 plants for 'Cheongyang' cultivars. Mosaic and mottle symptoms were

Table 1. Symptom development in red peppers by TMV-U1, PMMoV and PepMoV or their mixed inoculum

Commercial cultivars	Symptoms produced by				
	TMV	PMMoV	PepMoV	TMV-U1+PepMoV	PMMoV+PepMoV
'Manitta'	LD/–[a]	LD/–	–/M	LD/Mo	LD/Mo
'Bugang'	LD/–	LD/–	–/M	LD/Mo	LD/Mo
'Wangshilgun'	LD/M	LD/Mo	–/M	LD/M, Mo, D, S	LD/M, Mo, D, S
'Cheongyang'	LD/M	LD/Mo	–/M	LD/M, Mo, D, S	LD/M, Mo, D, S

[a]Inoculated leaves/upper leaves, LD: leaf drop, M: Mosaic, Mo: Mottle, –; Symptomless, S: Stunt, D: Death, Mal: Malformation, Inoculated leaves/Upper leaves.

shown in the 'Wangshilgun' cultivar. However, in the resistant cultivars of 'Manitta' and 'Bugang', only mottle symptom was produced (Fig. 1E).

Plants death was shown at 15 days post inoculation by the mixed virions of PMMoV and PepMoV in 7 and 6 out of 8 plants for 'Cheongyang' and 'Wangshilgun', respectively. Only mottle symptom was induced on the upper leaves of 'Manitta' and 'Bugang' cultivars, which were estimated to be resistant cultivars (Fig. 1F, Table 1).

Ultrastructure of single infection. The cells infected with TMV-U1 had large numbers of tobamovirus particles and accumulated in the cytoplasm and vacuole. TMV-U1 had the typical ultrastructues of tobamovirus as the stacked-band structure in cytoplasm, but several layers of band structures were not observed (Fig. 2A). The stacked-band structures were composed of virus particles having 300 nm long in cytoplasm. The multiple spiral aggregates (SA) were also observed (arrow) frequently in vacuole (Fig. 2B). Virus particles presented scatteredly in xylem vessle. The typical ultrastructures of TMV-U1 were shown together in the cells of 2 pepper cultivars, 'Wangshilgun' and 'Cheongyang', but not observed in 'Manitta' and 'Bugang' (Table 2).

PMMoV tobamovirus particles were packed in cytoplasm and scattered in vacuole of upper leaf cells with systemic infection. PepMoV had the typical ultrastructues of tobamovirus as the several layering stacked-band structure in cytoplasm. Virus particles presented scattered in xylem vessel and in phloem. The typical ultrastructures of PMMoV were shown together in the cells of 2 pepper cultivars, 'Wangshilgun' and 'Cheongyang', but Those in 'Manitta' and 'Bugang' was not observed (Table 3).

PepMoV potyvirus produced the typical cytoplasmic inclusions of pinwheels, scrolls, lamminated aggregates and tubes in cells of pepper cultivars, 'Wangshilgun', 'Cheongyang', 'Manitta' and 'Bugang' (Fig. 2C). A cytoplasmic ultrstructure of potyvirus was that virus particles of PepMoV sectioned crossly or obliquely were arrayed lineally in the tonoplast.

The typical ultrastructures of PepMoV observed as the complex of tube-like elements forming a loose amorphous cytoplasmic inclusion (Fig. 2D). The virus particles of PepMoV were presented as rarely in the cytoplasm with the potyvirus inclusions but not existed in xylem and phloem (Tables 2 and 3).

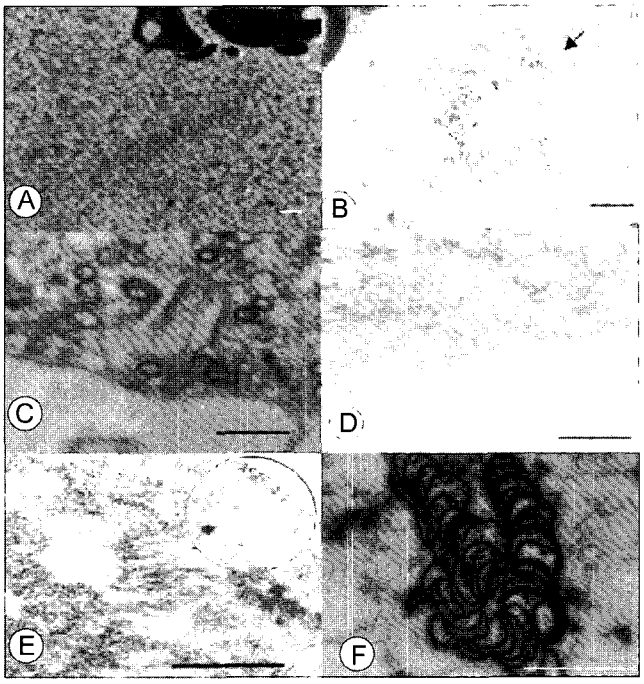

Fig. 2. Ultrastructures of red pepper cells with single and mixed infection. (A) The cells showed the typical ultrastructura characteristics of stacked-band structure in cytoplasm infected with *Tobacco mosaic virus*-U1, (B) The ultrastructure of multipl spiral aggregates in vacuole infected with *Tobacco mosaic virus* U1, (C) Typical inclusions of pinwheel, scroll and short curv laminated aggregate in cytoplasm infected with *Pepper mottl virus*, (D) Typical ultrastructure shown as amorphous inclusion i cytoplasm infected with *Pepper mottle virus*, (E) The angled-laye aggregates like ladder figure was observed in cytoplasm witl mixed infection by combination of *Tobacco mosaic virus*-U1 an *Pepper mottle virus*, (F) Typical inclusions of pinwheel and viru particles abundantly in inner part inclusions were observed i cytoplasm by mixed infection. Bars represent 500 nm.

Ultrastructure of mixed infection. The leaf cells of 4

Table 2. Cytopathic effects by infection of TMV-U1, PepMoV and TMV-U1+PepMoV

Virus[a]	Ultrastructure	Amount[b] of cytological morphologies induced in			
		Manitta	Bugang	Wangshilgeon	Cheongyang
TMV-U1	Band	–	–	+	++
	Particle	–	–	+++	+++
PepMoV	Pinwheel	+++	++	+++	++
	Scroll	++	+	+++	+++
	Laminated aggregate	++	++	++	++
	Particle	+	+	+	++
	Amorphous inclusion	–	–	+	+
TMV-U1 + PepMoV	Pinwheel	+	+	+++	++
	Scroll	++	+	+++	+
	Laminated aggregate	++	+	++	++
	Particle (potyvirus)	+	+	+++	+++
	Band (tobamovirus)	–	–	++	++
	Particle (tobamovirus)	–	–	+++	+++
	Amorphous inclusion	–	–	++	++
	ALA (angled-layer aggregates)	–	–	–	+

[a] TMV-U1: *Tobacco mosaic virus*-U1, PMMoV: *Pepper mild mottle virus* and PepMoV: *Pepper mottle virus.*
[b] Number of ultrastructure was decided by observation through the electron microscope. A total over 4 grids per block were observed. More than two blocks out of fives blocks per a combination were sectioned. Symbols indicate as follows: +; degree of amount by observation through electron microscope, –; not be observed.

Table 3. Cytopathic effects by infection of PMMoV[a], PepMoV and PMMoV+PepMoV

Virus	Ultrastructure	Amount[b] of cytological morphologies induce in			
		Manitta	Bugang	Wangshilgeon	Cheongyang
PMMoV	Band	–	–	++	+
	Particle	–	–	+++	+++
PepMoV	Pinwheel	+++	++	+++	++
	Scroll	++	+	+++	+++
	Laminated aggregate	++	++	++	++
	Particle	+	+	+	++
	Amorphous inclusion	–	–	+	+
PMMoV + PepMoV	Pinwheel	+++	+++	+++	+++
	Scroll	++	++	++	++
	Laminated aggregate	+++	++	+	+
	Particle (potyvirus)	+	+	++	++
	Vesiculation	–	–	+++	+++
	Band (tobamovirus)	–	–	++	++
	Particle (tobamovirus)	–	–	+++	+++
	Amorphous inclusion	–	–	+++	–

[a] TMV-U1: *Tobacco mosaic virus*-U1, PMMoV: *Pepper mild mottle virus* and PepMoV: *Pepper mottle virus.*
[b] Number of ultrastructures was decided by observation through electron microscope. A total over 4 grids per block were observed. More than two blocks out of fives blocks per a combination were sectioned. Symbols indicate as follows: +; degree of amount by observation through electron microscope, –; not be observed.

pepper cultivars, 'Wangshilgun', 'Cheongyang', 'Manitta' and 'Bugang' infected with TMV-U1 and PMMoV had the same virus particles or inclusions of each of those viruses. The two different viruses were located simultaneously in the cytoplasm. The typical ultrastructure of PepMoV as an amorphous inclusion was more frequently observed in cytoplasm than single infection. Amorphous inclusions were presented with other potyvirus inclusions in cytoplasm. The potyvirus inclusions induced by PepMoV were increased abundantly in the cytoplasm compared to those in single infection. The angled-layer aggregates like ladder figure made by tobamovirus particles were observed in cytoplasm of 'Cheongyang' cultivar (Fig. 2E). Pinwheels were developed well and had the virus particles abundantly at the inner part of the inclusion. Massed virus particles were observed in cytoplasm than single infection (Fig. 2F).

Virus particles were accumulated in phloem and xylem vessels (Table 2).

The leaf cells of 4 cultivars of peppers, 'Wangshilgun', 'Cheongyang', 'Manitta' and 'Bugang' infected with PMMoV and PepMoV had the same virus particles or inclusions of each of the viruses. Chloroplasts were vacuolated severely as affected by mixed infection. PMMoV had the typical ultrastructues of tobamovirus as the stacked-band structure presented in cytoplasm more than single infection. The typical ultrastructure of PepMoV as an amorphous inclusion was frequently observed in cytoplasm more than single infection. The virus particles were presented at inner part of scroll or pinwheel inclusions more than single infection. Amorphous inclusions were presented with other potyvirus inclusions in cytoplasm. The spiral aggregate (SA) was also observed in vacuole. Virus particles were accumulated severely in phloem and xylem vessel (Table 3).

Discussion

Sixty six viruses have been reported to infect peppers in the world. Among these, twelve viruses are reported in Korea. However, a few results have been reported for mixed infection. Symptom expressions and cytopathology were distinct depending on virus combinations of different viruses and the host species. Several factors involved in synergism have been studied by the ultrastuctural characteristics, virus synthesis, movements of viral agents and virus localization due to the mixed infections. However, a few results were known as the titer enhancement and the positive effect to the partner virus in virus locations in cells and tissues. For synergism of unrelated viruses in mixed infections, the each of the viruses may play a role of helper component for the replication of the partner virus, providing symptom enhancement.

The inclusions of potyvirus are involved in coat protein synthesis and viral RNA replication for virion assembly in tobacco vein motting potyvirus (Ammar et al., 1994). In case of single infection for the *Pepper mottle virus* potyviruses, the potyvirus particles were located at near or center of the inclusions. In mixed infection with tobamovirus and potivirus, potyvirus particles were mostly substituted for the tobamovirus particles (Cho, 1998). These results suggest that potyvirus inclusions probably affected on the coat protein synthesis and viral RNA replication for virion assembly in tobamovirus.

TMV-U1 tobamovirus particles rarely formed the spiral aggregate (SA) in vacuole. The SA was observed in crucifer plants that exhibited specific mixed virion aggregate infected with TuMV and RMV (Cho, 2002).

PMMoV tobamovirus induced generally typical ultrastructure made up stacked-band structures in cytoplasm and scattered in vacuoles of infected cells. Angled-layer aggregate (ALA) is formed in cytosol by ToMV, PMMoV and ORSV and TMV strains of acuba (Warmke, 1974), U5 (Shalla, 1968) and peanut clump (Francki et al., 1985; Thouvenel and Fanquet, 1981). However, ALA was not observed in cytosol infected with PMMoV. Despite the TMV-U1 could not produce angled-layer aggregates ultrastructureally, TMV-U1 in a cell infected mixedly with PepMoV made angled-layer aggregates, however, in the single infection, TMV-U1 did not make the angled-layer aggregates. From these studies, two unrelated viruses of PepMoV and TMV-U1 were influenced each other and then TMV-U1 might be recombinated protein and nucleic acid compositions, followed by producing angled-layer aggregates known as a specific ultractructure of U5-TMV and so forth.

Amorphous inclusion (AI) was observed in cytoplasm infected with PepMoV. The AI has been reported in PepMoV, PVY, PRSV, TVMV, WMV2, TuMV and MWMV (Baunoch et al., 1990). Those of PepMoV consist of finely convoluted tubules and those of PVY contain rod-like structures, but those of PRSV and other viruses have no definite substructure (Edwardson, 1974). AIs are mostly aggregates of helper component protein (Hiebert et al., 1984). The helper component of many potyviruses may occur within infected plants, but does not aggregate massively in vivo to form AIs. It is now well established that HC-protein facilitates the transmission of potyviruses by aphids (Govier and Kassanis, 1974), possibly by mediating the binding of virus particles to aphid stylets (Lopez-Abella et al., 1981). HC-protein also functions as a protease which autocatalyses the processing of its C-terminus (Carrington et al., 1989) and part of the cell-to cell movement complex (Robaglia et al., 1989). Potyviruses produce the most interesting cytopathic inclusions of pinwheels, scrolls, tubes and laminated aggregates in cytosol or nuclei in cells (Francki et al., 1985).

From the above result, TMV and PMMoV were changed in the expression of virulence by the partner potyvirus, and the virulence might be altered synergistically by partner potyvirus isolates.

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