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Ultrastructural Differences in Mixed Infections of Six *Turnip mosaic virus* and One *Ribgrass mosaic virus* Isolates in Crucifers

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Six isolates of *Turnip mosaic Potyvirus* (TuMV) namely, TuMV-CA7 from oriental cabbage, TuMV-TU and TuMV-TU2 from turnip, TuMV-RA from rape, TuMV-ST from stock, and TuMV-R9 from radish, and *Ribgrass mosaic Tobamovirus* (RMV-FG22) from oriental cabbage were isolated. Three kinds of characteristics of the six TuMV isolates were sorted by bioassay: TuMV-CA7 and TuMV-TU isolates infected mostly oriental cabbages; TuMV-ST, TuMV-TU2, and TuMV-R9 infected radishes; and TuMV-RA infected both oriental cabbages and radishes. Mixed infections of crucifers were RMV-FG22+TuMV-CA7, RMV-FG22+TuMV-TU, RMV-FG22+TuMV-RA, RMV-FG22+TuMV-ST, RMV-FG22 +TuMV-TU2 and RMV-FG22+TuMV-R9. Crops used were 'Tambok' cultivar resistant to TuMV, 'SSD63' susceptible inbred line of oriental cabbage, pure line of leaf mustard and 'Daeburyungyeorum' cultivar of radish. New specific ultrastructures of nonagon-like ring (NLR) and spiral aggregates (SA) by mixed infection with TuMV and RMV were formed in cells of crucifer plants. The NLR was made by a TuMV surrounded loosely by nine RMV particles, and the SA was formed spirally by full mixed of two virus particles. The SA had some NLR in its center, which was observed from cross sectioned SA. Host plants with specific ultrastructures expressed synergistic symptoms. Specific ultrastructures of NLR and SA were formed in combinations of RMV-FG22 and in TuMV-CA7, TuMV-TU, or TuMV-RA that could infect oriental cabbages. However, no specific ultrastructures and mixing of the two virions in the same cell were observed in combinations of RMV-FG22, and TuMV-ST, TuMV-TU2, or TuMV-R9 isolates having virulence in radishes.

Keywords : crucifer, mixed infection, RMV, synergism, TuMV, ultrastructure.

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Virus diseases cause considerable decrease in yield because control is difficult, and sometimes results in exorbitant economic damage, especially by mixed infections of two different viruses. Two unrelated viruses express synergistic symptoms on their common host which they mixedly infected. The disease, having synergistic external symptoms of cowpea stunt (Anderson et al., 1994), watermelon necrosis (Cho, 1998) and oriental cabbage necrotic stunt (Kim et al., 2001) were broke breaks out by the interaction between two heterogeneous plant viruses. Specific arrangements of octagon or nonagon by the mixed virions having different characteristics were observed in infected host cells octagon, 1 *Cucumber mosaic virus*: 8 *Bean common mosaic virus*, in cowpea stunt disease; hexagon, 1 *Bean yellow mosaic virus*: 6 *Cowpea mosaic virus*, in bean stunt disease (Carr and Kim, 1983); and nonagon, 1 *Watermelon mosaic virus*-2: 9 *Cucumber green mottle mosaic virus*, in watermelon necrosis disease (Cho 1998; Cho et al., 2000; Cho 2002; Kim et al., 2000). In cells of oriental cabbage showing necrotic stunt by dual infection of *Turnip mosaic virus* (TuMV) and *Ribgrass mosaic virus* (RMV), the two different virions were fully mixed (Kim et al., 2001). Nonagon-like ring (NLR) that is similar to nonagon but the circle is loose, 1:9 arrangement of one TuMV particle surrounded by 9 RMV particles, is made in the center of spiral aggregate (SA) (Cho et al., 2002). These specific arrangements of octagon, hexagon, nonagon, and NLR affect the synergistic external symptoms of virus disease. In case of nonagon, it might be a marker of synergism, that is, formation of nonagon in cells by mixed infection might induce synergism and economical damage on cucurbit (Cho, 1998; Cho et al., 2000; Kim et al., 2000).

Six TuMV isolates and an RMV-FG22 isolate were examined in crucifers, and some combinations depending on the hosts showed various synergistic effects. In the hosts infected mixedly with TuMV and RMV, a specific ultrastructure serves as a general marker of synergism, and there exists a relationship between synergism and specific ultrastructures. This study investigated the effects of external

and internal symptoms by interactions among synergism, specific ultrastructures, and kinds of hosts by mixed infections.

Materials and Methods

Viruses and cultivars. RMV-FG22 and six TuMV isolates TuMV-CA7, TuMV-TU, TuMV-RA, TuMV-ST, TuMV-TU2 and TuMV-R9 classified by bioassay were inoculated singly and mixedly on resistant oriental cabbage (*Brassica campestris* ssp. *pekinensis*) 'Tambok' and susceptible 'SSD63' inbred line, leaf mustard (*B. juncea*) of a pure line of 'Ganghwa' and radish (*Raphanus sativus*) 'Daeburyungyeorum' cultivar.

Electron microscopy. The single and double infections were confirmed by leaf dip method through electron microscopy. Upper leaves of singly and mixedly infected plants were collected at 2 weeks after inoculation. Systemically infected tissues were fixed over 2 hours at 4°C in 2.5% glutaraldehyde with Millonig's phosphate buffer, pH 7.0, and post-fixed using 2% osmium tetroxide for 90 minutes after having clearly rinsed with Millonig's phosphate buffer. The tissues were allowed to soak in the 1.0% uranyl acetate overnight in the refrigerator after washing with distilled water. Dehydration was done with an ascending series of 50-100% ethyl alcohol in six steps for 50 minutes each. The dehydrated tissues were embedded in Spurr resin and hardened at 60°C overnight. Ultra thin sections of 80 nm thickness were double stained with 2.0% uranyl acetate and 0.5% lead citrate for 20 minutes and 10 minutes, respectively.

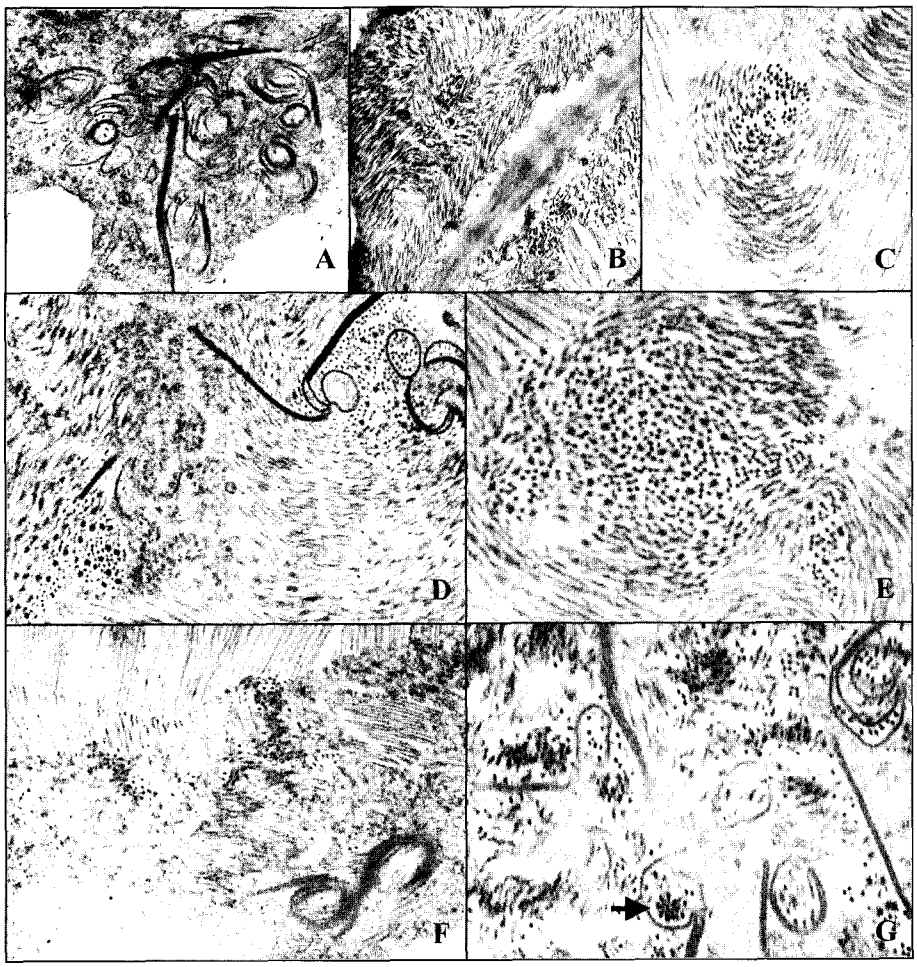

Fig. 1. Scroll, laminated aggregates and pinwheels were observed in cytosols of mesophyll, epidermal, xylem and phloem parenchyma cells (**A**) singly infected with TuMV. RMV particles showed stacked-band structure, and groups of RMV particles showed somewhat elastic and spiral aggregates (SA; **B**). The specific ultrastructures of NLR and SA were induced in the combinations of TuMV-CA7+RMV-FG22 (**C**), TuMV-TU+RMV-FG22 (**D**), and TuMV-RA+RMV-FG22 (**E**) on leaf mustard. In the central area of SA, the structures of NLR by two different viruses were shown in cross section (E), which was made by the mixed virions of TuMV-RA+RMV-FG22 in oriental cabbage 'SSD63'. In combinations of TuMV-ST+RMV-FG22, TuMV-R9+RMV-FG22, and TuMV-TU2+RMV-FG22 on leaf mustard, the specific ultrastructures of SA and NLR were hardly observed. TuMV-ST+RMV-FG22 did not evenly mix the two virus particles in a cell, and TuMV-R9+RMV-FG22 (**F**) and TuMV-TU2+RMV-FG22 (**G**) formed few octagon-like structures (arrow) by potyvirus particles.

Results

Single infection. The typical pot virus inclusions were observed in mesophyll, epidermal, and xylem and phloem parenchyma cells infected singly with six isolates of TuMV (Fig. 1A). Leaf mustard and oriental cabbage 'SSD63' contained relatively more inclusions and virion particles than oriental cabbage 'Tambok' and radish. RMV made stacked-band structure of about 300 nm in width of band, and particles scattered in vacuoles and xylem vessels. Groups of RMV particles were shown to be somewhat elastic and spiral aggregates (Fig. 1B).

Double infection. Cytopathologies were induced differently depending on the interactions among six TuMV isolates, RMV-FG22, and the hosts (Table 1). The ultrastructures of NLR and SA were observed in cells infected with the two different viruses but not in all combinations nor in all kinds of plants.

In oriental cabbage 'Tambok', combinations of TuMV-CA7+RMV-FG22 and TuMV-TU+RMV-FG22 showed two mixed virions, but did not form specific ultrastructures in the same cell. In other combinations of TuMV-RA+RMV-FG22, TuMV-ST+RMV-FG22, TuMV-TU2+RMV-FG22, and TuMV-R9+RMV-FG22, only RMV-FG22 particles were found in 'Tambok' cultivar (Table 2).

In susceptible oriental cabbage 'SSD63' (Table 3), the combinations of TuMV-CA7+RMV-FG22, TuMV-TU+RMV-FG22, TuMV-RA+RMV-FG22, and TuMV-TU2+RMV-FG22 caused some NLR and several SA structures. However, in the combination of TuMV-R9+RMV-FG22, the two virus particles were not mixed in the same cell. TuMV-ST+RMV-FG22 combination had only tobamovirus particles.

Leaf mustard (Table 4), susceptible to the two viruses,

Table 1. Ultrastructures induced by mixed infections of six combinations in crucifer

Crop	T-CA7[a] + RMV[b]	T-TU + RMV	T-RA + RMV	T-ST + RMV	T-TU2 + RMV	T-R9 + RMV
Brassica campestris ssp. *pekinensis* 'Tambok'	*M*+[c]	*SM*+	M–	M–	M–	M–
	R++	*R*+++	R+++	R+++	R+++	R+++
	T++	*T*+++	T–	T–	T–	T–
B. campestris ssp. *pekinensis* 'SSD63'	*SM*+	*SM*++	*SM*+++	M–	*M*++	M–
	R+++	*R*+++	*R*+++	R+++	*R*++	R++
	T++	*T*+++	*T*+++	T–	*T*++	T+
Raphnus sativus (Radish)	M–	M–	M–	M–	M++	M–
	R–	R++	R++	R+++	R+++	R++
	T–	T–	T++	T+++	T++	T+++
B. juncea (Leaf mustard)	*SM*+++	*SM*+++	*SM*+++	*M*++	*M*+++	*SM*++
	R++++	*R*++++	*R*++++	*R*++++	*R*++++	*R*++++
	T+++	*T*+++	*T*++++	*T*++	*T*+++	*T*+++

[a] Isolates of *Turnip mosaic virus* (TuMV), *Potyviridae*.
[b] Isolates of *Ribgrass mosaic virus* (RMV), *Tobamovirus*: RMV-FG22.
[c] A total of 4-8 grids were observed. Symbols indicate as follows: M = mixed virions; SM = spiral aggregates and mixed virions; R = RMV; T = TuMV (inclusion bodies and virus particles; old and italic letters represent occurring synergism in external symptoms); +, degree of amount by observation through electron microscope.

Table 2. Ultrastructures induced by mixed infections in *B. campestris* ssp. *pekinensis* 'Tambok'

Ultrastructure	T-CA7		T-TU		T-RA		T-ST		T-TU2		T-R9	
	S[a]	+RMV[b]	S	+RMV	S	+RMV	S	+RMV	S	+RMV	S	+RMV
Nonagon	–[c]	–	–	–	–	–	–	–	–	–	–	–
Spiral aggregate	–	–	–	–	–	–	–	–	–	–	–	–
Mix of the two virions	–	+	–	+	–	–	–	–	–	–	–	–
Potyvirus particles	–	+	++	++	+	–	–	–	–	–	–	–
Potyvirus inclusion body	–	+++	++	++	++	–	–	–	–	–	–	–
Tobamovirus particles	–	++	–	++	+	+	–	+	–	+	–	+

[a] Single infection of TuMV isolate.
[b] Mixed infection of TuMV isolate and RMV-FG22.
[c] Degree of amount by observation through electron microscope.

Table 3. Ultrastructures induced by mixed infections in *B. campestris* ssp. *pekinensis* 'SSD63'

Ultrastructure	T-CA7[a]		T-TU		T-RA		T-ST		T-TU2		T-R9	
	S[b]	+RMV[c]	S	+RMV	S	+RMV	S	+RMV	S	+RMV	S	+RMV
Nonagon	–[d]	+	–		–	+	–	–	–	–	–	–
Spiral aggregate	–	–	–	–	–	+++	–	–	–	–(+)[e]	–	–
Mix of two virions	–	+	–	–	–	+++	–	–	–	++	–	–
Potyvirus Particles	++	–	+	+	–	+++	–	–	+++	++	+	++
Potyvirus inclusion body	+++	+++	+++	+++	–	+++	–	–	+++	+++	++	+
Tobamovirus Particles	–	+++	–	–	–	+++	–	+++	–	++	–	++

[a] Isolates of *Turnip mosaic virus* (TuMV), *Potyviridae*.
[b] Single infection of TuMV isolate.
[c] Mixed infection of TuMV isolate and RMV-FG22.
[d] Degree of amount by observation through electron microscope.
[e] – (+): hardly could be seen.

Table 4. Ultrastructures induced by mixed infections in leaf mustard

Ultrastructure	T-CA7[a]		T-TU		T-RA		T-ST		T-TU2		T-R9	
	S[b]	+RMV[c]	S	+RMV	S	+RMV	S	+RMV	S	+RMV	S	+RMV
Nonagon	–[d]	++	–	+++	–	++	–	–	–	–	–	+
Spiral aggregate	–	++	–	+++	–	+++	–	+	–	+++	–	+
Mix of two virions	–	+++	–	+++	–	+++	–	++	–	+++	–	+++
Potyvirus Particles	+	++	+	+++	+++	+++	+++	+	+	++	++	++
Potyvirus inclusion body	+++	+++	+++	+++	+++	++++	+++	++	++	+++	+++	+++
Tobamovirus Particles	–	++++	–	++++	–	++++	–	+++	–	++++	–	++++

[a] Isolates of *Turnip mosaic virus* (TuMV), *Potyviridae*.
[b] Single infection of TuMV isolate.
[c] Mixed infection of TuMV isolate and RMV-FG22.
[d] Degree of amount by observation through electron microscope.

Table 5. Ultrastructures induced by mixed infections in radish

Ultrastructure	T-CA7[a]		T-TU		T-RA		T-ST		T-TU2		T-R9	
	S[b]	+RMV[c]	S	+RMV	S	+RMV	S	+RMV	S	+RMV	S	+RMV
Nonagon	–[d]	–	–	–	–	–	–	–	–	–	–	–
Spiral aggregate	–	–	–	–	–	–	–	–	–	–	–	–
Mix of two virions	–	–	–	–	–	–	–	–	–	++	–	–
Potyvirus Particles	–	–	–	–	–	++	–	++	++	++	–	+++
Potyvirus inclusion body	–(+)[e]	–	–	–	–	++	–	+++	+++	++	+	++
Tobamovirus Particles	–	–	–	++	–	++	–	+++	–	+++	–	++

[a] Isolates of *Turnip mosaic virus* (TuMV), *Potyviridae*.
[b] Single infection of TuMV isolate.
[c] Mixed infection of TuMV isolate and RMV-FG22.
[d] Degree of amount by observation through electron microscope.
[e] – (+): hardly could be seen.

contained several of both virus particles, NLR and SA structures were in all type cells. In the combinations of TuMV-CA7+RMV-FG22 (Fig. 1C), TuMV-TU+RMV-FG22 (Fig. 1D), and TuMV-RA+RMV-FG22 (Fig. 1E), SA and NLR structures were observed with extraordinary amount in cells. No specific ultrastructures were observed in TuMV-ST+RMV-FG22, TuMV-R9+RMV-FG22 (Fig. 1F), and TuMV-TU2+RMV-FG22 (Fig. 1G). However, in TuMV-R9+RMV-FG22, a few SAs in xylem vessel were found, while TuMV-TU2+RMV-FG22 formed a little octagon-like structure (arrow) by potyvirus particles (Fig. 1G).

In radish, RMV-FG22 isolates replicated very well when they were co-inoculated with TuMV-RA, TuMV-ST and

TuMV-R9, but mixed virions or specific ultrastructures were not observed. In TuMV-TU2+RMV-FG22 combination, however, the two virions were found just as a mixed form (Table 5).

Discussion

In combinations of RMV-FG22 and the six TuMV isolates, external symptoms and specific mixed viral aggregates had similar patterns with that of results of external symptoms by single infection of TuMV on differential crops. Generally, the specific ultrastructures of nonagon-like ring (NLR) and spiral aggregate (SA) existed in cells by mixed infections with RMV-FG22 and three of isolates TuMV, identified to infect mostly oriental cabbages, TuMV-CA7, TuMV-TU and TuMV-RA. Another three isolates which infected radish, TuMV-ST, TuMV-TU2 and TuMV-R9, rarely formed the specific mixed virion aggregates in cells infected doubly with RMV-FG22. However, in oriental cabbage Tambok, three combinations, TuMV-CA7+RMV-FG22, TuMV-TU+RMV-FG22, and TuMV-RA+RMV-FG22 did not form specific aggregates of NLR and SA in cells even though two combinations of TuMV-CA7+RMV-FG22 and TuMV-TU+RMV-FG22 produced a little synergism. In radish 'Daeburyungyeorum', all of the combinations did not form the specific aggregates in host cells. This phenomenon suggests that oriental cabbage Tambok and radish were highly resistant to TuMV and RMV-FG22. However, the leaf mustard was extremely susceptible to the two infecting viruses, and all combinations of TuMV+RMV-FG22 expressed intense synergistic symptoms.

Most hosts exhibited specific mixed virion aggregates of SA and/or NLR expressed synergistic symptom. There is a general trend that intensity of synergism is proportioned amount of specific ultrastructures, and that all plants show synergistic external symptoms. In particular, some hosts which expressed weak synergism did not always produce the specific ultrastructural aggregates of SA and NLR in their cells. In some cases, synergistic external symptom occurred even when the specific mixed viral aggregates did not form within the host cells. Additionally, in susceptible hosts, more specific ultrastructures were formed in more combinations. It is suggested that difference of external symptom expression and ultrastructures depends not only on the characteristics of hosts but also on the virion interactions between the co-infecting viruses.

In susceptible hosts, TuMV particles with RMV particles occur abundantly in xylem vessels, as well as in sieve tubes and in potyvirus inclusions. When two unrelated viruses posses a close affinity in a doubly infected cell, the inclusions of pinwheel, scroll, or cylinder which induced specifically by only potyviruses can associate with the other different viruses. Potyviruses have been reported to be the major factor in the synergistic interactions in serious virus diseases, and using molecular biology, it has been determined that the genome of potyvirus is the origin of the synergisms (Shi et al., 1997; Pruss et al., 1997; Vance et al., 1995). Synergism induced by mixed infection of TuMV isolates and RMV-FG22 partner virus, therefore, is suggested to be a result of complicated interactions among the two different viruses and their hosts, with the potyvirus genome being the major cause of the synergism.

TuMV-R9+RMV-FG22 and TuMV-TU2+RMV-FG22 formed a little octagon-like structure by potyvirus particles in leaf mustard. This may suggest that the potyvirus changed its characteristics on its coat protein or somewhere, such as in the case of angled-layer aggregates by CGMMV infected mixedly with WMV-2 on cucurbit (Cho, 1998; Cho et al., 2000).

It is very important that the specific mixed virion aggregates in cells may be used as a tool to aid in screening co-infected plants. These specific aggregates formed in mixed infected tissues but not in single infected tissues may be used as an indicator to perceive the possibility of synergism because specific aggregates by particles of two unrelated viruses in cells result from interactions between the two viruses and help each other in the same cell.

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