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Symptomatology of *Citrus mosaic sadwavirus* (CiMV) in Some Citrus Cultivars and Effect of CiMV Infection on Citrus Fruit Quality

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Citrus mosaic sadwavirus **(CiMV) is a closely related virus with the *Satsuma dwarf virus* (SDV) along with *Navel orange infectious mottling virus* (NIMV), *Natsudaidai dwarf virus* (NDV), and *Hyugagatsu virus* (HV). The present study found that the typical symptoms of CiMV-infected citrus fruits include the appearance of dark blue speckles or ringspots on fruit rinds and the browning of oil glands in the spots as rind coloring began. As rind coloring progressed, the spots gradually faded, whereas the browning of the oil glands worsened to the point that the tissues surrounding the oil glands became necrotic. In very early satsuma mandarins (*Citrus unshiu* 'Miyamoto Wase') and 'Setoka' cultivar (*C. hybrid* 'Setoka') of late-maturity citrus, the symptomatic fruits were eventually dropped. And in early satsuma mandarin (*C. unshiu* 'Miyakawa Wase'), the peel hardness of the virus-infected fruit (1,618.3 ± 305.5, g-force) was more than twice as hard as that of the healthy fruit (636.5 ± 39.1, g-force). The ratio of flesh weight to total fruit weight was higher for the healthy fruit (77.3 ± 1.7%) than for the infected fruit (70.7 ± 0.6) and peel puffing was more severe in the infected fruit (2.9 ± 0.4 mm) than in the healthy fruit (0.9 ± 0.2 mm). The soluble solids content in infected citrus fruits was less values than the healthy fruit by 0.5-1.5 °Brix. These findings reveal that CiMV infection on citrus trees reduces the fruit quality of citrus.**

Keywords **:** *Citrus mosaic sadwavirus*, fruit quality, symptom

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Citrus is one of the most important fruit crops worldwide, and the cultivation of late-maturity citrus cultivars including 'Setoka' (*Citrus hybrid* 'Setoka'), 'Kanpei' (*C. hybrid* 'Kanpei'), 'Ehime kashi No. 28' (*C. hybrid* 'Ehime kasha 28 gou'), and 'Shiranuhi' (*C. hybrid* 'Shiranuhi') is increasing, especially in Korea, where such cultivars are becoming economically important. However, because most citrus cultivars are propagated by grafting, viral pathogens that are transmitted by grafting can cause economic problems. In particular, late-maturity citrus cultivars are considered to be virus-sensitive. It has been reported that approximately 30 viruses or virus-like agents and 6 viroids were found in citrus trees worldwide (Ito et al., 2002; Korkmaz et al., 2000). Four viruses, namely *Citrus tristeza virus* (CTV), *Citrus tatter leaf virus* (CTLV), *Citrus mosaic sadwavirus* (CiMV), and *Satsuma dwarf virus* (SDV), and 5 viroids, *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III), *Citrus viroid IV* (CVd-IV), and *Citrus viroid OS* (CVd-OS) have been reported to infect citrus trees in Korean (Hyun et al., 2009, 2017). The study screened 155 orchards for viral infection, using multiplex PCR, and detected either SDV or CiMV in 35.2% of the trees tested: 43.7% of 'Setoka' trees, 40.0% of 'Kanpei' trees, 32.6% of 'Ehimekashi No. 28' trees, and 26.8% of 'Shiranuhi' trees (Hyun et al., 2017). CiMV of them is known to directly damage fruits including spotting and blotching of the rinds (Ito et al., 2004; Iwanami and Koizumi, 2000).

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CiMV is a member of the genus *Sadwavirus*, closely related virus group with the SDV along with *Navel orange infectious mottling virus* (NIMV), *Natsudaidai dwarf virus* (NDV), and *Hyugagatsu virus* (HV) (Ito et al., 2004; Iwanami 2010). In previous study, SDV and CiMV isolates were distinctively divided into two groups based on phylogenetic analysis of PP2 gene cloned from 22 viral isolates from Korea, and it was found that CiMV and SDV isolates from Korea shared 95.5-96.2% and 97.1-97.7% sequence identity with isolates from Japan, respectively (Hyun et al., 2017). Importantly, it was reported that both the total fresh weight and fruit yield of very early satsuma mandarin (*C. unshiu* 'Miyamoto Wase') plants infected with SDV and CiMV were ~60% and 25-45% lower, respectively, after four years of infection, when compared to healthy plants (Imada et al., 1980). However, even though many studies have investigated detection methods and genes for citrus viruses, few have assessed the effect of the viruses, especially on fruit quality (Ito et al., 2002, 2004; Iwanami et al., 1999). Therefore, this study was carried out to investigate CiMV symptoms according to citrus cultivars and the effects of CiMV on quality of citrus fruit in Korea.

We observed CiMV typical symptoms on very early satsuma mandarin, early satsuma mandarin (*C. unshiu* 'Miyagawa Wase'), 'Setoka', and 'Kiyomi' (Fig. 1). CiMV was detected all trees showing typical symptoms by multiplex PCR assay (data not presented). The typical symptoms included the appearance of dark blue speckles or ringspots on fruit rinds and the browning of oil glands in the spots as rind coloring began. As the coloring

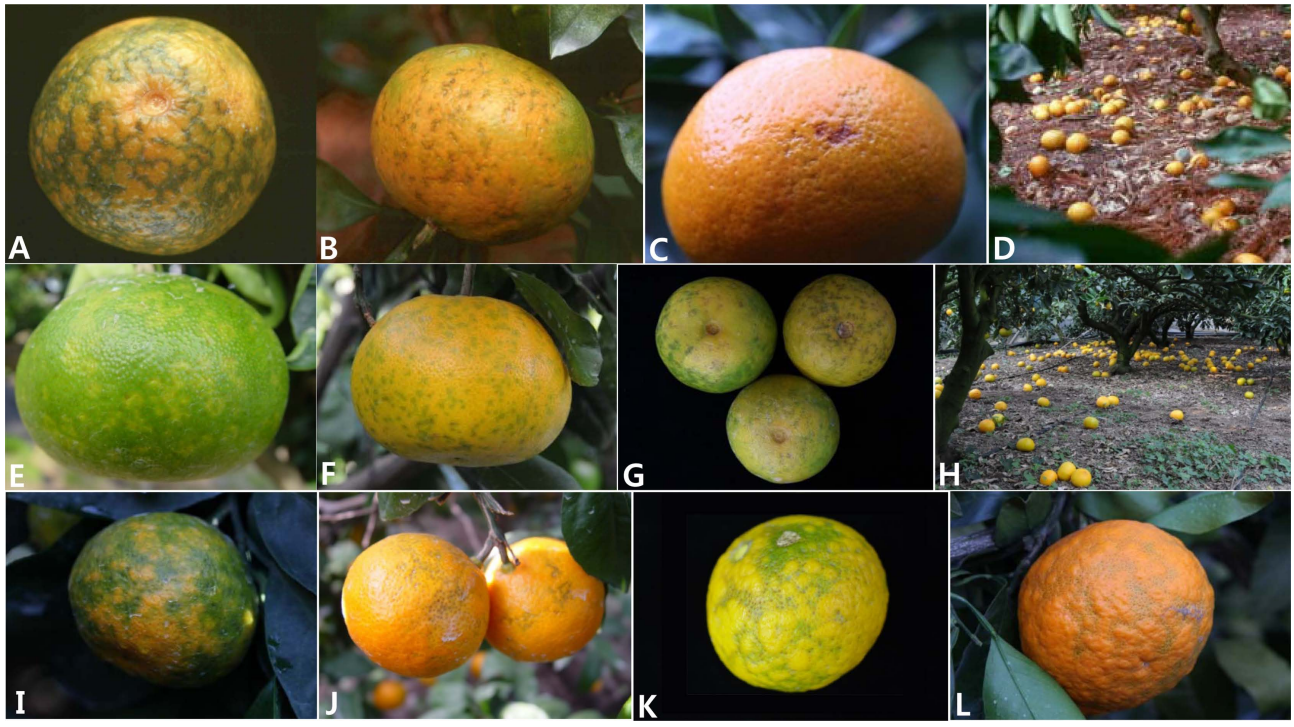

Fig. 1. Typical symptoms shown on citrus cultivars infected with *Citrus mosaic sadwavirus* (CiMV). (A-D) Very early satsuma mandarin. (E-H) 'Setoka' citrus cultivar. (I, J) Early satsuma mandarin. (K, L) 'Kiyomi' citrus cultivar.

Table 1. Virus isolates and detection of citrus viruses

Virus isolate	Origin	Virus detection				Reference
		CTV	CTLV	SDV	CiMV	
SM-1	Early satsuma mandarin, Jeju	+	–	–	+	Hyun et al. (2017)
SM-26	Early satsuma mandarin, Jeju	+	–	–	+	Hyun et al. (2017)
Jung-CiMV-3	Early satsuma mandarin, Jeju	+	–	–	+	Hyun et al. (2017)
Nam-CiMV	Early satsuma mandarin, Jeju	+	–	–	+	Hyun et al. (2017)
Sehwa	Early satsuma mandarin, Jeju	+	–	–	+	In this study

CTV, *Citrus tristeza virus*; CTLV, *Citrus tatter leaf virus*; SDV, *Satsuma dwarf virus*; CiMV, *Citrus mosaic sadwavirus*.

Table 2. Primers used to detect the 4 citrus viruses in uniplex or multiplex PCR

Target (virus)	Primers		Amplified gene	Product size (bp)	Reference
	Name	Sequence (5′ → 3′)			
CTV	CTV-Po-F	5′-GTGGCCAATAGGTCCGTAGA-3′	CP	412	Hyun et al. (2017)
	CTV-Po-R	5′-CGGGTCTCAACCTAGCCATA-3′			
CiMV	Sadwa(F)	5′-ACGTTCTTTCCAAGGGGAGT-3′	PP2	818	Hyun et al. (2017)
	Sadwa(R)	5′-CTCCATCAAGGGAGTTTGGA-3′			
SDV	SDV(2014)-F	5′-CAACACATCGGGAGGAAACT-3′	PP2	745	Hyun et al. (2017)
	SDV(2014)-R	5′-AGCATGGAAGATGGACCTTG-3′			
SDV/CiMV	PP2-3(F)	5′-GCACGGTCTCTCACTCAGGGA-3′	PP2	1,139	In this study
	PP2-4(R)	5′-TACCTGCAAATATATCGCAGGTTG-3′			
CTLV	CTLV(2013)-F	5′-CGAAAACCCCTTTTTGTCCT-3′	CP	607	Hyun et al. (2017)
	CTLV(2013)-R	5′-ATAGACCCGGCAAAGGAACT-3′			
Actin	Actin-F	5′-TCCACCATGTTCCCAGGTAT-3′	Actin	210	Hyun et al. (2017)
	Actin-R	5′-CATCTCTGTCTGCCACCTGA-3′			

CTV, *Citrus tristeza virus*; CiMV, *Citrus mosaic sadwavirus*; SDV, *Satsuma dwarf virus*; CTLV, *Citrus tatter leaf virus.*

progressed, the spots gradually disappeared, but browning of the oil glands became worse and eventually the tissues surrounding the oil glands became necrotic (Fig. 1). The five isolates, SM-1, SM-26, Jung-CiMV-3, Nam-CiMV, and Sehwa, were collected from each of early satsuma mandarin trees showing CiMV typical symptoms in Namwon, Jeju to assay fruit quality (Table 1). The scions from each of the satsuma mandarin trees were grafted onto trifoliate orange rootstock, maintained in a greenhouse and used to detect viruses. For diagnosis of CTV, CTLV, and SDV/CiMV, multiplex PCR was performed using LiliF Citrus Virus Muliplex PCR Kit (catalog No. IP11075, iNtRON Biotechnology Inc., Seongnam, Korea) with CTV-Po, PP2-3/4, CTLV-2013, and actin primer sets (Table 2, Fig. 2). And SDV and CiMV was specifically detected by uniplex PCR with SDV-2014 and Sadaw primer sets (Table 2), respectively, using Accupower Hotstart PCR pemimix (catalog No. K-5051, Bioneer, Daejeon, Korea). Total RNA for diagnosis was extracted from approximately 20 mg of young citrus leaves using TRIzol Reagent (catalog No. 15596-026, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA was dissolved in 100 μl FORMAzol solution (Molecular Research Center, Cincinnati, OH, USA) for stabilization of RNA solubilization and stored in –20°C or –70°C. cDNA was prepared using a TOPscript cDNA synthesis kit (catalog No. EZ105S, Enzynomics, Daejeon, Korea) according to the manufacturer's protocol. The PCR amplifications were performed in a programmable thermocycler (model T-Gradient Thermoblock, Biometra, Göttigen, Germany), using an initial denaturation step of 95°C for 2 min followed by 35 amplification cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 2 min,

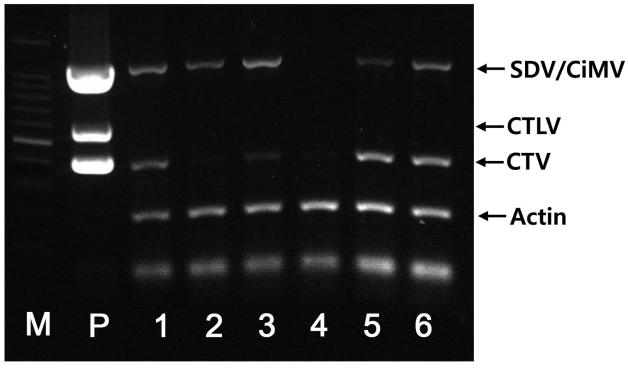

Fig. 2. Simultaneous detection of citrus viruses using multiplex PCR. Lane M: 100-bp DNA ladder; lanes 1-6: virus isolates from orchard. SDV, *Satsuma dwarf virus*; CiMV, *Citrus mosaic sadwavirus*; CTLV, *Citrus tatter leaf virus*; CTV, *Citrus tristeza virus*.

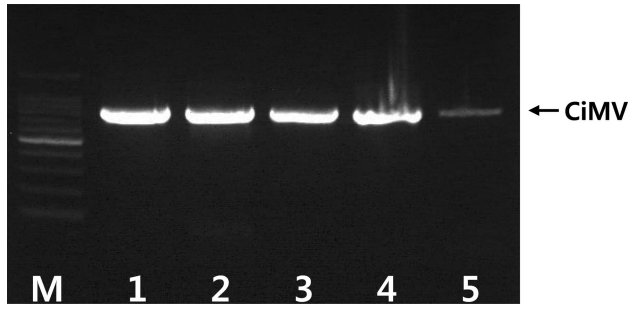

Fig. 3. Uniplex PCR for detection of of *Citrus mosaic sadwavirus* (CiMV) using Sadwa primer set. Lane M: 100-bp DNA ladder; lanes 1-5: Isolate SM-1, SM-26, Jung-CiMV-3, Nam-CiMV, and Sehwa, respectively.

and a final extension at 72°C for 10 min. After PCR, 12 µl of the product was electrophoresed in a 1.0% agarose gel in 1× TAE buffer for 30 min at 3 V/cm and visualized by ethidium bromide staining. A 100-bp DNA Ladder (catalog no. 3407A, Takara, Tokyo, Japan) was used as a molecular weight markers. As a result, the five trees were coinfected with CiMV and CTV (Table 1, Figs. 2 and 3). And, a total of 100 fruits exhibited typical symptoms were harvested from the four infected trees (isolate SM-1, SM-26, Jung-CiMV-3, and Nam-CiMV) of early satsuma mandarin in early December, and both peel hardness and degree of puffing were compared to those of symptomless fruits from healthy trees. Peel hardness was measured using a TA-XT2 texture analyzer (Stable Micro System, Godalming, UK) according to the manufacture's protocol. The peel hardness of the virus-infected fruit (1,618.3 ± 305.5, g-force) was more than twice as hard as that of the healthy fruit (636.5 ± 39.1, g-force) (Table 3). Furthermore, the ratio of flesh weight to total fruit weight was higher for the healthy fruit (77.3 ± 1.7%) than for the infected fruit (70.7 ± 0.6) (Table 3). Meanwhile, degree of peel puffing was measured as the interval between the pericarp and underlying segments. Peel puffing was more severe in the infected fruit (2.9 ± 0.4 mm) than in the healthy fruit (0.9 ± 0.2 mm) (Table 3).

One hundred fruits were harvested from each of three infected (Jung-CiMV-3, Nam-CiMV, and Sehwa) and three healthy trees both on October 27, during the early stage of coloring, and November 16, 2015, the late of coloring. And then both the titratable acidity (TA) and soluble solids content (SSC) of the fruits were analyzed. SSC was recorded using a refractometer (PR-101, Atago Co., Osaka, Japan). TA was also determined by adjusting the pH to 8.1 with 0.1 N NaOH after adding 20 ml distilled water in 5 ml fruit juice. In fruits harvested in October 27, the SSC and TA of the healthy fruit was 9.1 ± 0.2 °Brix and 1.20 ± 0.11%, respectively, whereas that of the infected fruit was 8.37 ± 0.31 °Brix and 1.20 ± 0.14%, respectively (Table 4). In fruits harvested in November 16, the SSC and TA of the healthy fruit was 10.43 ± 0.36 °Brix and 1.30 ± 0.15%, respectively, whereas that of the infected fruit was 8.87 ± 0.44 °Brix and 1.17 ± 0.26%, respectively (Table 4). Thus, the results of the present study confirmed that CiMV reduces the commercial quality of citrus fruits and affects internal quality characteristics, such as SSC, even though the trees were coinfected with CiMV and CTV because these CTV isolates were mild strain by enzyme-linked immunosorbent assay using MCA-13 antibody (data not presented). Practically, though CTV was detected in 72.1% of 775 surveyed trees, there were no any CTV symptoms (Hyun et al., 2017). And it is not to be said that CTV did not affect the expression of CiMV at all because the most CTV were mild strains, but since the typical symptoms of CTV did not appear but only the typical symptoms of CiMV, the symptoms of trees investigated in this study might be mainly induced by CiMV infection. However, even if CTV is mild strain, the possibility of affecting the virus severity by coinfection with CiMV cannot be ruled out.

Previous studies have reported that the typical symptoms of CiMV infection, in certain cultivars, such as satsuma mandarin, include spotting and blotching of the fruit rind (Iwanami and Koizumi, 2000). Indeed, the present study, which surveyed citrus fruits in the Jeju area, found that the typical symptoms of CiMV-infected fruits included the appearance of dark blue speckles or ringspots on fruit rinds and the browning of oil glands in the spots as rind coloring began. As the coloring progressed, the spots gradually

Table 3. Effect of *Citrus mosaic sadwavirus* (CiMV) infection on the quality of citrus fruit

	Peel hardness (g-force)[a]	Flesh weight (%)[b]	Peel puffing (mm)[c]
Healthy fruit	636.5 ± 39.1 a	77.3 ± 1.7 a	0.9 ± 0.2 a
Infected fruit	1,618.3 ± 305.5 b	70.7 ± 0.6 b	2.9 ± 0.4 b

[a]Peel hardness was assayed using a TA-XT2 texture analyzer (Stable Micro System, Godalming, UK).
[b]Flesh weight (%) was calculated as (flesh weight/fruit weight) × 100%.
[c]Degree of peel puffing was measured as the spacing (mm) between the peel and the underlying segments.

Table 4. Effect of *Citrus mosaic sadwavirus* (CiMV) on the sugar and acid contents of citrus fruit

Survey date	Soluble solids content (°Brix)		Titratable acidity (%)	
	Healthy fruit	Infected fruit	Healthy fruit	Infected fruit
Oct 27	9.10 ± 0.24 a[a]	8.37 ± 0.31 b	1.20 ± 0.11 a	1.20 ± 0.14 a
Nov 16	10.43 ± 0.36 a	8.87 ± 0.44 b	1.30 ± 0.15 a	1.17 ± 0.26 a

[a]Numbers within a column followed by different letters are significantly different ($P = 0.05$) according to Duncan's multiple range test.

disappeared, but browning of the oil glands became worse and eventually the tissues surrounding the oil glands became necrotic (Fig. 1). In very early satsuma mandarins and 'Setoka' cultivar of late-maturity citrus, many of the symptomatic fruits were eventually dropped (Fig. 1D and H). Even though many citrus trees are infected with CiMV, there were trees that showed no typical symptoms. In fact, during some years, some trees did not exhibit any typical symptoms, even though CiMV infection was known. Although unconfirmed, it is possible that the inconsistent display of symptoms is a result of virus density or environmental conditions. It has been reported CiMV isolate Ci-968 causes mosaic patterns on citrus fruit rinds, whereas infection by the other CiMV isolates (*i.e.*, ND-1, LB-1, and Az-1) does not, and plural CiMV variants have been detected from a single citrus tree (Ito et al., 2007; Iwanami et al., 2001). Many trees were infected with CiMV, but typical symptoms were observed on only some trees. This is possible that non-pathogenic CiMV isolates are there.

The present study confirmed that CiMV infection on citrus cultivars reduce the quality of citrus fruits by reducing SSC, exacerbating peel puffing, and reducing flesh weight. Therefore, CiMV-infected trees should be removed from citrus orchards so that the infected fruits should not be distributed to the market for the competitiveness of the citrus industry.

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