Plant Pathol. J. 21(4) : 377-382 (2005)

The Plant Pathology Journal

Occurrence of *Chrysanthemum stunt viroid* in Chrysanthemum in Korea

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(Received on October 6, 2005; Accepted on November 17, 2005)

Infection rate of *Chrysanthemum stunt viroid* (CSVd) in 64 commercial chrysanthemum cultivars cultivated in Korea ranged from 9.7 to 66.8%. Symptoms on leaves of CSVd-infected chrysanthemum included yellow spots, chlorosis, vein clearing, vein bending and crumpling. CSVd induced flower malformation in 'Scot', color change in 'Sharotte', and color breaking in 'Sharon'. CSVd caused reduction of plant height, leaf size, flower size and the flowers number by 32-50%, 26-35%, 14-36% and 14-75%, respectively. In conclusion, CSVd affected plant height, leaf size and flower quality in chrysanthemum plants.

Keywords : *Chrysanthemum stunt viroid*, *Dendranthema grandiflorum*, infection rate

Chrysanthemum is an important cut-flower world-wide. They are grown in all Provinces of Korea and are sold as cut flowers or as pot flowers. It accounts for about 31 percent of the total cut-flower cultivation area of 2,614 ha in Korea. Sales totaled 120,640 million won in 2004 (Ministry of Agriculture and Forestry, 2004). In addition to a increase of domestic sales, the amount of export to Japan is increasing every year; in 2004 totaled $9.3 million (Ministry of Agriculture and Forestry, 2004), accounting for 19.1% of the total amount of floricultural crops exported.

Two viroids were reported in chrysanthemum world-wide (Bouwen and Zaayen, 1995). These are *Chrysanthemum stunt viroid* (CSVd; Diener and Lawson, 1973; Dusi et al., 1990; Hooftman et al., 1996; Kusunoki, 1993; Shiwaku, 1996a) and *Chrysanthemum chlorotic mottle viroid* (Diener et al., 1977; McGovern et al., 1988; Navarro and Flores, 1997; Peña et al., 1999). Of these two viroids, the most important one is CSVd. CSVd causes yellowing, early flowering, shortening of plant height by 2/3 to 1/2, reduction of leaf size and flower size (Dusi, 1990; Kusunoki et al., 1993).

CSVd was firstly observed in America in 1947, North America and Australia in 1951, the Netherlands in 1952, Belgium in 1972, Japan in 1977 and Brazil in 1990. Nowadays the pathogen occurs prevalently in the world (Lawson, 1987; Sakamoto, 1995). In Korea CSVd was found for the first time in 1997 from chrysanthemum 'Chunkwang' (Chung et al., 2001). Recently, dwarf symptom in chrysanthemum plants has been spread to most of cultivars and the damages have increased.

The objective of the present study was to determine the incidence of CSVd and to clarify the damages caused by it in commercial chrysanthemum cultivars in Korea.

Materials and Methods

Disease incidence. The observations on the occurrence of CSVd were conducted in 2005. Leaf samples were randomly collected from 64 commercial chrysanthemum cultivars at 35 fields in 9 main cultivation regions of Busan, Changwon, Kimhae, Masan, Gumi, Yesan, Yeoncheon, Goyang and Taean, Korea. Diagnosis was conducted using reverse transcription and polymerase chain reaction (RT-PCR).

Growth. To investigate the effects of CSVd on cut-flower quality, seven chrysanthemum cultivars 'Chunkwang', 'Owolcheong', 'Baekgwang', 'Seolpung', 'Subangnyeok', 'Angaesoguk' and 'Pyuma' were graft-inoculated with CSVd; Those seven cultivars were not infected with *Tomato aspermy virus* (TAV), *Chrysanthemum virus* B (CVB) or *Chrysanthemum chlorotic mottle viroid* (ChChMoVd). CSVd infection was confirmed by RT-PCR at 2 months after inoculation. Infected plants were propagated by cutting and were used for experiments. Length of cut-flower, flower size and the number of flowers per plant were determined at flowering start date. Vase life was determined from the date of dipping cut-flowers into the 2 liters of water to the start date of wilting outermost flower leaves. Length of cut-flower used for vase life ranged from 45 to 50 cm.

Effects of CSVd on plant height and leaf size were determined with cultivars 'Kasandra', 'Sharotte' and 'Delmont', which were naturally infected with CSVd. Those three cultivars were not infected with TAV, CVB or ChChMoVd. Plant height was measured from soil surface to the top of stem. Leaf size was measured with fully

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expanded leaves including petiole.

Extraction of RNA. RNA was prepared with 0.1 g of leaves using CF11 cellulose according to a method described previously (Shiwaku et al., 1996b). Pellet was resuspended in 50 ul of nuclease-free water.

Primers and RT-PCR conditions. A pair of primer was synthesized on the basis of CSVd (GeneBank accession no. AF394452) to amplify DNA fragment of 354 bp. The sequence of reverse primer (5'-TTCTTTCAAAGCAGCAGGGT-3') was complementary to nucleotides 36-55 of the CSVd and that of forward primer (5'-AAAGAAATGAGGCGAAGAAG-3') was homologous to nucleotides 56-75 of the CSVd.

Complementary DNA (cDNA) synthesis was accomplished as follows: One ul of RNA and 1 ul of 10 pm reverse primer was heated at 70°C for 5 min followed by adding 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 5 mM $MgCl_2$, 1 mM each dNTP, 1 ul of MuLV reverse transcriptase (Applied Biosystems, USA) and 1 ul of RNase inhibitor (1 U/ul) on ice and incubated at 37°C for 1 hr.

The PCR reaction mixture contains 5 ul of cDNA, 1 × PCR buffer, 0.5 mM of each dNTP, 1 ul of 10 pm each primer, 4 mM $MgCl_2$ and 2.5 U AmpliTaq DNA polymerase (Applied Biosystems, USA). Forty PCR cycles were conducted in PTC-0220 Perlitier Thermal Cycler (MJ Research, MA, USA). The thermal conditions were as follows: denaturation at 94°C for 30 sec (2 min for the first cycle), annealing at 53°C for 1 min and extension at 72°C for 1 min.

Table 1. Infection rate of Chrysanthemum stunt viroid in spray typed new chrysanthemum cultivars

Cultivars	No. of plants investigated	Infection rate (%)
Euro yellow	35	28.6
Euro white	22	0.0
Monarisa (Pink)	15	0.0
Monarisa (White)	7	23.5
Feeling Green	33	12.1
Feeling White	10	0.0
Luses	11	0.0
Hokusei	10	0.0
Sei hil	9	0.0
Sei verona	29	0.0
Sei claire	9	0.0
Sei noadarasi	13	0.0
Sei siba	14	100.0
Sei siba (Red)	14	100.0
Yuka	35	0.0
Vyking	25	20.0
Art	20	10.0
King fisher	23	0.0
Ibis	35	5.7
Ardilo	10	0.0
Lollipop	20	0.0
Lineker (Pink)	33	20.0
Lineker (Puple)	10	0.0
Lineker (Dark)	10	0.0
Lineker (White)	20	0.0
Lineker (Amma)	20	0.0
Red agumma	10	0.0
Any	10	0.0
Land	10	0.0
Red rosa	10	0.0
Moonlight	24	0.0
Furore gold	15	6.6
Yoko ono	20	20.0
Biarritz	15	0.0
Furore	15	0.0
Chopin	10	33.3
Barcadi	10	0.0
Ford	14	0.0
Angela	4	0.0
Average	–	9.7

Table 2. Infection rate of Chrysanthemum stunt viroid in old chrysanthemum cultivars

Plant type	Cultivars	No. of plants investigated	Infection rate (%)
Spray	Angaesoguk	20	20.0
	Ihwa	5	0.0
	Geumbora	7	28.6
	Sharotte	8	61.1
	Mirae	6	83.3
	Seolleim	6	100.0
	Geumsu	15	60.0
	Hongdan	5	93.3
	Geumpungcha	30	90.0
	Mipungcha	25	90.0
	Delmont	20	70.0
	Deseura	25	100.0
	Argus	25	50.0
	Asma	25	100.0
Standard	Mascot	2	100.0
	Diamond	15	80.0
	Okbong	15	100.0
	Bonghwang	20	100.0
	Sintaegeuk	20	100.0
	Goldenking	20	100.0
	Sinma	36	9.4
	Baekseon	12	0.0
	Baekgwang	20	0.0
Average		–	66.8

Electron microscopy. For observation of anatomical changes of chrysanthemum tissues by infection with CSVd, stem and petiole of 'Owolcheong' were cut into small pieces by hand dissection and fixed in 1% Karnovsky's fixative solution, pH 7.2 for 4 h. Postfixation was conducted in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2 for 2 h at 4°C. The material was dehydrated in concentration gradients of ethanol (50%, 75%, 90%, 95% and 100% for 30 min each step) and then dehydrated two times in a 100% amyl acetate. Thin sections of 75 nm were produced using RMS-MTX microtome. The specimens were gold coated and were examined with a scanning electron microscope (Hitachi S-2460N, Japan).

Results

Disease incidence. CSVd was detected in 31 cultivars out of 64 cultivars investigated. Infection rate ranged from 9.7% to 66.8% depending on cultivars. Percentages of chrysanthemum plants infected with CSVd in cultivars propagated for a long time in Korea was 66.8% (Table 1). Infection rate of new cultivars imported from Japan and Europe for the last 3 years was 9.7% (Table 2).

Symptomatology. Foliar symptoms caused by natural infection with CSVd: 'Sharotte' and 'Vyking' showed chlorotic yellow spots and necrotic yellow spots under high

Fig. 1. Symptoms induced by natural infection with *Chrysanthemum stunt viroid* on chrysanthemum plants. (A) 'Sharotte' showing yellow spots under high temperature over 32-35 degrees, (B) 'Vyking' revealing necrotic big yellows spots under high temperature of over 32-35 degrees, (C) 'Sintaegeuk' showing crumpling, (D) and (E) 'Chunkwang' revealing yellowing with vein bending and vein clearing, respectively.

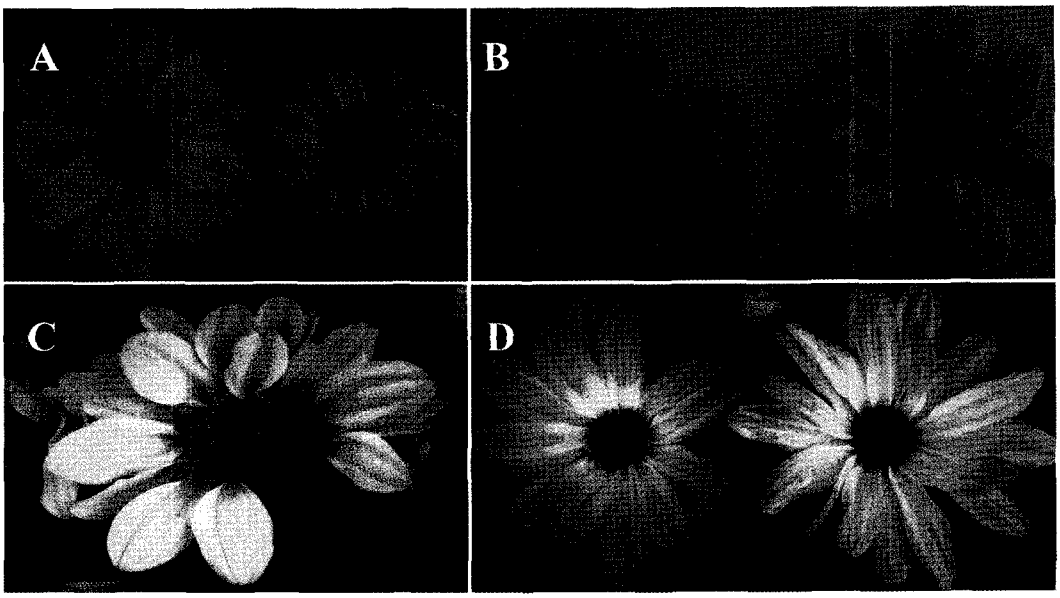

Fig. 2. Symptoms caused by natural infection with *Chrysanthemum stunt viroid* on chrysanthemum flowers. (A) 'Geumsu' showing reduction of size, left, healthy; right, infected, (B) 'Sharotte' showing color change, left, healthy; right, infected, (C) 'Scot' revealing malformation, and (D) 'Sharon' showing color breaking, left, healthy; right, infected.

Fig. 3. Chrysanthemum 'Monarisa' afftected with CSVd. Front chrysanthemum plants are infected with *Chrysanthemum stunt viroid,* showing reduction of plant height, and the rear ones are healthy.

temperature of over 32-35 degrees in summer, respectively; 'Sintaegeuk' showed crumpling, and 'Chunkwang' revealed vein clearing or chlorosis (Fig. 1). CSVd reduced flower size of 'Geumsu', and changed flower color of 'Sharon' to single yellow without red. CSVd caused flower malformation in 'Scot', and flower color breaking in 'Sharon' (Fig. 2).

CSVd reduced plant height of 'Sharotte', 'Kasandra' and 'Delmont' by 50.3%, 41.5% and 31.8%, and leaf size by 34.6%, 26.9% and 25.8%, respectively (Table 3). CSVd also caused reduction in cut-flower length by 9.0% to 51%, flower size by 14% to 36%, and the number of flowers per plant by 14% to 75% (Table 4). CSVd delayed flowering in 'Chunkwang' and 'Angaesoguk'. Whereas, CSVd accelerated flowering in 'Seolpung' and 'Owolcheong', and it did not exert influence on flowering in 'Baekgwang', 'Subangnyeok' and 'Pyuma' (Table 4). CSVd diminished vase life of most of chrysanthemum cultivars tested (Table 4).

Table 3. Effect of Chrysanthemum stunt viroid on the growth of spray typed chrysanthemum plants

Cultivars	Division	Height of plant (cm)	Length of leaf (cm)
Sharotte	Healthy	46.3	10.4
	Diseased	23.0	6.8
	Significance	**[a]	**
Kasandra	Healthy	47.5	9.3
	Diseased	27.8	6.8
	Significance	**	**
Delmont	Healthy	50.0	10.1
	Diseased	34.1	7.7
	Significance	**	**

[a] T test *significant P=0.05, **significant P=0.01

Anatomy of CSVd-affected tissue. Histological examination of cross sections of infected stem showed abnormal development of the cambium cells (Fig. 4A). This pathological change was not found in tissues of healthy plants (Fig. 4B). The pith of petiole was appeared to be poorly developed compared to that of healthy one (Fig. 4C, D).

Table 4. Effects of Chrysanthemum stunt viroid on the quality of chrysanthemum flowers

Cultivars	Division	Cut-flower length (cm)	Flower diameter (cm)	No. of flowers/plant	Flowering start date	Vase life (days)
Chunkwang	Healthy	73.3	8.9	18	Oct.30	15
	Diseased	35.6	6.5	10	Nov.08	7
	Significance	**[a]	**	**	–	–
Owolcheong	Healthy	96.8	9.4	16	Oct.12	16
	Diseased	87.8	6.9	6	Oct.09	7
	Significance	*	**	**	–	–
Baekgwang	Healthy	65.0	10.5	10	Sept.20	16
	Diseased	60.0	9.6	10	Sept.20	16
	Significance	NS	NS	NS	–	–
Seolpung	Healthy	115.0	11.5	20	Oct.30	16
	Diseased	68.8	7.4	5	Oct.23	7
	Significance	**	**	**	–	–
Subangnyeok	Healthy	84.4	11.2	5	Oct.30	16
	Diseased	65.3	8.2	4	Oct.30	7
	Significance	*	**	NS	–	–
Angaesoguk	Healthy	85.7	1.9	183	Nov.02	15
	Diseased	44.5	1.5	67	Nov.06	7
	Significance	**	**	**	–	–
Pyuma	Healthy	123.7	5.0	21	Oct.30	15
	Diseased	82.3	4.3	18	Oct.30	7
	Significance	**	**	*	–	–

[a]T test, **significant P=0.01, NS non significant.

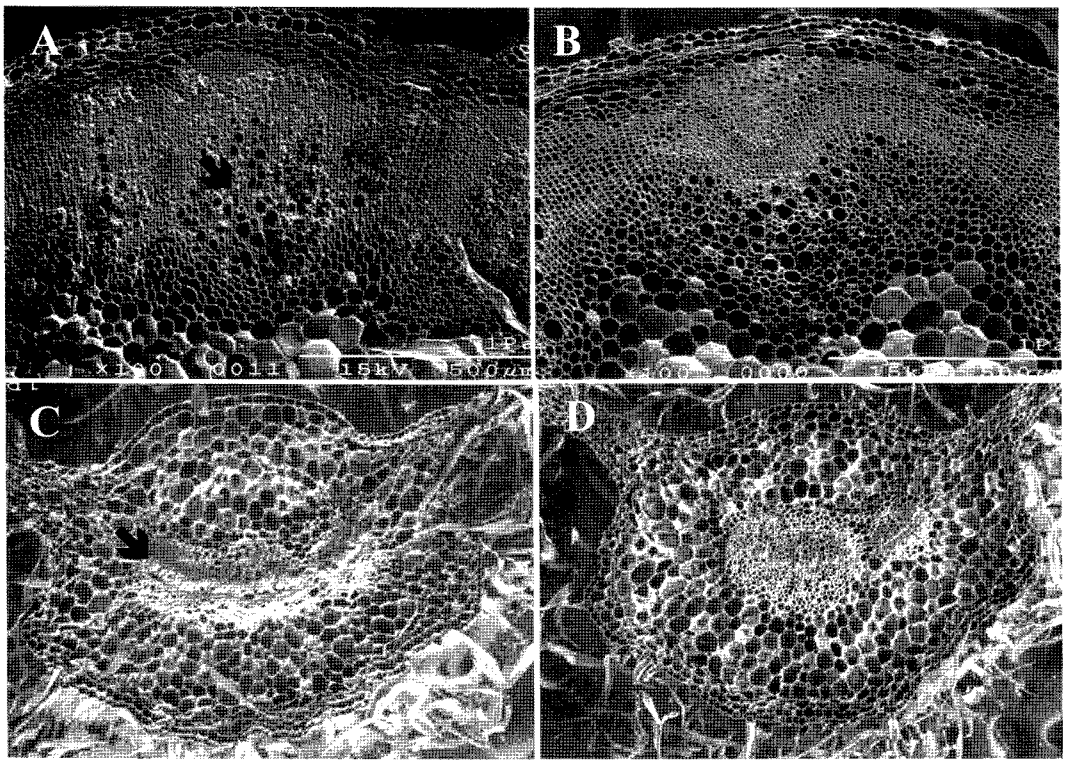

Fig. 4. Transverse sections of stem and petiole of chrysanthemum 'Owolcheong'. (A) Abnormal development of conductive cells of stem (arrow) by CSVd infection, (C) poor development of the pith of petiole (arrow) by CSVd infection, (B) and (D) healthy.

Discussion

As increase of the production area of chrysanthemum increase from 784 ha in 2001 to 820 ha in 2004 (including potted chrysanthemum) (Ministry of Agriculture and Forestry, 2004), CSVd has spread to most of chrysanthemum cultivars in Korea. When CSVd was identified in 1997, it was occurred only in 'Chunkwang', but recently CSVd is widespread.

Prevalent of CSVd was most unlikely by 'Chunkwang', but could result from cuttings imported from Europe or Japan for the last 2 to 3 years because 'Chunkwang' had been cultivated in limited places including Masan and Changwon in Gyeongnam Province, and a few places in Gyeonggi Province. Furthermore 'Chunkwang' had not been cultivated in those places anymore due to deterioration in quality by viroid or viral disease accumulated during long cultivation period, or due to 'Sinma' that substituted 'Chunkwang'.

Nucleotide sequences of full-length CSVd were determined with 3 cultivars of 'Sei siba', 'Euro yellow' and 'Feeling green' (data not shown). We assumed that they might have differences in their nucleotide sequences, because they were bred from different countries. Unexpectedly, all clones from the 3 different cultivars showed the same nucleotide sequences as the one isolated from 'Chunkwang' (GeneBank accession no. AF394452) (Chung et al., 2001), suggesting it was dominant strain worldwide.

The symptoms which developed after infection with CSVd were dependent on the cultivar. Foliar symptoms were less common and often chlorosis of leaves was the only indication of infection. Leaf spots or flecks were also sometimes seen in some cultivars. Leaf size was reduced in proportion to the reduction of plant height. In many cultivars the main symptom was stunting, with a reduction of up to 51% in overall height in mature plants, resulting in losing their commercial value completely.

The other common symptoms were floral, with infected plants having reduced flower size. Flowers were not fully opened or early wilted compared to healthy ones. This observation was assumed to result from abnormal development of the conductive cells of stem tissues. Corruption of the vascular cambium cells of stem tissues by CSVd was previously reported (Morelli and Nelson, 1987). They proposed that vascular cambium was the first and the most severely affected tissues by CSVd infection.

The objective of the present study was to determine the incidence of CSVd in commercial chrysanthemums, and to clarify the damages caused by it in Korea. CSVd was prevalent in commercial chrysanthemum cultivars and damages were severe depend on the fields. To prevent spread of CSVd, further experiments associated with transmission are required.

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