Notes

Viral Infection of Tissue Cultured Orchids and Evaluation of Damages

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Most orchids are propagated by tissue culture. To survey the viral infection of tissue cultured Orchids, total RNA was extracted from *in vitro* *Cymbidium* and *Phalaenopsis* spp. collected from companies producing tissue-cultured orchids, and RT-PCR analysis was conducted with primer pairs specific to *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ring spot virus* (ORSV), which are infecting wide range of orchid genera. The bulb size of *Cymbidium* infected with CymMV and ORSV was compared with healthy one at 10 months after planting *in vitro* orchids in the glasshouse. The CymMV or ORSV infection in 97 *Cymbidium* and 55 *Phalaenopsis* plants was 84.5 and 89.1%, respectively. Mixed infection was found in 52.6 and 47.3% of *Cymbidium* and *Phalaenopsis* tested, whereas virus-free orchids were 15.5 and 10.9%, respectively. The CymMV and ORSV reduced the bulb size by 2.7-50% depending on the cultivars of *Cymbidium*. The both viruses caused yellowing, mottle and mosaic with or without necrosis in 4 *Cymbidium* cultivars.

Keywords : CymMV, ORSV, RT-PCR, tissue culture, virus

Orchid, one of the most important potted floricultural crops in Korea, commands a cultivation area of 332 ha in 2005. It accounts for 25.8% in terms of the potted floricultural crop cultivation area. Thirty kinds of viruses have been reported on orchids worldwide (Lawson and Hsu, 1995). Among them, *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot virus* (ORSV) are the most prevalent in Korea (Chang et al., 1991; Park et al., 1998; Ryu et al., 1998). CymMV causes chlorotic or necrotic symptoms on orchid leaves and flowers. The infected flowers become deformed and exhibit color breaking in *Cymbidium*. ORSV induces streak or striped mosaic or ringspots on leaves (Chang et al., 1991).

Most orchids are generally propagated by tissue culture methods of axillary buds and shoot meristems. For vegetative propagation of *Phalaenopsis* by tissue culture, inter-

nodal sections of flower stalks are usually used worldwide including Korea (Homma and Asahira, 1985). In *Cymbidium*, shoot meristems are usually used, and they are multiplied upto 10,000 plants *in vitro*. There are limit in number in propagation by tissue culture, because over than 10,000 plants per meristem might cause genetic variation. From the reason new meristem must be excised from field-grown orchids for every 10,000 plants.

To investigate virus infection status of *in vitro* *Cymbidium* and *Phalaenopsis*, RT-PCR was carried out with primer pairs specific to CymMV and ORSV. Primer pairs were as follows; CymMVK-F (5'-ACAATAATTTGAAAT-AATCATGGGA-3') and CymMVK-R (5'-AAAACCACACGCTTATTAAGTTTG-3'), and ORSVK-F (5'-ACGCA-CAATCTGATTCGTATTGAA-3') and ORSVK-R (5'-TAT-CAACGTTATTTTCCTAAATAT-3'). For duplex RT-PCR in one reaction tube, 10 ng of total RNA in 8 μ L of nuclease free water and 1 μ L of 10 pmole CymMVK-R and ORSVK-R primers for reverse transcription were heated at 70°C for 5 min followed by adding 4 μ L of 5 $\times$ reaction buffer, 2.5 mM MgCl₂, 0.25 mM of each dNTP, 1 μ L of Improm II reverse transcriptase (Promega, USA) on ice, and incubating at 37°C for 1 hr. PCR amplification was performed in a 50 μ L volume containing 20 μ L of the cDNA solution, 0.2 mM of each dNTP, 2 mM MgCl₂, 10 pmole reverse and forward primers for each virus, 2.5 units of GoTaq DNA polymerase (Promega, USA), and the 10 μ L of 5 $\times$ PCR buffer. The thermocycler program consisted of 1 cycle at 94°C for 2 min, 30 cycles at 94°C for 30 sec, 50°C for 1 min, 72°C for 1 min, followed by a final extension at 72°C for 10 min. Total 97 *Cymbidium* and 55 *Phalaenopsis* collected from companies propagating orchids by tissue culture in Korea were surveyed by RT-PCR as described above. The amplified products were 716 bp for CymMV and 528 bp for ORSV (Fig. 1).

The CymMV and/or ORSV infection in *Cymbidium* and *Phalaenopsis* was 84.5 and 89.1%, whereas virus-free plants were lower to 15.5 and 10.9%, respectively (Fig. 1, Table 1). High percentage of orchids was mixed infected by CymMV and ORSV in this survey. ORSV was more prevalent than CymMV (Table 1). This result was coincident with the observation of Park et al. (1998) where infection

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Fig. 1. Detection of CymMV and ORSV from *in vitro* orchids by duplex RT-PCR. The cDNA fragments synthesized from viral RNAs were amplified in one tube with primer pairs both of CymMVK-F and CymMVK-R for CymMV and ORSVK-F and ORSVK-R for ORSV detection. The sizes of PCR product are 716 bp and 528 bp, respectively. All orchid samples were collected from orchid cultivation company in Korea. A. *Cymbidium* sp. Lane 1-16: A company, lane 17-25: B company, lane 26-33: C company and lane 34-47: D company. B. *Phalaenopsis* sp. Lane 1-45: E company, lane 46-55: F company.

Table 1. Virus infection rate of CymMV and ORSV in tissue cultured *Cymbidium* sp. and *Phalaenopsis* sp.

Orchids	Source of sample ^b	No. of plants tested	Infection rate (%) ^a				
			CymMV	ORSV	CymMV and ORSV	Virus infected	Virus free
<i>Cymbidium</i> sp.	A	16	0.0	100.0	0.0	100.0	0.0
	B	9	0.0	11.1	77.7	88.8	11.2
	C	28	0.0	50.0	0.0	50.0	50.0
	D	44	0.0	0.0	100.0	100.0	0.0
Sub-total		97	0.0	40.0	52.6	84.5	15.5
<i>Phalaenopsis</i> sp.	E	45	0.0	40.0	46.7	86.7	13.3
	F	10	50.0	0.0	50.0	100.0	0.0
Sub-total		55	9.0	32.7	47.3	89.1	10.9
Total		152	8.3	33.5	45.7	87.5	12.5

^a RT-PCR was used for detection of CymMV or ORSV.

^b Companies producing tissue cultured orchids.

rate of ORSV and CymMV in field collected orchids was 15.5 and 22.4%, respectively. This data indicated not only virus infected *Cymbidium* and *Phalaenopsis* have been used for tissue culture but also CymMV and ORSV do not easily removed by meristem tip culture at least in *Cymbidium*, because presently *Cymbidium* has been propagated *in vitro* by excision of meristem tips in Korea. In Thailand, CymMV and ORSV were detected in 65.4% and 0% of *Dendrobium*. On the contrary to Korea, CymMV was a prevalent virus in *Dendrobium* in Thailand (Kehntry et al., 2006).

Tissue culture of meristem tips have been popularly used for obtaining virus-free plants. Although meristem was excised from field-cultivated plants, it would not guarantee to be virus-free condition. When meristem tip culture was firstly used to produce virus-free plants, it was assumed that the regenerated plants were healthy since viruses did not invade the meristematic tissues of the bud (Kassanis, 1965), despite the fact that *Tobacco mosaic virus* (TMV) was reported to be present in meristems of tomato (Sheffield, 1942). CymMV was eliminated from CymMV and ORSV-infected meristem tissue pieces less than 0.2-0.5 mm,

Table 2. Comparison of the bulb size in *Cymbidium* infected with CymMV and/or ORSV

Cultivar	Division	No. of plants investigated	Diam. of bulb (mm)
Moon flight	Healthy	3	25.4±0.1
	CymMV	1	12.4
	ORSV	4	16.8±0.3
	CymMV+ORSV	4	12.2±0.2
Yellow butterfly	Healthy	1	23.2
	CymMV	1	13.8
	ORSV	2	15.9±0.1
	CymMV+ORSV	6	17.2±0.3
Moon stone	Healthy	4	22.6±0.2
	CymMV	4	14.5±0.4
	ORSV	0	–
	CymMV+ORSV	6	22.0±0.4
Silk road	Healthy	0	–
	CymMV	1	16.1
	ORSV	4	20.7±0.2
	CymMV+ORSV	0	–
Total		41	–

however, the ORSV persisted (Ishii, 1974). However the survival of meristem tissue pieces less than 0.2-0.5 mm was little or no unless grafting tissue pieces onto callus tissue (Ishii, 1974). Removing CymMV and ORSV by meristem tip culture is practically impossible. Accordingly virus free orchids must be used for mass production *in vitro*.

Next, we assessed the symptoms and growth affected by CymMV and ORSV infection in naturally CymMV and/or ORSV-infected *in vitro Cymbidium* sp., grown from tissue cultured plants. A total of 41 *Cymbidium* sp. plants of 4 cultivars were surveyed by RT-PCR and acclimated in the glasshouse. The growth was assessed by measuring the bulb diameter at 10 months after acclimation. The whole plants and the symptoms on leaves were photographed at the same period as the measuring the bulb size. CymMV and ORSV reduced the diameter of bulbs ranged from 2.7 to 52.0% depending on the cultivars.

In ‘Moon flight’, virus affected the growth of bulb severely in the order of double infection by CymMV and ORSV, CymMV single and ORSV single infections. Peculiarly, in both ‘Yellow butterfly’ and ‘Moonstone’, CymMV single infection more severely affected the growth of bulb than double infection (Table 2). However, we don’t think there was any interference between ORSV and

Fig. 2. Symptomatology in 4 *Cymbidium* cultivars infected with CymMV and/or ORSV. The *Cymbidium* cultivars showed reduction in growth vigor and revealed symptoms of yellowing, mottle and necrosis on leaves. A. ‘Moon flight’, B. ‘Yellow butterfly’, C. ‘Moon stone’ and D. ‘Silk road’.

CymMV. Further experiments are need with more replication. Common result from all *Cymbidium* cultivars tested was that CymMV affected more severely than ORSV, and CymMV and/or ORSV reduced the bulb size and growth vigor in all *Cymbidium* cultivars tested (Fig. 2). Typical viral symptoms such as yellowing with mottle was observed on leaves of cultivars Moon flight, Yellow butterfly and Moon stone doubly infected with both viruses, and black necrosis on leaves of 'Silk road' single infected with CymMV (Fig. 2).

This study showed that high percentage of *in vitro* orchids was infected with CymMV and/or ORSV. This result indicated that most tissue culture companies of orchids do not use virus-free plants for tissue culture, because simple excision of meristem tips from virus-infected *Cymbidium* is mostly resulted in fail to remove CymMV and ORSV. Result of the reduction of bulb size and growth vigor in *Cymbidium* by infection with CymMV or/and ORSV in this study showed the need for producing virus-free *Cymbidium* to produce high-quality plants and export to other countries with strict quarantine conditions.

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