Detection of viruses infecting Lilium spp. by RT-PCR and Real-Time PCR

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Abstract
In order to enhance the Italian lily bulb production, a breeding program was carried out at CRA-VIV in Pescia (PT - Italy) during the last years. Asiatic hybrids (*Lilium* x *elegans* Thunb.), lily cultivars and other native species were involved in the program. The obtained lily lines, which presented interesting traits, were preserved in a collection. After years of vegetative propagation, some of the new selections showed symptoms referable to viral infections. Virus diseases represent some of the most dangerous threats of *Lilium*, so the application of fast and effective diagnostic techniques for early detection is very important. The aim of the present study, in the frame of a phytosanitary survey of the lily collection, is to investigate the presence and incidence of Cucumber Mosaic Virus (CMV), Lilium Symptomless Virus (LSV), Lily Mottle Virus (LMoV) and two tospoviruses (Impatiens Necrotic Spot Virus, INSV, and Tomato Spotted Wilt Virus, TSWV).
Among the 60 samples object of this study, infections by LSV and CMV were frequently observed. Also LMoV was detected in a smaller amount of samples. All of the samples were negative to INSV and TSWV.

INTRODUCTION

Among the bulbous plants, lilies (*Lilium* spp.) are in demand in the floriculture industry both as potted plants and cut flowers (Dole and Wilkins, 2005) and ranked within the top 10 flowers in the export market. The genus *Lilium* consist of about 80 species which can be classified in 7 sections. The three commercially important divisions of lily include Easter lily (*Lilium longiflorum*), Asiatic and Oriental hybrids. Recently also some hybrids intergroups were added, such as: *Longiflorum* x Asiatic (LA), Oriental x Trumpet (OT), *Longiflorum* x Oriental (LO) and Oriental x Asiatic (OA).

The main limiting factor in lilies cultivation is their susceptibility to virus diseases; almost all lilies are propagated vegetatively, and infected bulbs used for propagation represent sources of further diffusion of viruses. Viral infections cause relevant economic losses in lily production around the world. The extent of damage does, however vary widely depending on the susceptibility of different lily cultivars towards specific viruses (Lawson, 2011). The most commonly occurring viruses infecting lily are Cucumber Mosaic Virus (CMV), Lily Symptomless Virus (LSV) and Lily Mottle Virus (LMoV) (Asjes, 2000).

The symptoms of virus infections comprise reduced growth, vein-clearing and light green stripes between the veins, gray or brown necrotic spots and flower colour breaking,

affecting the quality and yield of cut flowers production (Lawson, 1981; Raju and Olsen, 1985). Symptoms may be stronger if two or more viruses infect lily plants together.

In the present paper diagnostic techniques such as RT-PCR and Real Time PCR were performed and optimized for the diagnosis of five viruses, including CMV, LSV, LMoV and two tospoviruses (TSWV and INSV) already reported to infect ornamental crops in Tuscany (Triolo *et al.*, 1991; Materazzi and Triolo, 2001).

MATERIALS AND METHODS

In the lily collection held at CRA-VIV (Pescia, Pistoia – Italy), during spring–summer 2011, symptoms referable to virus infections (chlorotic and/or necrotic stripes, gray or brown necrotic spots, flower stems and/or basal leaves deformations) were observed.

Leaves and flower tissues from 60 symptomatic and symptomless plants belonging to 20 different lily clones (three plants for each clone) were sampled. Total RNA was extracted using *RNeasy Plant Mini Kit* (Qiagen) protocol, modified according to MacKenzie *et al*. (1997). 2 g. of tissue were ground using a *Tissue lyzer* (Qiagen) adding 5-7 ml of g*rinding buffer* (4 M guanidine isothiocyanate, 0.2 M sodium acetate pH 5.0, 25 mM EDTA, 2.5% PVP-40 and 2.0% Sodium bisulfite added just before use). The solution (1 ml) was transferred in a 1.5 ml tube, added 100 µl of 20% sarkosyl and incubated at 56°C with agitation for 10 min. After 2 min. centrifugation, 600-650 µl were transferred to a *QIAshredder spin column* (Qiagen) placed in a 2 ml collection tube. The following steps of RNA extraction were according to the manufacturer's protocol.

The extracted RNA was then retro-transcribed into cDNA using the *iScript cDNA Synthesis kit* (Biorad); in a 0.5 ml tube, 16 µl of reaction mix, consisting of 11 µl of sterile water, 4 µl of 1X *iScript* buffer and 1 µl of *iScript* retrotrascriptase, were added to 4 µl of RNA. The following thermal protocol was performed: 1 cycle at 25 °C for 5 min, 1 cycle at 42 °C for 30 min and 1 cycle at 85 °C for 5 min.

RT-PCR end point

Reverse transcription-polymerase chain reaction (RT-PCR) with different primer pairs specific to each investigated virus was performed for the detection of CMV, LSV, LMoV, INSV and TSWV (Tab. 1). Briefly, 2 µl of cDNA was amplified in 25 µl of reaction mixture containing 1X GoTaq Flexi Buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of sense and antisense primers and 1 U of GoTaq HotStart DNA Polymerase (Promega).

PCR amplifications were at first performed according to the original protocols described in literature.

Protocols were then uniformed to unique amplification conditions in order to allow a faster singleplex detection of the investigated viruses: initial denaturation at 94 °C for 2 min, then 30 cycles at 94 °C for 30 sec., annealing at 55 °C for 45 sec. and extension at 72 °C for 45 sec., with a final extension at 72°C for 7 min. The same cDNA samples were tested again with the new cycling conditions.

10 µl of PCR product were then mixed with 1 µl of 10X loading dye and electrophoresed in 2% agarose gels in TBE buffer. A 100 bp DNA ladder was used on gel to approximatively determine the lenght of the amplified product. Gels were stained with GelRed (Biotium) and observed under UV light.

Real Time PCR

The aforementioned primer pairs (Tab. 1) were also used in Real Time PCR assays, with the uniformed cycling conditions.

For each sample, 5 µl of cDNA were amplified in a total volume of 20 µl containing 1x *QuantiFast SYBR Green PCR Master Mix* (Qiagen) and 0.5 µM of each primer. Reactions were performed in a *iQ5* Real-Time thermocycler (Biorad).

After amplification, the melting curve test was performed by continuous temperature increase starting from 65°C up to 95°C (0.5°C increase/each step, for 10 seconds). Calculation of the melting temperature for each amplicon (*Tm*) was done directly by the equipment software.

RESULTS

RT-PCR end point

Among the 60 samples tested, results obtained with original protocols showed 40 samples (66.7%), belonging to 17 clones, infected with CMV; 53 (88.3%), belonging to 20 clones, positive to LSV and 4 (6.7%), belonging to 2 clones, infected with LMoV. None of the tested samples resulted infected by INSV and TSWV.

The same samples, tested with the new amplification conditions for CMV, LSV and LMoV showed results perfectly matching the ones obtained with standard protocols, confirming the sensitivity and specificity of the tests (Tab. 2; Fig. 1, 2, and 3).

Among the 60 tested samples, only 7 (11.7%) provided negative results to the five viruses investigated.

Results obtained showed 12 samples singularly infected by LSV, while no single infections by CMV and LMoV were detected.

Mixed infections were observed in 41 samples: 37 of them were co-infected with CMV+LSV, 1 co-infected by LSV+LMoV and 3 samples were positive to CMV+LSV+LMoV.

Real Time PCR

The same primers used for RT-PCR were used to perform a Real Time PCR assay. All of the samples positive to the end-point PCR assay were confirmed to be infected. Also, the number of positive samples was higher for CMV (53/60, 88.3%) and LMoV (8/60, 13.3%), indicating a higher sensitivity of this assay. For each infected sample, the SYBR Green melting curve analysis showed a single peak, indicating its specificity (Tab. 2; Fig. 1, 2, and 3).

Only 1 of the 7 negative samples obtained by end point RT-PCR confirmed such status after the Real Time PCR assay. Also, 6 samples singularly infected by CMV were detected, while all of the 8 LMoV positive samples were co-infected with CMV+LSV. No samples provided positive reaction to TSWV or INSV.

CONCLUSION

Management of diseases induced by viruses is a critical point in the propagation and production of lilies. Therefore, reliable and fast detection of viruses is crucial in order to handle of healthy plant material.

The uniformed cycling conditions used in this study for the diagnosis of CMV, LSV and LMoV provided results perfectly matching the ones obtained with the original protocols already described in literature. Also, the use of Real Time technique provides further sensibility and rapidity, with no need of post-amplification processing.

The analysis performed on 60 samples of the CRA-VIV *Lilium* varietal collection showed a negative phytovirological status. In fact, despite the absence of infections by TSWV and INSV, the plants tested were frequently infected by CMV, LSV and, to a minor

extent, LMoV. Only 7 samples proved to be negative to the 5 viruses when tested by RT-PCR, while only 1 of them confirmed such status after the Real Time PCR assay.

Also, considering that the spread of aphid-borne CMV, LSV and LMoV generally occurs very rapidly, the adoption of measures to prevent vector-mediated diffusion of the viruses is of fundamental importance.

AKNOWLEDGEMENTS

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Tables

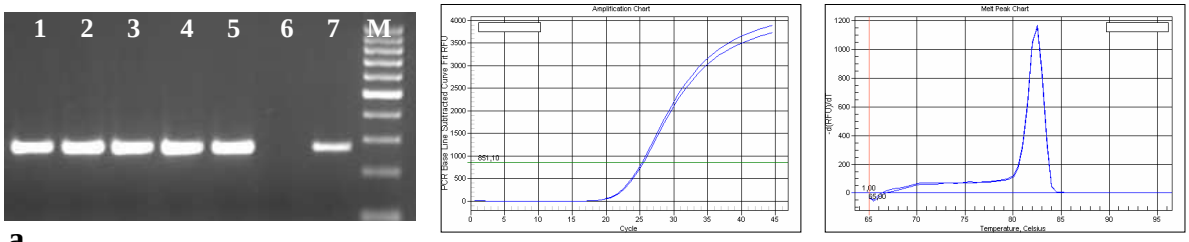
Table 1. Primers for reverse transcription-polymerase chain reaction (RT-PCR) and Real Time PCR amplification.

Virus	Primer sequence (5'-3')	Amplicon size	Reference
CMV	5'- ACTCTTAACCACCCAACCTT -3' 5'- AACATAGCAGAGATGGCGG -3'	280 bp	Lumia *et al.*, 2001
LSV	5'–AAGGATCCATGCAATCAAGACCAAGA–3' 5'–AAGAGCTCTCATCCATTATTTGCGTA–3'	900 bp	Singh *et al*., 2005
LMoV	5'-GCAAATGAGACACTCAATGCTG-3' 5'-CGTGCGTGAAGTAACTTCATAG-3'	651 bp	Lim *et al.*, 2009
INSV	5'GGATGTAAGCCCTTCTTTGTAGTGG-3' 5'-CCTTCCAAGTCACCCTCTGATTG-3'	364 bp	Liu *et al.*, 2009
TSWV	5′-AATTGCCTTGCAACCAATTC-3′ 5′-ATCAGTCGAAATGGTCGGCA-3′	267 bp	Mumford *et al*., 1996

Table 2. Results of RT-PCR and Real Time PCR assays for the detection of the 5 viruses (CMV, LSV, LMoV, INSV and TSWV) on 60 lily plants belonging to 20 clones.

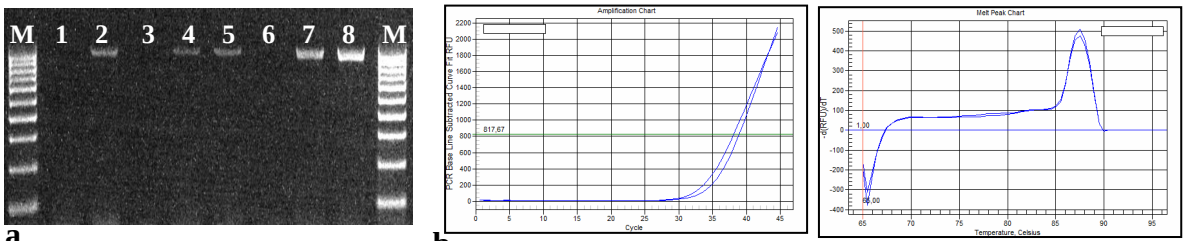
		RT-PCR end point					Real Time PCR				
N.	**Sample**	**CMV**	**LSV**	**LMoV**	**INSV**	**TSWV**	**CMV**	**LSV**	**LMoV**	**INSV**	**TSWV**
1.	121/1	+	+	-	-	-	+	+	-	-	-
2.	121/2	+	+	-	-	-	+	+	-	-	-
3.	121/3	-	+	-	-	-	+	+	-	-	-
4.	71/1	-	+	-	-	-	+	+	-	-	-
5.	71/2	+	+	-	-	-	+	+	-	-	-
6.	71/3	-	+	-	-	-	-	+	-	-	-
7.	618/1	-	-	-	-	-	+	-	-	-	-
8.	618/2	+	+	-	-	-	+	+	-	-	-
9.	618/3	-	-	-	-	-	+	-	-	-	-
10.	219/1	-	+	-	-	-	-	+	-	-	-
11.	219/2	-	+	-	-	-	-	+	-	-	-
12.	219/3	-	+	-	-	-	-	+	-	-	-
13.	187/1	+	+	-	-	-	+	+	-	-	-
14.	187/2	+	+	-	-	-	+	+	+	-	-
15.	187/3	-	+	-	-	-	+	+	+	-	-
16.	556/1	+	+	-	-	-	-	+	-	-	-
17.	556/2	-	+	-	-	-	+	+	-	-	-
18.	556/3	+	+	-	-	-	+	+	-	-	-
19.	557/1	+	+	-	-	-	+	+	-	-	-
20.	557/2	+	+	-	-	-	+	+	-	-	-
21.	557/3	+	+	-	-	-	+	+	-	-	-
22.	10/1	+	+	-	-	-	+	+	-	-	-
23.	10/2	+	+	-	-	-	+	+	-	-	-
24.	10/3	+	+	-	-	-	+	+	-	-	-
25.	39/1	-	-	-	-	-	-	-	-	-	-
26.	39/2	-	-	-	-	-	+	-	-	-	-
27.	39/3	-	+	-	-	-	+	+	-	-	-
28.	19/1	+	+	-	-	-	+	+	-	-	-
29.	19/2	+	+	-	-	-	+	+	-	-	-
30.	19/3	+	+	-	-	-	+	+	-	-	-
31.	149/1	-	-	-	-	-	+	-	-	-	-
32.	149/2	+	+	-	-	-	+	+	-	-	-
33.	149/3	-	-	-	-	-	+	-	-	-	-
34.	513/1	+	+	-	-	-	+	+	-	-	-
35.	513/2	+	+	-	-	-	+	+	-	-	-
36.	513/3	+	+	-	-	-	+	+	-	-	-
37.	207/1	+	+	-	-	-	+	+	+	-	-
38.	207/2	+	+	+	-	-	+	+	+	-	-
39.	207/3	+	+	-	-	-	+	+	+	-	-
40.	107/1	+	+	-	-	-	+	+	-	-	-
41.	107/2	+	+	-	-	-	+	+	-	-	-
42.	107/3	+	+	-	-	-	+	+	-	-	-
43.	40/1	+	+	-	-	-	+	+	-	-	-
44.	40/2	+	+	-	-	-	+	+	-	-	-
45.	40/3	+	+	-	-	-	+	+	-	-	-
46.	98/1	+	+	-	-	-	+	+	-	-	-
47.	98/2	+	+	-	-	-	+	+	-	-	-
48.	98/3	+	+	-	-	-	+	+	-	-	-
49.	352/1	-	-	-	-	-	+	-	-	-	-
50.	352/2	+	+	-	-	-	+	+	-	-	-
51.	352/3	+	+	-	-	-	+	+	-	-	-
52.	134/1	-	+	-	-	-	+	+	-	-	-
53.	134/2	-	+	-	-	-	-	+	-	-	-
54.	134/3	-	+	-	-	-	+	+	-	-	-
55.	196/1	+	+	-	-	-	+	+	-	-	-
56.	196/2	+	+	-	-	-	+	+	-	-	-
57.	196/3	+	+	-	-	-	+	+	-	-	-
58.	117/3	+	+	+	-	-	+	+	+	-	-
59.	117/2	+	+	+	-	-	+	+	+	-	-
60.	117/1	-	+	+			+	+	+	-	-
Total positive samples		**40**	**53**	**4**	**0**	**0**	**53**	**53**	**8**	**0**	**0**

Figures:



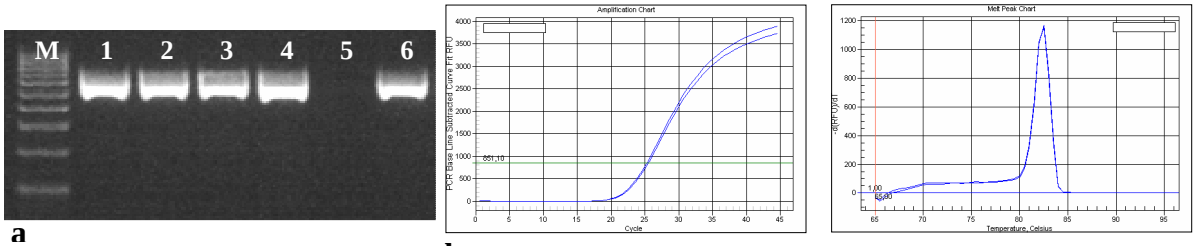
a **b** **c**

Fig. 1. **a)** RT-PCR: Gel electrophoresis of PCR products obtained for CMV detection: lanes 1-5: positive samples; lane 6: negative control; lane 7: positive control; M: 100bp Ladder; **b)** Real Time Amplification curve of a CMV positive sample; **c)** Melting curve peak of a CMV positive sample.



a **b** **c**

Fig. 2. **a)** RT-PCR: Gel electrophoresis of PCR products obtained for LSV detection: lane 1: negative control; lane 2: positive control; lanes 3, 6: negative samples; lanes 4, 5, 7, 8: positive samples; M: 100bp Ladder; **b)** Real Time Amplification curve of a LSV positive sample; **c)** Melting curve peak of a LSV positive sample.



a **b** **c**

Fig. 3. **a)** Gel electrophoresis of PCR products obtained for LMoV detection: lanes 1-4: positive samples; lane 5: negative control; lane 6: positive control; M: 100bp Ladder; **b)** Real Time Amplification curve of a LMoV positive sample; **c)** Melting curve peak of a LMoV positive sample.