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Production of virus-free plants of *Lilium* spp. from bulbs obtained in vitro and ex vitro

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ABSTRACT

Lilium tissue culture is used for mass propagation of *elite* material as well as for obtaining virus-free plants. The aim of the present study was to evaluate the effectiveness of meristem tip culture from microbulbs obtained in vitro and from bulblets obtained via scaling macropropagation – either with or without thermotherapy and chemotherapy treatments applied before meristem tip culture – to obtain virus-free *Lilium* spp. plants. To this end, microshoot regeneration from meristematic tips was first tested and two diagnostic techniques were compared after meristem tip culture treatment. Different alternatives were assayed in several *Lilium* hybrids to obtain virus-free plants. Effective virus elimination was possible using meristematic tips extracted from bulblets produced ex vitro by scaling, a procedure that has not been previously reported in *Lilium*. The number of virus-free plants obtained, which depended on the genotype as well as on the virus present in the original material, reached ~100% via meristem tip culture with or without pre-thermotherapy treatment at 35 °C. Meristem tip culture produced 100% of LMoV-free plants in *Lilium longiflorum* 'Snow Queen' and LA hybrid 'Lacorno', also CMV-free plants in Asiatic hybrid 'Navona' and LA hybrid 'Fangio', and LSV-free plants in LA 'Royal Respect'. The LSV infection rate decreased in Asiatic hybrid 'Visconti' when thermotherapy was applied ex vitro before meristem tip culture. Chemotherapy applied during in vitro bulb differentiation prior to meristem tip culture led to a complete elimination of LSV in the LO hybrid 'Triumphator'. Ex vitro chemotherapy was ineffective in virus elimination even when applied at high concentrations.

1. Introduction

Lilium tissue culture is used for mass propagation of *elite* material and to obtain virus-free plants (Marinangeli, 2003). Meristem tip culture has been used successfully for virus elimination in some *Lilium* genotypes, the degree of effectiveness depending on the virus and on the host plant (Lawson and Hsu, 1996; Aswath et al., 2001). The most common viruses that infect *Lilium* are *Lily symptomless virus* (LSV), *Lily mottle virus* (LMoV) and *Cucumber mosaic virus* (CMV) (Asjes, 2000). Previous research showed that LSV was eliminated from *Lilium* spp. by (i) early excision of the shoots regenerated from scale sections in vitro (Allen et al., 1980), (ii) meristem tip culture (Asjes et al., 1974; Allen and Anderson, 1980; Allen et al., 1980; Nesi et al., 2009), and (iii) a combination of meristem tip culture and thermotherapy (Nesi et al., 2009). Meristem tip culture was used to eliminate LMoV (Blom-Barnhoorn and Van Aartrijk, 1985) whereas chemotherapy jointly with callus culture was used to eliminate CMV from *Lilium longiflorum* (Ozaki et al., 1996; Xu et al., 2000). In addition, thermotherapy was effective in eliminating LSV from an Asiatic hybrid (Nesi et al., 2009), but it is not known to be effective in eliminating CMV and LMoV.

LSV elimination from *Lilium* depends not only on the treatment applied but also on the hybrid infected. Furthermore, although LSV-free plants were obtained in Asiatic hybrids through in vitro culture of regenerated shoots from internal scales of infected bulbs (Allen et al., 1980), it was not possible to eliminate LSV from *L. longiflorum* 'Ace' and 'Nellie White' with the same procedure (Linderman et al., 1976; Allen et al., 1980), therefore meristem tip culture was necessary to obtain virus-free plants (Allen and Anderson, 1980). Ribavirin (40 μM) (Virazole®) in meristem tip culture medium reduced the percentage of *L. longiflorum* 'Arai' plants infected with LSV and/or TBV from 61.4% to 35.4%. However, it was ineffective in eliminating the virus from Asiatic hybrid 'Enchantment' (Blom-Barnhoorn and Van Aartrijk, 1985). In an attempt to eliminate LSV from two Asiatic hybrids by means of meristem tip culture and

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Table 1
Medium composition for in vitro culture of *Lilium* spp.

Culture medium components	Meristem tip culture per liter	Microbulb induction and multiplication	Microbulb growth
MS salts (Murashige and Skoog, 1962)	1×	1×	1×
MS Vitamin mixture	1×	1×	1×
Myo-inositol	0.10 g	0.10 g	0.10 g
Naphthaleneacetic acid (NAA)	–	0.03 g	0.10 g
Kinetin	–	–	0.10 g
Sucrose	30 g	30 g	90 g
Agar	8 g	8 g	8 g
pH	5.7	5.7	5.7

Table 2
Primers used for *Lily symptomless virus* (LSV), *Lily mottle virus* (LMoV) and *Cucumber mosaic virus* (CMV) diagnosis. F: forward, R: reverse.

Virus	Primer	Sequences	Fragment (bp)
LSV	F	5′-GAYGARYTYTTYAARATGAARGT-3′	483
	R	5′-ARYTGYTTRTGYGCRTTRTG-3′	
LMoV	F	5′-CARTTYGARACYTGGTAYAAYGC-3′	513
	R	5′-TGCATRTTYTTRTTRACRTCRTC-3′	
CMV	F	5′-ACCCTRAARCCRCCDRAAATWGA-3′	408
	R	5′-CGYTGRTGYTCRAYGTCRACRTG-3′	

in vitro thermotherapy, Nesi et al. (2009) observed that whereas one of the hybrids was LSV-free after a meristem tip culture procedure, for the other hybrid a post-treatment of in vitro thermotherapy and a second meristem tip culture were necessary to reach the same status, thus demonstrating that the effectiveness of the procedures followed for virus elimination depends on the genotype. Virus elimination also depends on the treatment applied and the virus present. It was observed that without meristem tip culture LSV and LMoV were reduced in bulb scales of *Lilium* × *parkmanii* treated at 30 °C, the treatment being less inhibitory for LMoV than for LSV. Likewise, 40 μM ribavirin markedly reduced the titer of LSV but not of LMoV (Cohen et al., 1985). Through in vitro culture of scales of *L. longiflorum* 'Georgia' and Oriental hybrid 'Casa Blanca' without a meristem tip culture stage and a simultaneous treatment of chemotherapy and thermotherapy, it was observed that the increase in concentration of the antiviral compound reduced both the growth and the number of bulblets obtained. Also, scales kept at 35 °C for four weeks produced a lower number of bulblets than the control at 25 °C (Xu and Niimi, 1999). The in vitro culture of *L. longiflorum* scales including 50 μM ribavirin reduced the infection rate with LSV and had no effects on the infection with LMoV (Xu and Niimi, 1999).

Further research showed that after the first stage of meristem tip culture in *Lilium brownii* 'Colchesteri' infected with LSV and LMoV, the bulbs were infected with at least one of the viruses. In contrast, the second stage of meristem tip culture in a medium containing the antiviral compound 2,4-dioxohexahydro-1,3,5-triazine (DHT) was effective in eliminating LSV but was ineffective in eliminating LMoV (Masuda et al., 2011). Moreover, whereas antivirals DHT and Virazole reduced the LSV and CMV infection rate in a callus culture of *L. longiflorum* (Xu et al., 2000), they were ineffective in eliminating LMoV from *L. longiflorum* and *L. brownii* 'Colchesteri' (Xu and Niimi, 1999; Masuda et al., 2011).

In summary, LSV was eliminated by meristem tip culture, with or without in vitro chemo- and thermotherapy, elimination of CMV was possible via meristem tip culture and in vitro chemotherapy, and elimination of LMoV was only possible by meristem tip culture, although with a low degree of effectiveness. Independently of the virus present, the effectiveness of the techniques was highly dependent on the genotype. In all the above-mentioned studies, meristem tip culture was performed in microbulbs obtained by in vitro culture. Thermotherapy and chemotherapy treatments were also carried out during a stage of either in vitro microbulb culture or in vitro meristem tip culture.

In view of the above, the purpose of the present study was to evaluate the effectiveness of meristem tip culture from microbulbs obtained by in vitro culture and from bulblets obtained via ex vitro scaling. Both thermotherapy and chemotherapy treatments were applied before meristem tip culture, i.e. during microbulb and bulblet differentiation in vitro and ex vitro, respectively, to obtain virus-free *Lilium* spp. plants.

2. Materials and methods

Microshoot regeneration from meristematic tips was first tested and two diagnostic techniques were compared. Bulbs infected with LSV, LMoV and CMV were used as plant material to adjust the virus eradication techniques. In order to obtain the initial material, leaf samples and then bulb scales from each hybrid were analyzed by DAS-ELISA for LSV, LMoV and/or CMV detection. Infected bulbs were used for virus eradication experiments. In all cases, thermotherapy and/or chemotherapy treatments were applied before meristem tip culture during microbulb and bulblet differentiation in vitro and ex vitro, respectively. The medium composition for meristem tip culture, bulb induction and multiplication and microbulb growth is shown in Table 1. In all the trials, meristematic tips with the first scale primordium were extracted aseptically under a stereomicroscope (Olympus SZ61TR, Olympus Optical Co., Japan) using a sterile hypodermic needle N° 21, and were transferred to the meristem tip culture medium and maintained at 25 ± 2 °C under a photoperiod of 16 h of light (RFA 48 μmol m^{-2} s^{-1}). The microshoots obtained were cultivated on microbulb growth medium in darkness at 25 ± 2 °C. Microbulbs were subsequently planted in 60 well multi-cell trays (55 ml volume per well) filled with commercial substrate based on peat and perlite (Grow Mix, Terrafértil S.A., Moreno, Argentina) and maintained in a greenhouse at 18–25 °C day/night temperature.

Virus diagnosis in each trial was performed by DAS-ELISA according to the general protocol described by Clark and Adams (1977) using commercial kits from BQ Support (Lisse, The Netherlands) as described in Chinestra et al., 2010. Reverse Transcription Polymerase Chain Reaction technique (RT-PCR) was followed in the first trial in order to compare its detection sensitivity with DAS-ELISA after meristem tip culture. To this end, total RNA was isolated using RNeasy Plant Mini Kit (Qiagen) and was stored at −80 °C. Complementary DNA (cDNA) synthesis was performed using iScript™ cDNA Synthesis Kit (Bio-Rad) following the manufacturer's instructions. For PCR reaction, cDNA product (2 μL) was added to a 20 μL reaction mixture containing 0.2 mM each of dNTPs, 0.5 μM of each primer, 2 μL 10× Taq polymerase buffer, 1.5 mM $MgCl_2$, and 2 U Taq DNA polymerase. Degenerate pairs of primers for LSV, LMoV and CMV diagnosis were selected from Niimi et al., 2003 (Table 2) and synthesized by Ruralex Fagos (Buenos Aires, Argentina). PCR reaction was carried out in a PXE 0.2 Thermal Cycler under the following conditions: initial denaturation at

Table 3
Contamination and regeneration of 'Snow Queen', 'Nello', 'Litoween', 'Triumphator' and 'Serano' microshoots from meristematic tips after 90 days of in vitro culture. Different letters indicate a significant difference between hybrids with $p < 0.05$ by Pearson's chi-square test.

Hybrids	No. of meristematic tips cultured	No. of contaminated cultures (%)	No. of regenerated plants (%)
L. l 'Snow Queen'	150	2 (1.3) a	90 (60.8) b
Asiatic 'Nello'	30	1 (3.3) a	29 (100) a
LA 'Litouwen'	30	2 (6.6) a	22 (78.6) b
LO 'Triumphator'	30	3 (10.0) a	19 (70.4) b
OT 'Serano'	30	0 (0) a	19 (63.3) b

Table 4
Number of virus-free microshoots of LO hybrid 'Triumphator' and number of plants infected with LSV, LMoV or CMV after meristem tip culture kept in vitro for 90 days and tested by DAS-ELISA.

Hybrid	No. of microshoots tested	No. of microshoots infected with each virus (%)			No. of virus-free microshoots (%)[a]
		LSV	LMoV	CMV	
LO 'Triumphator'	11	1 (9.09)	2 (18.2)	1 (9.09)	7 (63.6)

[a] Virus-free plants showed the same results using RT-PCR reaction.

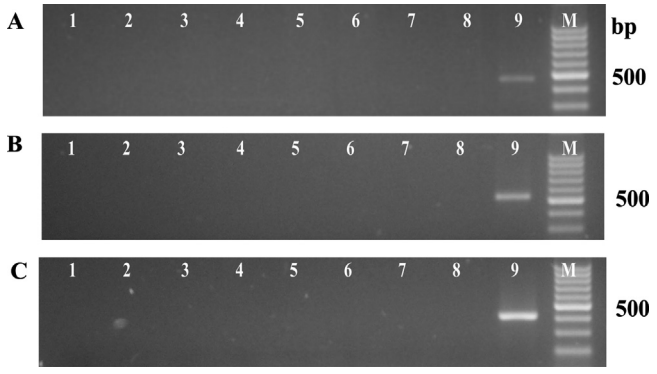

Fig. 1. Samples analyzed with degenerate primers for (A) LSV (483 pb), (B) LMoV (513 pb) and (C) CMV (408 pb) diagnosis. Negative control (1), samples (2–8), positive control (9), DNA Ladder (Genbiotech) Molecular marker 100 pb (M).

95 °C for 3 min, 35 cycles at 94 °C for 30 s, annealing at 55 °C for 45 s for LSV and LMoV primers, and 58 °C for 45 s for CMV primers, extension at 72 °C for 45 s and final extension at 72 °C for 7 min. PCR products were assessed on a 2% (w/v) agarose gel (Genbiotech) in a TAE (1×) buffer (40 mM Tris base, 5 mM sodium acetate, 1 mM EDTA, pH 8.0), and were stained with SYBR Safe® 1% (Invitrogen). Electrophoresis was performed at 80 V for 110 min. Bands were visualized in a blue LED light transilluminator.

In each trial, the differences in the number and average fresh weight of the bulblets obtained during scaling were analyzed by Fisher's LSD test. Culture contamination, shoot regeneration, total infection and infection with each virus were analyzed by Pearson's Chi-Squared test. Fisher's exact test was used when the expected values were below five in more than 20% of the cells of the contingency table. Data were analyzed using InfoStat software (Di Rienzo et al., 2011).

2.1. Evaluation of plant regeneration from meristematic tips extracted from microbulbs and comparison of DAS-ELISA and RT-PCR techniques

Bulb scales of *L. longiflorum* 'Snow Queen' and *Lilium* hybrids Asiatic 'Nello', LA 'Litouween', LO 'Triumphator' and OT 'Serano' were cultivated and kept in vitro. Bulb scales were sterilized by immersion in 70% ethanol for 1 min and then in a sodium hypochlorite solution (0.12 g L^{-1} of active chlorine) with 0.5 ml L^{-1} of Tween 20 for 15 min. They were subsequently rinsed three times with sterile distilled water. Transverse scale sections (~3 mm thick) were placed on microbulb induction medium and kept in darkness at 25 ± 2 °C until the microbulb differentiation stage.

Extraction and culture of meristematic tips were carried out as described above. Microshoot regeneration was tested for all the hybrids whereas the diagnostic techniques were evaluated in the LO hybrid 'Triumphator' cultures with bulbs infected with all three viruses, as detected by DAS-ELISA prior to meristem tip culture. The percentage of microshoots with each virus was evaluated 90 days after meristem tip culture. To compare the diagnostic techniques after meristem tip culture, a subsample of DAS-ELISA negative plants was analyzed using RT-PCR.

2.2. Meristem tip culture of bulblets obtained by ex vitro scaling

Bulbs of *L. longiflorum* 'Snow Queen', Asiatic hybrid 'Navona' and LA 'Fangio' infected with LSV, LMoV and CMV were used for scaling propagation. Bulb scales were sterilized by immersion in a sodium hypochlorite solution (0.22 g L^{-1} of active chlorine) for 15 min and then in a solution of carbendazim (Carbendaglex CS 50%, Gleba) 0.1% a.i., Captan (Captan Tomen WP 80%, Cheminova) 0.16% a.i., and Carbofuran (Furadan CS 48%, FMC) 0.05% a.i. for 15 min. Scales were placed in plastic containers between layers of *Sphagnum* peat moss (Simonetta, Tierra del Fuego, Argentina) for 5 weeks in darkness at 25 ± 2 °C to induce bulblet differentiation. The number and average fresh weight of the bulblets were recorded after scaling.

Prior to meristematic tip extraction, the bulblets were sterilized by immersion in 70% ethanol for 1 min and then in a sodium hypochlorite solution (1.2 g L^{-1} of active chlorine) with 0.5 ml L^{-1} of Tween 20 for 15 min. They were subsequently rinsed three times with sterile distilled water. Extraction and culture of meristematic tips and ex vitro culture of microbulbs were carried out as described above. The newly fully developed leaves were analyzed by DAS-ELISA for LSV, LMoV and CMV detection. The percentage of virus-infected plants was evaluated after six months in greenhouse culture.

2.3. Meristem tip culture of bulblets subjected to thermotherapy during ex vitro scaling

Bulbs of Asiatic hybrid 'Visconti' and LA hybrids 'Fangio' and 'Lacorno' infected with LSV, LMoV and CMV were used for scaling propagation. Bulb scales were sterilized and put in plastic containers between layers of peat for 5 weeks at 25 °C (control), 35 °C and 40 °C (thermotherapy) in darkness to induce bulblet differentiation. The number and average fresh weight of the bulblets were recorded after scaling. The bulblets were sterilized prior to meristematic tip extraction. The percentage of virus-infected plants was

evaluated by DAS-ELISA after in vitro culture and after six months in greenhouse culture.

2.4. Meristem tip culture of bulblets subjected to thermotherapy and chemotherapy during ex vitro scaling

Bulbs of LA hybrid 'Royal Respect' with single infection of LSV or CMV were used for scaling propagation. Bulb scales were sterilized and cultured on damp absorbent paper in plastic trays in darkness for 4 weeks at 25 and 35 °C to induce bulblet differentiation. In addition, treatments with different antiviral concentrations were carried out at 0, 100 and 300 μM ribavirin (Virazole®). Fifty scales per treatment were cultured. The bulblets were sterilized prior to meristematic tip extraction. The number and average fresh weight of the bulblets obtained by scaling, as well as the percentage of microshoot regeneration from meristematic tips, were all recorded. The percentage of virus-infected plants was evaluated by DAS-ELISA after six months in greenhouse culture.

2.5. In vitro chemotherapy and thermotherapy with and without meristem tip culture

Bulb scales of LO hybrid 'Triumphator' infected simultaneously with LSV and LMoV were sterilized and cultured in vitro. After two multiplication cycles, two microscales were taken from each microbulb and were divided longitudinally. Each half of the scale was cultured at 25 or 35 °C for 4 weeks on microbulb induction medium, with or without 20 μM ribavirin which was sterilized by filtration and was added to the culture medium dispensed in petri dishes with 25 ml each. Fifty scales were used per treatment. Half of the microbulbs regenerated in each treatment were chosen at random and planted ex vitro in order to test the effects of chemo- and thermotherapy treatments without meristem tip culture. The remaining microbulbs were used for meristem tip culture. After six months of in vitro culture, the microbulbs obtained by meristem tip culture were also cultured ex vitro. In both cases, plants were evaluated by DAS-ELISA after six months in greenhouse culture.

3. Results

3.1. Plant regeneration from meristematic tips extracted from microbulbs and comparison of DAS-ELISA and RT-PCR techniques

No significant differences in culture contamination were found between hybrids (Table 3). The percentage of shoot regeneration from meristematic tips was high, although it was not homogenous among the hybrids ($p < 0.05$), with 'Nello' showing the highest percentage (100%) and 'Snow Queen' the lowest (60.3%) (Table 3). In the LO 'Triumphator' hybrid, 63.6% of virus-free plants were obtained and only single infections with LSV, LMoV and CMV were found in low amounts (Table 4). Plants with negative DAS-ELISA results were also negative when analyzed using RT-PCR reaction (Fig. 1).

3.2. Meristem tip culture from bulblets obtained by ex vitro scaling

No significant differences were found between hybrids either in the number of bulblets per scale or in the average fresh weight of the bulblets obtained by scaling (Table 5).

The number of samples infected with each virus before meristem tip culture, with either single or co-infections, is shown in Table 6. The average percentage of virus-free plants obtained following the DAS-ELISA test differed between 'Snow Queen' and 'Navona' ($p < 0.005$) and between 'Fangio' and 'Navona' ($p < 0.01$). In contrast, no differences were detected between 'Snow Queen' and 'Fangio' ($p = 0.94$) which were found to have ~87% of virus-free plants (Table 6).

LSV was the most difficult virus to eliminate in the three hybrids analyzed although the percentage eliminated varied among them ($p < 0.01$). It was followed by LMoV and CMV (Table 6). The effectiveness of LSV and LMoV elimination by meristem tip culture was not homogenous between the hybrids 'Snow Queen' and 'Navona' ($p < 0.05$).

3.3. Meristem tip culture from bulblets subjected to thermotherapy during ex vitro scaling

The temperature during scaling affected bulblet differentiation and growth. The scales kept at 40 °C did not produce any bulblets. Moreover, the number of bulblets per scale and their fresh weight were higher when scales were cultured at 25 °C than when cultured at 35 °C ($p < 0.05$) (Table 7).

The number of samples infected with each virus before scaling and after six months in greenhouse culture of microbulbs obtained by meristem tip culture is shown in Table 8.

In the Asiatic hybrid 'Visconti', significant differences between the thermotherapy treatment and the control were found for LSV elimination ($p < 0.05$). The viral infection rate at 25 °C in relation to the total number of bulbs originally infected with this virus was 15.9%, whereas at 35 °C all plants were virus-free.

Furthermore, the differences between treatments were not significant for LSV, LMoV or CMV eradication in 'Lacorno' and 'Fangio' hybrids ($p > 0.2$). In the LA hybrid 'Lacorno', no infection with LMoV was found at 25 and 35 °C, thus indicating that meristem tip culture on its own led to virus elimination. In the LA hybrid 'Fangio', meristem tip culture either alone or with thermotherapy pre-treatment led to CMV elimination in 100% of the plants analyzed. Although meristem tip culture at 25 °C was highly effective for total virus elimination in the three hybrids tested, thermotherapy at 35 °C resulted in 100% of virus-free plants in 'Visconti' ($p < 0.01$), whereas in the 'Fangio' and 'Lacorno' hybrids there were no significant differences compared to the control condition at 25 °C ($p \geq 0.5$). Moreover, in all three hybrids evaluated, the infection rate was highest after six months in greenhouse culture in comparison with the analysis conducted after in vitro culture (Table 9).

3.4. Meristem tip culture from bulblets subjected to thermotherapy and chemotherapy during ex vitro scaling

In the LA 'Royal Respect' hybrid, the number and average fresh weight of bulblets obtained were lower in the thermotherapy treatment than in the control group ($p < 0.05$). Furthermore, chemotherapy effects were different at 25 and 35 °C ($p < 0.05$). In the thermotherapy treatment, the 100 and 300 μM ribavirin treatments produced a lower number of bulblets with a lower biomass than the control at 0 μM rivabirin (Table 10). In addition, many of the bulblets obtained at 35 °C were very small and, in several cases, the meristematic zone was necrotic (data not shown), therefore it was only possible to obtain a low number of meristematic tips from these bulblets.

Average contamination was low during meristem tip culture (7.1%). Chemotherapy at 35 °C significantly affected ($p < 0.05$) the regeneration rate of microshoots from meristematic tips (Table 10).

Regarding virus elimination, LSV was not detected in either the treatments or in the controls, thus the chemotherapy and thermotherapy effects could not be confirmed. In the case of CMV elimination, no significant differences were found between the thermotherapy treatment and the control at 25 °C ($p = 0.4$) in which 93% of plants were virus-free (Table 11). In addition, chemotherapy

Table 5
Bulblet regeneration during scaling and average fresh weight of bulblets obtained in *Lilium longiflorum* 'Snow Queen', Asiatic 'Navona' and LA 'Fangio' hybrids. Different letters indicate a significant difference between the hybrids with $p < 0.05$ by Fisher's LSD test.

Hybrids	No. of scales used for scaling	No. of bulblets obtained	Bulblets per scale	Bulblet weight (mg)
L. 'Snow Queen'	87	100	1.15 a	343 a
As 'Navona'	99	116	1.17 a	335 a
LA 'Fangio'	40	46	1.15 a	315 a

Table 6
Virus elimination through meristem tip culture of bulblets obtained by scaling in *Lilium longiflorum* 'Snow Queen', Asiatic 'Navona' and LA 'Fangio' hybrids initially infected with LSV, LMoV and/or CMV and plants remaining infected with each virus. Virus detection was carried out by DAS-ELISA after six months in greenhouse culture. Different letters indicate a significant difference with $p < 0.05$ by Pearson's chi-square test or Fisher's exact test with expected value <5.

Hybrids	Virus present before meristem tip culture	No. of plants infected with each virus before meristem tip culture	No. of plants infected with each virus after meristem tip culture (%)[b]	Percentage of virus-free plants[c]
L. 'Snow Queen' 52[a]	LSV	52	6 (11.5) b	86.5 a
	LMoV	20	0 (0.0) b	
	CMV	32	1 (3.1) a	
As 'Navona' 39	LSV	18	8 (44.4) a	58.9 b
	LMoV	28	8 (28.6) a	
	CMV	9	0 (0.0) a	
LA 'Fangio' 31	LSV	7	2 (28.6) ab	87.1 a
	LMoV	18	2 (11.1) ab	
	CMV	10	0 (0.0) a	

[a] Total number of plants per hybrid.
[b] Percentage of plants infected with each virus. Lower case letters 'a' and 'b' indicate differences between the hybrids for each virus.
[c] Percentage of virus-free plants for each hybrid. Lower case letters 'a' and 'b' indicate differences between the hybrids in the total percentage of virus-free plants obtained.

Table 7
Bulblet regeneration during scaling and average fresh weight of bulblets obtained at 25 and 35 °C in LA 'Lacorno', LA 'Fangio' and Asiatic 'Visconti' hybrids. Different letters indicate a significant difference between the thermotherapy treatment and the control in each hybrid with $p < 0.05$ by Fisher's LSD test.

Hybrids	*T* (°C)	No. of scales in scaling	No. of bulblets obtained	No. of bulblets per scale	Bulblet weight (mg)
Asiatic 'Visconti'	25	100	168	1.68 a	186 a
	35	52	22	0.42 b	58 b
LA 'Lacorno'	25	37	54	1.46 a	154 a
	35	45	27	0.6 b	61 b
LA 'Fangio'	25	21	25	1.19 a	236 a
	35	48	30	1.04 a	175 b

Table 8
Virus elimination through meristem tip culture of bulblets obtained by scaling at 25 and 35 °C in Asiatic 'Visconti', LA 'Lacorno' and LA 'Fangio' hybrids initially infected with LSV, LMoV and/or CMV, and plants remaining infected with each virus. Virus detection was carried out by DAS-ELISA. The total percentage of plants infected after six months in greenhouse culture is shown. Different letters indicate a significant difference in the total percentage of virus-free plants between the thermotherapy treatment and the control in each hybrid with $p < 0.05$ by Pearson's chi-square test or Fisher's exact test with expected value <5.

Hybrids	Virus present before meristem tip culture	*T* (°C)	No. of plants analyzed for each virus per treatment	No. of plants infected with each virus after treatments (%)	Percentage of virus-free plants	
					25 °C	35 °C
Asiatic 'Visconti' 116[a]	LSV	25	94	15 (15.9)	78.7 b	100 a
		35	22	0 (0)		
	CMV	25	21	5 (23.8)		
		35	11	0 (0)		
LA 'Lacorno' 49	LSV	25	23	1 (4.3)	94.1 a	100 a
		35	13	0 (0)		
	LMoV	25	34	0 (0)		
		35	15	0 (0)		
	CMV	25	11	1 (9.1)		
		35	3	0 (0)		
LA 'Fangio' 31	LSV	25	13	2 (15.3)	84.2 a	91.6 a
		35	8	0 (0)		
	LMoV	25	13	2 (15.3)		
		35	8	1 (12.5)		
	CMV	25	19	0 (0)		
		35	12	0 (0)		

[a] Total number of plants per hybrid.

Table 9
Virus detection by DAS-ELISA after in vitro culture and after six months in greenhouse culture in plants obtained by meristem tip culture of bulblets produced by scaling at 25 and 35 °C in Asiatic 'Visconti', LA 'Lacorno' and LA 'Fangio' hybrids initially infected with LSV, LMoV and/or CMV.

Hybrids	Virus present before meristem tip culture	*T* (°C)	No. of plants analyzed for each virus per treatment	No. of microshoots infected after in vitro culture	No. of plants infected after six months of greenhouse culture
Asiatic 'Visconti'	LSV	25	94	4	15
		35	22	0	0
	CMV	25	21	5	5
		35	11	0	0
LA 'Lacorno'	LSV	25	23	0	1
		35	13	0	0
	LMoV	25	34	0	0
		35	15	0	0
	CMV	25	11	1	1
		35	3	0	0
LA 'Fangio'	LSV	25	13	0	2
		35	8	0	0
	LMoV	25	13	1	2
		35	8	1	1
	CMV	25	19	0	0
		35	12	0	0

Table 10
Chemotherapy and thermotherapy effects on the number and fresh weight of bulblets obtained by scaling from 50 scales per treatment, and on the in vitro microshoot regeneration in *Lilium* LA 'Royal Respect'. Different letters indicate a significant difference between the chemotherapy treatments and the control carried out at 25 and 35 °C with $p < 0.05$ by Fisher's LSD test.

T (°C)	Ribavirin (μM)	No. of bulblets produced ex vitro	No. of bulblets per scale	Bulblet weight (mg)	No. of meristematic tips extracted	No. of microshoots regenerated in vitro (%)
25	0	85	1.7 a	176 b	60	38 (63.3) a
	100	77	1.54 a	165 b	75	42 (56) a
	300	92	1.84 a	223 a	78	49 (62.8) a
35	0	76	1.52 a	83 a	53	17 (32.0) a
	100	27	0.54 b	37 b	29	11 (37.9) a
	300	28	0.56 b	26 b	45	2 (4.4) b

Table 11
Chemotherapy and thermotherapy effects during scaling on the number of virus-free plants and of those infected with LSV and CMV after meristem tip culture in LA 'Royal Respect' hybrid. Bulbs at the initial stage of experimentation showed single infections with each virus. Virus detection was carried out by DAS-ELISA after six months in greenhouse culture. Different letters indicate a significant difference with $p < 0.05$ by Pearson's chi-square test or Fisher's exact test with expected value <5.

Virus present	*T* (°C)	Ribavirin (μM)	No. of plants analyzed for each virus and treatment	Results after meristem tip culture	
				No. of plants infected (%)	Percentage of virus-free plants
LSV	25	0	7	0 (0)	100 a
		100	11	0 (0)	
		300	17	0 (0)	
	35	0	10	0 (0)	100 a
		100	3	0 (0)	
		300	–	–	
CMV	25	0	17	1 (5.8) a	92.9 a
		100	19	1 (5.2) a	
		300	21	2 (9.5) a	
	35	0	3	0 (0)	100 a
		100	4	0 (0)	
		300	–	–	

applied during ex vitro scaling, followed by meristem tip culture, was ineffective in eliminating CMV at 25 °C ($p = 0.84$) (Table 11).

3.5. *In vitro chemotherapy and thermotherapy with and without meristem tip culture*

Microscales subjected to in vitro thermotherapy at 35 °C, either with or without chemotherapy, did not regenerate microbulbs. In contrast, at the base of all the microscales cultured at 25 °C, at least one microbulb was produced.

At 25 °C, the regeneration of microbulbs from microscales cultured in vitro followed by meristem tip culture produced a high percentage of virus-free plants without any significant differences between the chemotherapy treatment and the control ($p = 0.63$) (Table 12). Regarding each virus in particular, chemotherapy was effective in total LSV elimination as compared with the control group ($p < 0.05$) which reached a percentage of 16.7% of

Table 12
Chemotherapy effects on the number of virus-free plants and of those infected with LSV, LMoV and CMV with or without meristem tip culture in LO hybrid 'Triumphator'. Bulbs at the initial stage of experimentation showed mixed infections of LSV and LMoV. Virus detection was carried out by DAS-ELISA after six months in greenhouse culture. Different letters indicate a significant difference with $p < 0.05$ by Pearson's chi-square test or Fisher's exact test with expected value <5.

Treatment	Ribavirin (μM)	No. of plants tested	No. of infected plants (%)[a]		No. of virus-free plants[b] (%)	
			LSV	LMoV		
With a meristem tip culture stage	0	18	3 (16.7) a	4 (22.2) a	13 (72.2) a	28 (68.3) A
	20	23	0 (0.0) b	8 (34.8) a	15 (65.2) a	
Without a meristem tip culture stage	0	26	21 (80.8) a	11 (42.3) a	4 (15.4) a	12 (23.5) B
	20	25	14 (56.0) b	10 (40.0) a	7 (28.0) a	

[a] Number and percentage (in parentheses) of plants infected with each virus. Some plants showed co-infections with more than one virus (data not shown). Lower case letters 'a' and 'b' indicate differences between the chemotherapy treatment and the control in the number of plants infected with each virus, with and without a meristem tip culture stage.
[b] Number and percentage (in parentheses) of virus-free plants. Lower case letters 'a' and 'b' indicate differences between the chemotherapy treatment and the control in the number of virus-free plants, with and without a meristem tip culture stage. Capital letters 'A' and 'B' indicate differences in the total number of virus-free plants obtained with and without a meristem tip culture stage.

LSV-infected plants (Table 12). LMoV could not be totally eliminated via meristem tip culture of microbulbs differentiated in vitro, and chemotherapy produced no effect in LMoV elimination ($p = 0.38$) (Table 12).

Although meristem tip culture was highly effective in virus elimination ($p < 0.0001$), it was also possible to obtain *Lilium* virus-free plants by in vitro culture without meristem tip culture but only in low amounts (Table 12).

When chemotherapy was applied during microbulb development and microbulbs were just planted ex vitro without undergoing a meristem tip culture stage, there were no significant differences in the total infection rate between the chemotherapy treatment and the control ($p = 0.27$). In addition, when each virus was compared separately, differences in the infection rates of LSV were found ($p < 0.05$) but no differences were found for LMoV between the chemotherapy treatment and the control ($p = 0.86$) (Table 12).

4. Discussion

The variation between genotypes in the percentage of plant regeneration from meristematic tips recorded in the present study is in agreement with previous research (Cai et al., 1988) in which the survival rate of meristematic tips cultured in vitro was highly dependent on the genotype and varied from 42% to 88% in *Dahlia* spp.

Previous studies showed that the RT-PCR reaction in adult plants offers higher sensitivity than DAS-ELISA, thus allowing detection of viruses in serology-negative plants (Niimi et al., 2003; Sato et al., 2002; Sharma et al., 2005). Moreover, previous research that compared RT-PCR with DAS-ELISA after a treatment for LSV elimination from *Lilium* (Nesi et al., 2009) reported similar results to those collected in the present study, i.e. DAS-ELISA negative plants were also negative when analyzed by RT-PCR.

The wide range of percentages of virus elimination by meristem tip culture among the hybrids analyzed in the present study is in good agreement with results reported on Asiatic hybrids 'Enchantment' and 'Fire King', in which 74% and 56% of the plants tested were LSV-free (Asjes et al., 1974). However, they are higher than those reported by Allen and Anderson (1980) who observed that 16.7% and 25% of plants were LSV-free in *L. longiflorum* 'Ace' and 'Nellie White', respectively. In addition, the differences in the effectiveness of LSV and LMoV elimination by meristem tip culture among hybrids are in agreement with previous studies in which the percentage of virus elimination by meristem tip culture in *Lilium* depends not only on the virus but also on the hybrid infected (Asjes et al., 1974; Allen et al., 1980; Nesi et al., 2009).

Results regarding virus elimination in the Asiatic hybrid 'Visconti' indicate the possibility of optimizing the production of LSV-free plants using thermotherapy during ex vitro scaling prior to meristem tip culture. Variability of the effectiveness of thermotherapy in virus elimination among genotypes was previously reported, although it was applied to explants during in vitro culture (Nesi et al., 2009). Xu and Niimi (1999) reported a total infection rate of 80% at 25 °C and of 60% at 35 °C in Oriental hybrid 'Casa Blanca', whereas in *L. longiflorum* 'Georgia', they reported an infection rate of 88% at 25 °C whereas they found no microbulbs in the scales at 35 °C.

The temperature effects during ex vitro scaling on bulblet number and average fresh weight in the present study are in agreement with results reported by Xu and Niimi (1999) in which bulblets were lower in number at 35 °C than at 25 °C during in vitro scale culture of Oriental hybrid 'Casablanca' and of *L. longiflorum* 'Georgia'. Xu and Niimi (1999) revealed that the percentages of bulblet differentiation at 25 °C and at 35 °C were 78% and 0% for 'Georgia' and 80% and 43% for Oriental hybrid 'Casa Blanca', respectively. Moreover, the number of bulblets per scale at 25 and 35 °C was 2.3 and 0 for 'Georgia' and 1.3 and 0.6 for 'Casa Blanca' at 25 and 35 °C, respectively (Xu and Niimi, 1999).

Taking into account the negative effects of thermotherapy at 35 °C on the regeneration of bulblets from scales and the possible improvement in the effectiveness of virus elimination, it is clear that by starting scaling with a higher number of scales may compensate for its lower regeneration rate. In fact the benefit of a higher percentage of virus-free plants justifies the application of thermotherapy to some hybrids and, particularly, for LSV elimination. However, the low bulblet yield in scaling at 35 °C should be taken into account when analyzing the global effectiveness of the process.

Rivabirin applied under ex vitro conditions was not phytotoxic in 'Royal Respect' at 25 °C although it was at 35 °C. Previous research found that the number of bulblets of *L. longiflorum* 'Georgia' produced in vitro was lower when the ribavirin concentration was increased from 0.5 to 50 μM (Xu and Niimi, 1999). However, Blom-Barnhoorn and Van Aartrijk (1985) noted that in *L. longiflorum* 'Arai' the number of microbulbs was not affected by ribavirin at concentrations ranging from 0 to 40 μM, although this number decreased at concentrations of 400 μM. It is important to highlight that in the present study ribavirin was applied under ex vitro conditions and at concentrations varying between 100 and 300 μM without producing phytotoxicity at 25 °C, this being in support of applying stronger chemotherapy treatments to scaling than to in vitro cultures.

Chemotherapy during ex vitro scaling, followed by meristem tip culture did not improve virus removal. Previous research demonstrated that the percentage of plants infected with LSV and/or TBV

decreased from 61.4% to 35.4% at a concentration of 40 μM ribavirin in the in vitro meristem tip culture medium in *L. longiflorum* 'Arai', whereas the same concentration had no effect on the Asiatic hybrid 'Enchantment' (Blom-Barnhoorn and Van Aartrijk, 1985), thus indicating that the results could be affected by which hybrids were tested. In line with this, it must be taken into account that Blom-Barnhoorn and Van Aartrijk (1985), added ribavirin to the culture medium in vitro, whereas in the present study, ribavirin was added to a hydration solution of scales during ex vitro scaling.

The low number of virus-free plants obtained in the present study without a stage of meristem tip culture is in agreement with previous research (Allen et al., 1980) that reported a decrease in the percentage of LSV-infected plants in the Asiatic hybrid 'Red Carpet' by means of early excision of microbulbs obtained through in vitro culture without meristem tip culture. In line with this, in vitro chemotherapy favored LSV elimination even without a meristem tip culture stage and it had no effect on LMoV elimination. Furthermore, previous research reported that a concentration of 50 μM ribavirin applied during the differentiation of microbulbs from scales led to a decrease of LSV in *L. longiflorum* 'Georgia' but it had no effect on LMoV elimination, with 100% of infected plants after six months in greenhouse culture (Xu and Niimi, 1999). In a similar study, *Prunus necrotic ring-spot virus* (PNRSV) was eliminated from infected *Begonia x semperflorens* plants by in vitro culture with chemotherapy and thermotherapy without carrying out meristem tip culture (Verma et al., 2005).

Our comparison of the highest infection rate detected by DAS-ELISA after six months in greenhouse culture with that detected after in vitro culture shows the need for carrying out periodic testing of the material before producing stock I propagation material. This is in agreement with Xu and Niimi (1999) who, after carrying out in vitro chemotherapy and/or thermotherapy without meristem tip culture, observed that the percentage of infected plants detected by DAS-ELISA at the end of in vitro culture was lower than after six months in greenhouse culture. This is probably due to the high concentration of growth regulators in the medium, to other in vitro culture conditions or to other not well-established factors that may inhibit virus replication or keep a low viral concentration (Conci, 2004). Therefore, as DAS-ELISA analysis may produce negative results in infected plants evaluated immediately after in vitro culture, they must be periodically tested and evaluated after several months of greenhouse culture. Molecular techniques should also be implemented as reported for other crops (Ramgareeb et al., 2010; Neelamathi et al., 2014; Meena et al., 2014).

5. Conclusions

Virus elimination was successful using meristematic tips extracted from bulblets produced ex vitro by scaling, a procedure which has not been previously reported in *Lilium*. This method is more economical and it is independent of the high contamination rate that may occur in the tissue culture of bulb scales.

The percentage of virus-free plants obtained depended on the genotype and on the virus present in the original material and reached ~100% using meristem tip culture, with or without prethermotherapy treatment at 35 °C. Meristem tip culture from bulblets differentiated ex vitro by scaling produced 100% of LMoV-free plants in *L. longiflorum* 'Snow Queen' and LA 'Lacorno', also CMV-free plants in Asiatic hybrid 'Navona' and LA hybrid 'Fangio' and LSV-free plants in LA 'Royal Respect'.

Thermotherapy during scaling improved LSV virus elimination by meristem tip culture in Asiatic hybrid 'Visconti'. Under these conditions, both the average fresh weight and the number of bulblets obtained from scales decreased, this being an obstacle that could be solved by increasing the initial number of scales, which does not involve high costs. Moreover, scaling without peat allowed the development of bulblets at the base of the scales.

Chemotherapy applied during in vitro bulb differentiation prior to meristem tip culture led to a complete elimination of LSV in the LO hybrid 'Triumphator'. In contrast, ex vitro chemotherapy was ineffective in eliminating viruses, even when applied at high concentrations.

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