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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