Development, selection and propagation of interspecific hybrids of *Lilium*

Beatrice Nesi*, Sara Lazzereschi, Simona Pecchioli, Antonio Grassotti, Gianluca Burchi
Consiglio per la Ricerca e la sperimentazione in Agricoltura – Landscaping plants and Nursery Research Unit -Via dei Fiori n. 8, 51012 Pescia (PT) - Italy

Mariateresa Cardarelli
Consiglio per la Ricerca e la sperimentazione in Agricoltura – Centro di Ricerca per lo studio delle relazioni tra Pianta e Suolo, via della Navicella 2-4, 00184 Roma, Italy

Carolina Maria Cardona Suarez, Giuseppe Colla
Department of Agriculture, Forestry, Nature and Energy, University of Tuscia, via San Camillo De Lellis, 01100 Viterbo, Italy

email corresponding author: beatrice.nesi@entecra.it

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Abstract

In breeding programs of ornamental crops, interspecific hybridization is one of the most important way to introduce new genetic variation. The sexual barriers hampering interspecific hybridization have been distinguished into pre-fertilization and post-fertilization barriers. A breeding program on *Lilium* was carried out in Symplitaly project to overcome barriers of interspecific incompatibility and to originate hybrids with new ornamental traits (shape, size and colour of flower) and suitable to the Italian environment (tolerance to low temperatures and scarce light). Several crosses among Oriental hybrids, *L. longiflorum* OT hybrids, LA hybrids, Asiatic hybrids and wild species were made by using cut-style method. Ovary and ovule culture has been applied to overcome post-zygotic barriers. Fourteen days after pollination ovaries were cut and *in vitro* cultivated using two different substrates respectively with NAA 1 mg/L and IAA 0.5 mg/L. After 50-60 days more swollen ovules were taken out from ovary and cultivated on two substrates, with IBA 0.5 mg/L and NAA 0.1 mg/L, for 120-180 days. To confirm the hybrid origin of new bulblets, RAPD and SSR markers methods have been tested.

According the project, *in vitro* bulblet propagation of *Lilium* has been studied using an automated balloon type bubble bioreactor. In fact scaling-up in bioreactors and reduction in manual handling could provide an efficient and economic system for *in vitro* multiplication of bulb plants. Two different culture methods were compared: continuous immersion and temporary immersion in liquid medium (ebb and flood system). Results revealed that morphological traits and biomass accumulation were more efficient when culturing was performed under continuous immersion.

INTRODUCTION

In Italy ornamental bulbs are widely used as cut flowers, potted and garden plants. The most important bulb for cut flower production is certainly *Lilium*. Several *Lilium* varieties, divided into various commercial groups, are grown throughout the year in different Italian areas. Nowadays LA and Oriental hybrids are mainly cultivated with a reduced presence of *L. longiflorum* (Grassotti and Gimelli, 2011). In ornamental crops breeding, interspecific hybridization is the most important source of genetic variation. In order to introduce new variability in ornamental traits such as flower colours, flower shape and plant form, it is necessary to overcome interspecific crossing barriers. Both pre-fertilization and post-fertilization barriers restrict interspecific hybridization between the different sections of the genus *Lilium*. Several techniques, such as the cut style method, the grafted-

style method, and *in vitro* pollination techniques have been developed to overcome pre-fertilization barriers. However, even if fertilization is successful, post-fertilization barriers can inhibit the growth of hybrid embryos. *In vitro* pollination and rescue methods such as embryo culture, ovary-slice culture, and ovule culture have been developed to bypass these barriers (Lim and Van Tuyl, 2006).

The aim of this research was the development of a method to obtain a national production of interspecific *Lilium* hybrids overcoming the sexual barriers that occur among parental species and to assess their hybridity by molecular markers. An additional objective was to provide a feasible methodology for commercial production of *Lilium* in bioreactor comparing the continuous immersion system with the temporary immersion system. This technology could improve the growth of the plantlets and become an efficient model for automation and large-scale production of quality *Lilium* shoots.

Genetic markers represent a basic tool for an early characterization and identification of true interspecific hybrid genotypes. Several molecular markers were applied with different purposes to study the genus *Lilium*. ITS sequences were analyzed in *Lilium carniolicum* for phylogeny and systematic studies (Resetnik *et al.,* 2007) and for the evaluation of *Lilium* interspecific hybrids (Nesi, 2008; Nesi *et al.,* 2011). RAPD markers were used to confirm the hybrid origin of interspecific hybrids (Yamagishi *et al.,* 2002; Arshney *et al.,* 2001; Grassotti *et al.,* 2011); RAPD were also used as the starting point to obtain SCAR markers (Nesi *et al.,* 2011). Even ISSR markers have been particularly useful in revealing polymorphisms (Yamagishi *et al*., 2002). Nowadays microsatellite markers (SSR) are considered very interesting for their capacity to amplify a particular region of the genome using specific primers. In *Lilium philadelphicum* and *L. japonicum* specific microsatellites were isolated (Horning *et al*., 2003; Kawase *et al.,* 2010).

MATERIALS AND METHODS

Hybrid establishment

Several crosses among Oriental hybrids ('Nova Zembla' and 'Mother's Choice'), *L. longiflorum* (cv 'White Heaven'), OT-hybrids (cv 'All is all' and 'Robina'), LO hybrids (cv 'White Triumph' and 'White Triumph' (red throat, probabily 'Triumphator')), LA hybrids (cv 'Original Love', 'Hyde Park' and 'Brindisi'), Asiatic hybrids cv 'Gironde' and two Asiatic hybrids selected at CRA-VIV for interesting and innovative characters (409 and 00149), (pollenless, flower colour and shape etc.), and some wild species with a high potential (*L. regale, Lilium* cv 'Pink Perfection' and *L. candidum*) (Fig. 1), were made using cut-style method. Fourteen days after pollination, ovaries were cut and cultivated using MS salts and vitamins (pH 5.8), in two different media ($IAA_{0.5}$ and NAA_1) for 50-60 days (Fukai and Tsuji, 2004; Van Creij *et al.,* 2000) (Table 1). Then white swollen ovules, obtained from ovary culture, were cultivated in other two media (Fig. 2), $IBA_{0.5}$ and $NAA_{0.1}$, using MS salts and vitamins (pH 5.8), for 120-180 days (Nesi, 2008) (Table 1). Afterwards some embryos grown from ovules culture were transferred in $IBA_{0,5}$ e MS0 media for multiplication and enlargement (Nesi *et al.,* 2011) (Table 1).

New hybrids genetic evaluation

Hybrids genetic evaluation was done by means of RAPD and SSR markers. Genomic DNA was extracted from Oriental, *L. longiflorum*, LA, Asiatic hybrids, some wild species and hypothetical hybrids obtained. DNA was extracted 60 mg of fresh leaves using a *Invisorb Spin Plant Mini Kit* (Invitek - Germany) according to the manufacturer's protocol. Then it was quantified by means of a spectrophotometer *SmartSpec Plus* (BIORAD - USA).

The analysis was conducted first by RAPD markers, using OPA and OPB primers. The amplifications were done in a total volume of 25 ul of reaction mixture containing the reagents necessary at the following concentrations: 1X *buffer*, 1.5 mM $MgCl_2$, 0.2 mm of each dNTPs, 1 µM of primer, 2 U of Taq DNA polymerase (5 Prime - Germany) and 20 ng of DNA. The amplifications were performed in a thermocycler *Mastercycler* (Eppendorf - USA), according to the

protocol described below. After incubation for 3 min at 92 °C, the reaction mixture was subjected to 45 cycles according to the following profile: 92 °C for 15 sec, 35 °C for 30 sec, 72 °C for 1 min; amplification products were then incubated at 75 °C for 10 min, then at 65 °C for 10 min and subsequently stored at 4 °C. The amplification products were separated on a 2% agarose gel with 1X TBE buffer stained with 1mg/ml ethidium bromide and visualized by a UV light transilluminator. A 100 bp marker (5 Prime - Germany) was used as a reference. Each reaction was repeated twice and only reproducible bands were considered for analysis.

For SSR analysis, nine primer pairs including LpCA20104, LpCA5, LpCA870, LpGA9, LpGA210, LpGA4, Lja16T, Lja10A and Lja13A were selected based literature (Horning *et al.*, 2003; Kawase *et al.*, 2010). Characteristics of microsatellite loci are shown in Table 2. PCR was carried out in 20 µL reaction mix containing 1X buffer, 1.5 mM MgCl2, 0.2mm of each dNTPs, 0.5 µM of each primer, 20 ng of DNA and 1U of PerfectTaq DNA polymerase (5 PRIME - USA), according to the following amplification profile: 9 min at 92 °C, then 40 cycles at 92 °C for 30 sec, 30 sec at the annealing temperature and 1 min at 72 °C, with a final elongation at 72 °C for 5 min. PCR products were electrophoretically separated on 3% agarose gel in 1X TBE buffer and visualized by ethidium bromide staining.

Micropropagation in bioreactor

Twenty shoots [15 g fresh weight (FW) of inoculum] were transferred to 4 L balloon type bubble bioreactor (BTBB) with 1 L of MS liquid medium supplemented with 60 g/L sucrose, 4.4 µM BA and 1.1 µM IAA (pH 5.8). The net inside the bioreactor avoided the complete submersion of explants in the liquid medium. All bioreactors were maintained at 25°C±1°C in dark conditions. The continuous immersion was compared with the temporary immersion [ebb and flood system with 30 min of immersion every 2.5 h (0.1 air volume culture volume^{-1} min^{-1})]. After 4 weeks morphological parameters and dry weight (drying for 48 h at 70°C) were determined. Growth index (GI) was calculated as described by Russowski *et al.* (2006) [GI = (weight final – weight initial) weight initial^{-1}].

RESULTS AND DISCUSSION

Hybrid establishment and genetic evaluation

In two years of cultivation about 1800 crosses were made using cut-style method; cross combinations which gave bulblets are listed in table 3. Then 1000 ovaries were placed *in vitro* culture, after 50-60 days in ovary culture, about 8000 ovules were transferred. These ovules, maintained in the dark for 120-180 days of culture at 22-23 °C, produced approximately 60 new individuals (Table 3). The cut-style method was effective for overcoming pre-zygote barriers, while through *in vitro* ovaries and ovules cultures post-zygotic barriers could be solved. The best time for culturing ovaries removed by mother plants is between 7 and 14 days after pollination.

For an early identification of putative hybrids obtained by *in vitro embryo rescue* the application of polymorphic RAPD and SSR markers is necessary. For RAPD analysis, primers OPA11, OPA16, OPB04 and OPB05 were polymorphic and produced the best results. They allowed the identification of more polymorphisms in the parental amplification profile, with typical bands of the male and female parents. Figure 3 shows an example of electrophoretic profile with two parents and two hybrids; in the putative hybrids there are typical bands of both parents and some inherited only by the male parent. As regards the SSR analysis between tested primers only some (LpCA5 , LpGA210 and LpGA4) showed polymorphisms. In particular Fig. 4 shows the amplification profiles obtained with two different primer pairs; in both cases it is possible to identify bands typical of each parent present in the hybrids.

Micropropagation in bioreactor

Shoot length, fresh weight of each shoot and total fresh weight, and growth index were higher in continuous immersion with respect to temporary immersion (Table 4). No differences were observed for dry matter, presence of malformations and hyperhydricity (data not shown) (Lian *et al.*, 2003). Shoot diameter and rooting process were better in temporary immersion system. The better shoot performances in the continuous immersion bioreactor is linked to the fact that in continuous culture the plant tissues are continuously submerged permitting a maximum nutrient and hormones uptake which improved the growth of the plantlets (Lian *et al*., 2003; Yan *et al.,* 2010). Similar results were reported for *Spathiphyllum cannifolium* (Dewir *et al*., 2006), *Alocasia amazonica* (Jo *et al.*, 2008), *Allium sativum* (Kim *et al.*, 2004), Chrysanthemum (Hahn and Paek, 2005), *Oncidium* (Yang *et al*., 2010). All shoots survived after *ex vitro* transplantation under greenhouse conditions.

CONCLUSIONS

Proper identification of accession is a very important tool in genetic resources management. The results obtained in this project show the importance of biotechnological methods application in *Lilium* breeding. RAPD and SSR application in hybrid identification demonstrate to be a reliable and valid tool for an early identification of new hybrids during the selection process within the breeding program. Moreover also the application of continuous immersion bioreactors for the micropropagation of *Lilium* is a viable and efficient model for automation and large-scale production of quality shoots.

These methods could enlarge the varietal assortment and at the same time introduce new material interesting for the Italian environment.

ACKNOWLEDGEMENTS

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Tables

Table 1. Culture media used in ovary and ovule culture and for new embryos multiplication .

Media	Sugar	Hormons	Reference
	Ovary culture		
$IAA_{0.5}$	6 %	Zeatina 2.88 µM BA 0.22 µM IAA 2.85 µM	Fukai and Tsuji, 2004; Van Creij *et al.*, 2000
NAA_1	10 %	NAA 5.37 µM	
	Ovule culture		
$NAA_{0.1}$	5 %	NAA 0.53 µM	Nesi, 2008
$IBA_{0.5}$	3 %	IBA 2.46 µM	
	Multiplication and enlargement		
$IBA_{0,5}$	3 %	IBA 2.46 µM	Nesi *et al.*, 2011
MS0	3 %	-	

Table 2. SSR analysis: primers tested, annealing temperature and size of the amplified fragment.

Locus	Temp. (°C)	Fragment size (bp)	Reference
LpCA20104	63	178 – 242	Horning *et al.*, 2003
LpCA5	53	144 – 202	
LpGA9	65	73 – 97	
LpGA210	55	166 – 196	
LpGA4	63	191 – 289	
LpCA870	55	160 – 236	
Lja16T	54	153 – 190	Kawase *et al.*, 2010
Lja10A	54	158 – 178	
Lja13A	54	76 – 120	

Table 3. New material obtained from ovary and ovule culture.

Cross combination	Bulblets obtained	Ovary culture media	Total
♀ All is all x ♂W. Triumph O x LO	8	$IAA_{0.5}$	15
	7	NAA_1	
♀ All is all x ♂W. Triumph (red-throat flowers) O x LO	3	$IAA_{0.5}$	3
♀ All is all x ♂W. Haeven O x L	9	$IAA_{0.5}$	15
	6	NAA_1	
♀ W. Triumph x ♂ All is all LO x O	1	$IAA_{0.5}$	1
♀ Gironde x ♂ All is all A x O	1	NAA_1	1
♀ Mother's choice x ♂ Gironde O x A	1	$IAA_{0.5}$	2
	1	NAA_1	
♀ Robina x ♂ Original Love OT x LA	2	NAA_1	2
♀ 409 (A) x ♂ White Haeven A x L	1	$IAA_{0.5}$	1
♀ Nova Zembla x ♂W. Haeven O x L	2	$IAA_{0.5}$	3
	1	NAA_1	
♀ Nova Zembla x ♂W. Triumph O x LO	1	$IAA_{0.5}$	1
Cross combination using wild species			
♀ All is all (O) x ♂ Regale	3	$IAA_{0.5}$	4
	1	NAA_1	
♀ All is all (O) x ♂ Candidum	2	$IAA_{0.5}$	2
♀ 00149 (A) x ♂ Candidum	1	$IAA_{0.5}$	1
♀ Hyde Park (LA) x ♂ Candidum	8	$IAA_{0.5}$	9
	1	NAA_1	
♀ Brindisi (LA) x ♂ Candidum	1	$IAA_{0.5}$	1
♀ Robina (OT) x ♂ Candidum	2	$IAA_{0.5}$	2
♀ 409 (A) x ♂ Regale	1	$IAA_{0.5}$	1
♀ Nova Zembla (O) x ♂ Pink perfection	1	NAA_1	1
			65

Table 4. Effect of culture method on morphological traits of *Lilium* shoots obtained after four weeks in bioreactor. Different letters in a row indicate significant differences at p = 0.05 (Duncan's test).

Parameters	Bioreactor system	
	Continuous immersion	Temporary immersion
Shoots explant $^{-1}$(n.)	2.5 a	2.0 ab
Shoot length (cm)	15.0 a	9.5 b
Shoot diameter (cm)	1.0 b	1.3 a
Total fresh weight (g)	131.1 a	102.7 b
Shoot fresh weight (g)	5.1 a	3.9 b
Growth Index	12.0 a	9.3 b
Dry matter (%)	12.3 ns	11.1 ns
Malformations (%)	1.3 ns	2.5 ns
Roots shoot $^{-1}$ (n.)	1.8 b	5.3 a

Figures

Fig. 1. Images of some cultivars and wild species involved in the breeding program: a) 'Hyde Park', b) 'All is All', c) 'Robina, d) *L. Regale*, e) 'Gironde', f) 'White Heaven', g) 'White Triumph' (red throat), h) 'White Triumph', i) 'Pink Perfection', j) 'Brindisi', k) '409', l) '00149'.

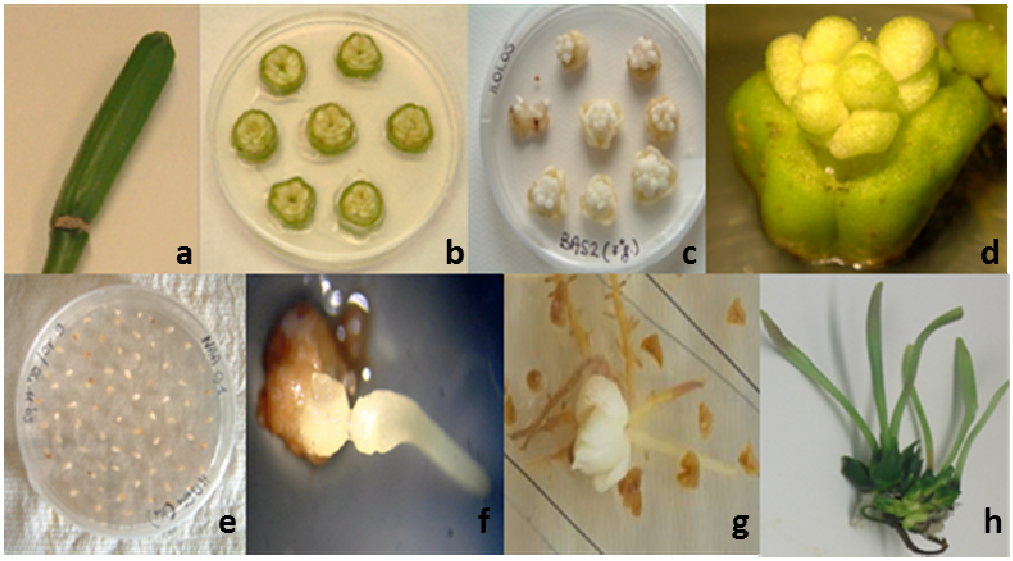

Fig. 2. Steps of *in vitro* rescue method for overcoming post-zygotic barriers: enlarged ovary (a); ovary slices in a petri dish (b); ovary slices with swollen ovules [(c) and (d)]; ovules culture (e); new bulblets from ovule culture at different growth stages [(f), (g) and (h)].

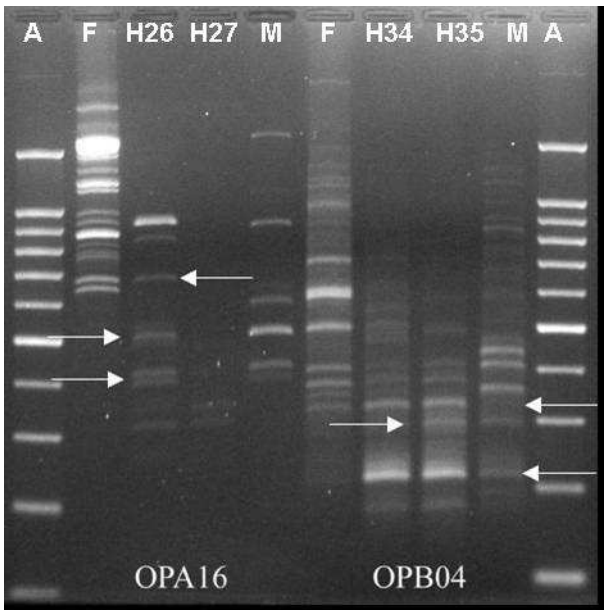

Fig. 3. RAPD analysis: amplification profiles using OPA16 (F= female parent; H26 and H27= hybrids; M= male parent, A = marker) and OPB04 primer (F= female parent; H34 and H35= hybrids; M= male parent, A = marker).

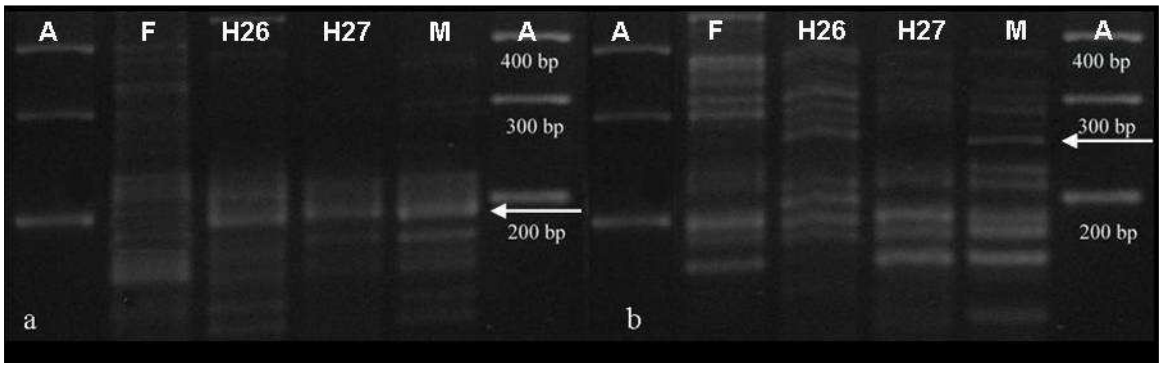

Fig. 4. SSR analysis: amplification profiles using LpGA210 (a) and LpGA4 (b) primer (F= female parent; H26 and H27= hybrids; M= male parent, A = marker).