THE ESTABLISHMENT OF SUSPENSION AND MERISTEM CULTURES FOR THE DEVELOPMENT OF A PROTOPLAST REGENERATION AND FUSION SYSTEM IN LILY

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Abstract

The present results indicate that established morphogenic suspension cultures can be obtained from crosses between cultivars of *L. longiflorum* and from a cross between Asiatic hybrid 'Orlito' x 'Connecticut King'. Meristem cultures were obtained from *L. longiflorum* 'Gelria' and Oriental hybrid 'Star Gazer'. Viable protoplasts can be isolated from these suspension and meristem cultures. The effect of different source tissues and of the culture conditions of the source tissues, on the plating efficiency and regeneration ability of protoplasts, are currently being analysed.

1. Introduction

The objectives of the present study are to develop a protoplast regeneration system in lily and to produce somatic hybrids through protoplast fusion between Oriental and Asiatic hybrids. To obtain plant material for the isolation of regenerable protoplasts, two different approaches are used: 1) the initiation of suspension cultures from callus induced on bulb scales or embryos and 2) the initiation of meristem cultures from vegetative apices.

2. Materials and methods

2.1. Plant material

Lilium species, hybrids and cultivars used in the present study, were obtained from the CPRO-species and cultivar collection (table 1).

2.2. Callus induction on medium with 2,4-D, picloram and dicamba

Mature embryos obtained from crosses of *L. longiflorum* 'Gelria' x 'Snow Queen', Asiatic hybrid 'Connecticut King' x 'Orlito' and Oriental hybrid 'Star Gazer' x 'Le Rêve' were cultured on N6 medium (Chu et al., 1975) with 10 µM of 2.4-dichlorophenoxyacetic acid (2,4-D), picloram or dicamba. On average 115 embryos were incubated on these different media. Bulb scales were cut into segments, on average 4, of about 2mm and on average 25 scale segments from *in vitro* grown bulbs of 'Gelria' , *L. henryi*, 'Star Gazer' and 'Connecticut King' were incubated on MS medium (Murashige and Skoog, 1962) with 5 to 50 µM picloram or dicamba.

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2.3. Callus induction on MS medium with NAA and BAP

Immature seeds from *L. longiflorum* 'White American' x 'Snow Queen', *L. henryi* x *L. henryi*, Oriental hybrid 'Star Gazer' x 'Capitol' and Asiatic hybrid 'Orlito' x 'Connecticut King' were cultured on MS medium with 2.75 μM 1-naphtylacetic acid (NAA). On average 444 immature seeds were incubated on this medium. On average 80 bulb scale segments from *in vitro* grown bulbs of *L. henryi*, 'Star Gazer' and 'Connecticut King', were incubated on MS media with a range of 2.2 to 22 μM 6-benzylaminopurine (BAP) and a range of 2.7 to 27 μM NAA. In preliminary experiments callus induction on bulb scale segments from *L. longiflorum* 'Gelria' was observed at lower concentrations of NAA and BAP. Bulb scale segments from 'Gelria' were therefore incubated on MS medium with a lower range of BAP and NAA: i.e. 0.4 to 8.9 μM BAP and 2.7 to 10.7 μM NAA

2.4. Initiation of regeneration from suspension cultures

On average 14 calli of about 3x3 mm large, from a *L. x formolongi* suspension culture, were incubated on solid MS medium with 0.3 to 67 g/l sucrose and 0.44 μM BAP (table 6). Calli were cultured under light conditions at 24°C and initiation of regeneration was scored after 4 weeks of culture. The suspension culture was kindly provided by Mii et al. (1994). Calli from suspension cultures of *L. longiflorum* 'Gelria' x 'Snow Queen' were cultured under the same conditions for initiation of regeneration. Calli from other suspension cultures were incubated on MS medium with l0g/l sucrose and 0.44 μM BAP, and were cultured as described.

2.5. Initiation of meristem cultures

Apices from *in vitro* cultured bulbs of Oriental hybrid 'Star Gazer', Asiatic hybrid 'Connecticut King' and *L. longiflorum* 'Gelria', were cultured separately in small tubes with liquid MS media with 1.1 to 4.4 μM BAP. The tubes, five in total for each culture medium, were placed on a slowly rotating wheel (2 rpm), and cultured under light conditions at 24°C (Sugiura, 1993).

2.6. Isolation of protoplast

Protoplasts were isolated from suspension cultures according to Mii et al. (1994). Protoplasts were cultured in liquid KM medium (Kao and Michayluk, 1975) or were embedded in alginate layers, floating in liquid medium.

3. Results

3.1. Initiation of suspension cultures from callus induced on medium with 2,4-D, picloram or dicamba

The best results with respect to callus induction were observed on N6 medium with picloram and dicamba. In addition an effect of genotype was observed: the highest frequencies of callus induction were observed on embryos from *L. longiflorum* 'Gelria' x 'Snow Queen' (table 2). Sustained callus growth was observed at a concentration of 1 to 5 μM picloram. Callus cultures obtained from calli of some seedlings of *L. longiflorum* 'Gelria' x 'Snow Queen', grew faster then those obtained from other crosses and suspension cultures could be established only from this cross.

Callus cultures were also obtained from bulb scale segments of different cultivars. The highest frequencies of callus induction were observed on medium with 10 μM picloram (table 3). Although frequencies of callus induction were rather high, callus growth itself was rather limited and a suspension culture could be obtained only from 'Gelria'.

3.2. Initiation of suspension cultures from callus induced on medium with NAA and BAP

To obtain suspension cultures from Asiatic and Oriental hybrids medium with NAA and with or without BAP was used to induce callus and to initiate suspension cultures.

3.2.1. Embryos

An effect of genotype was observed with respect to callus induction on embryos from different crosses: the highest frequencies of callus induction were observed on embryos from *L. longiflorum* 'White American' x 'Snow Queen' and from *L. henryi* x *L. henryi* (table 4). Callus cultures obtained from *L. longiflorum* 'White American' x 'Snow Queen', grew faster than those obtained from other crosses. Suspension cultures could be established with callus obtained from this cross and with callus obtained from the cross Asiatic hybrid 'Orlito' x 'Connecticut King'.

3.2.2. Bulb scales

Frequencies of callus induction observed on bulb scales from 'Gelria', *L. henryi,* 'Star Gazer' and 'Connecticut King' are presented in table 5. Frequencies of callus induction and growth rate on scale segments of 'Gelria' were highest on MS medium with 5.4 μM NAA and 2.2 μM BAP (table 5). Higher concentrations of BAP reduced the growth rate of the calli. Root formation was observed on medium with higher concentrations of NAA and the lowest concentration of BAP. MS medium with 5.4 μM NAA en 2.2 μM BAP was most suitable for sustained callus growth. Callus induction was very limited on scale segments of Asiatic hybrid 'Connecticut King'; a lower range of hormone concentrations should be tested for callus induction. The highest frequencies of callus induction on scale segments from Oriental hybrid 'Star Gazer' and *L. henryi*, were observed on MS medium with 5.4 to 27 μM NAA and 2.2 to 4.4 μM BAP (table 5). BAP reduced the growth rate of calli at higher concentrations. NAA induced root formation at higher concentrations. Callus growth on scale segments from these cultivars, was less important in comparison with callus growth on scales from 'Gelria'. MS medium with 5.4 μM NAA and 2.2 to 4.4 μM BAP was most suitable for sustained callus growth. In comparison to medium with NAA and BAP, the amount of callus formation was less important on medium with 10 μM picloram. However on medium with picloram higher frequencies of callus induction were observed: i.e. frequencies of 83, 76.2, 16 and 63% were observed on bulb scale segments of 'Gelria', *L. henryi*, 'Connecticut King' and 'Star Gazer' respectively.

Suspension cultures were initiated from callus cultures obtained from embryos and bulb scales. Suspension cell lines were obtained from *L. longiflorum* 'White American' x 'Snow Queen' and from Asiatic hybrid 'Orlito' x 'Connecticut King' and are subcultured in MS medium with 2.7 μM NAA. Suspension cultures derived from bulb scales are subcultured in MS medium with 5.4 μM NAA and 2.2 to 4.4 μM BAP. Suspension cultures obtained from embryo derived callus are growing faster and can be maintained for longer periods then suspension cultures obtained from bulb scale derived callus.

3.3. Initiation of regeneration from suspension cultures

The concentration of sucrose in the culture medium affected the efficiency of regeneration from callus of a suspension culture of *L. x formolongi*. The highest number of shoots was observed on medium with 3.3 to 10 g/l sucrose (Table 6). Enhanced efficiencies of regeneration were also observed, under equal culture conditions, for long-term established suspension cultures of *L. longiflorum* 'Gelria' x 'Snow Queen'. The regeneration ability of suspension cultures from different origins, is routinely tested on MS medium with 10 g/l sucrose and 0.44 μM BAP.

3.4. Initiation of meristem cultures

Vegetative apices developed slowly into callus-like tissues on which initiation of regeneration was frequently observed. After ±2 to 2.5 months of culture, 85% of the vegetative apices from 'Star Gazer' and 75% of the apices from 'Gelria', developed into meristematic callus clumps in MS medium with 1.1 to 3.3 μM BAP; only 20% of the vegetative apices from 'Connecticut King' developed meristematic tissues on medium with the 1.1 μM BAP.

3.5. Isolation of protoplasts from suspension and meristem cultures

Protoplasts were isolated from suspension cultures, 5 in total, initiated with callus induced on embryos from *L. longiflorum* 'Gelria' x 'Snow Queen'. These suspension cultures were able to regenerate shoots at a high frequency. Viable protoplasts were isolated from these suspension cultures. Viable protoplasts could also be isolated from meristem cultures of *L. longiflorum* 'Gelria' and Oriental hybrid 'Star Gazer'.

4. Discussion

Callus cultures could be obtained from all genotypes, from embryos and bulb scales. Qualitative differences were observed with respect to the frequency of callus induction and the growth of calli. A significant effect of genotype can be observed with respect to callus induction on embryos (tables 2 and 4). This effect is observed on culture media with different hormones: i.e. picloram or NAA. The highest frequencies of callus induction were thus observed on embryos obtained from crosses with cultivars of *L. longiflorum*. High frequencies of callus induction were also observed on embryos from the related cross *L. x formolongi* (Mii et al. 1994). This indicates that *L. x formolongi* and *L. longiflorum* are possibly more responsive with respect to callus induction than genotypes from the Asiatic and Oriental hybrids.

The best results with regard to callus induction were obtained with picloram and NAA with or without BAP. Enhanced callus growth was yet observed on medium with lower concentrations of picloram or NAA and BAP. Dicamba appeared to be rather toxic within the concentration range used in the present study. A lower range of hormone concentrations should be tried out to determine the optimal concentration for callus induction and sustained callus growth. Finally, 2,4-D appeared to be the least effective growth factor with regard to callus induction on embryos. Suspension cultures were obtained from crosses between cultivars of *L. longiflorum* and from the cross Asiatic hybrid 'Orlito' x 'Connecticut King'. The regeneration efficiency of established suspension cultures was enhanced by reducing the sucrose concentration of the culture medium. Optimization of medium and culture conditions would most probably further enhance the efficiency of regeneration from callus. This could be very useful to develop an

efficient regeneration system for protoplast derived microcalli and/or a procedure for vegetative propagation of a specific cultivar or hybrid.

Meristem cultures, initiated as described by Sugiura (1993), were obtained from Oriental hybrid 'Star Gazer' and *L. longiflorum* 'Gelria'. The Oriental hybrid 'Star Gazer' appeared to be more responsive for the induction of a meristematic type of callus, than 'Gelria' and 'Connecticut King'. A lower range of BAP concentrations should be tested to determine the optimal concentration for the initiation of meristem cultures from 'Connecticut King'. Sugiura (1993) regenerated plants from protoplasts, isolated from meristem cultures of *L. speciorubel* and *L. x elegans*. The isolation of viable protoplasts from meristem cultures in the present study, indicates that these results are reproducible with respect to protoplast isolation. Meristem cultures can thus be used as an alternative to suspension cultures for the development of a protoplast regeneration system.

To reach the final objectives of the present study, i.e. the production of somatic hybrids between Oriental and Asiatic hybrids, source tissues of different origins are available for the isolation and fusion of protoplasts: i.e. suspension cultures of Asiatic hybrid 'Orlito' x 'Connecticut King' and meristem cultures from Oriental hybrid 'Star Gazer'. The culture conditions of different source tissues and the culture conditions of protoplasts are currently being studied for their influence on the plating efficiency and regeneration ability of protoplasts.

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Table 1 - Lilium species, hybrids and cultivars used in the present study.

Name	Type
L. longiflorum	Cultivars: 'Gelria', 'Snow Queen', 'White American'
L. henryi	Species: accessions with CPRO- number 72202 and 72113-6;
L. x formolongi	Hybrid, cultivar 'Azuza'
'Star Gazer'	Oriental hybrid
'Capitol'	Oriental hybrid
'Le Rêve'	Oriental hybrid
'Connecticut King'	Asiatic hybrid
'Orlito'	Asiatic hybrid

Table 2 - The frequency (%) of callus induction on embryos from different crosses, cultured on N6 medium with 10 μM of 2,4-D, picloram or dicamba. Within each column, values are significantly different, unless marked with a common letter (chi-square test, $P < 0.001$).

Crosses	2,4-D	Picloram	Dicamba
L. longiflorum			
'Gelria' x 'Snow Queen'	6.7	98.5	96[a]
'Conn. King' x 'Orlito'	0	56.7	78.7[a]
'Star Gazer' x 'Le Rêve'	0	20	6.7

Table 3 - The effect of the growth regulators dicamba and picloram on the frequency (%) of callus induction on bulb scale slices from different cultivars.

Cultivars and species	Dicamba (μM)			Picloram (μM)		
	5	10	50	5	10	50
'Gelria'	88	70	63	100	80	100
L. henryi	46	25	15	46	61	38
'Connecticut King'	29	5	0	19	29	4
'Star Gazer'	13	8	3	52	65	46

Table 4 - The frequency (%) of callus induction observed on embryos from different crosses, cultured on MS medium with 2.75μM NAA. Values are significantly different unless marked with a common letter (chi-square test, $P < 0.01$).

Crosses				Frequency of callus induction
L. longiflorum	'White American'	x	'Snow Queen'	12.8[a]
L. henryi 72202	x 72113-6			11.6[a]
Asiatic hybrid	'Connecticut King'	x	'Orlito'	7.6[b]
Oriental hybrid	'Star Gazer'	x	'Capitol'	6.5[b]

Table 5 - The frequency (%) of callus induction on scale segments from 'Gelria', L. henryi, 'Connecticut King' and 'Star Gazer', cultured on MS medium with NAA and BAP. Callus formation is described as follows: +++, ++, +: more than 2/3, 1/3 or less than 1/3 of the calli are larger than 2.5mm².

BAP (μM)	NAA (μM)		
	2.7	5.4	10.7
'Gelria'			
0.4	59/++	59/+++	56/+++
2.2	49/+++	68/+++	51/++
4.4	34/++	53/++	28/++
8.9	25/++	13/++	18/++
	5.4	16.2	27
L. henryi			
2.2	36.0/+++	10.0/+	17.5/++
4.4	13.8/++	25.0/++	18.8/++
13.2	10.0/+	3.8/++	2.5/++
22	2.5/0	0.0/0	0.0/0
'Connecticut King'.			
2.2	4/+	0/0	0/0
4.4	0/0	0/0	0/0
13.2	0/0	0/0	0/0
22	0/0	0/0	0/0
'Star Gazer'.			
2.2	55/+	67/++	61/++
4.4	50/+	46/+	54/++
13.2	19/+	11/+	34/+
22	11/+	14/++	18/++

Table 6 - The effect of the sucrose concentration on the regeneration efficiency of callus from L. x formolongi. Within each column values are significantly different, unless marked with a common letter (chi-square test, $P < 0.001$).

Sucrose concentration	Number of shoots		
	total	per mm²	per clump
g/l	number	callus	of callus
67.0	26[a]	0.013	1.9[a]
33.0	41[a]	0.033	2.7[a]
10.0	88[b]	0.14	5.2[b]
3.3	73[b]	0.27	5.2[b]
0.3	0[c]	0.	0.0[c]