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Development of *in vitro* propagation protocol and *gus* expression studies on Asiatic lily hybrids (*Lilium* spp.)

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ABSTRACT

***In vitro* propagation experiments were carried out to study the effect of media, genotype and explant on micropropagation in three cultivars (Prato, Brunello and Dreamland) of Asiatic *Lilium* hybrids MS basal medium supplemented with 1 mg/l NAA induced best culture establishment and bulblet multiplication in all the three cultivars of Asiatic hybrid of *Lilium*. Best rooting of *in vitro* derived bulblets was recorded in modified MS medium supplemented with 1 mg/l NAA and 90 g/1sucrose. The potting mixture of vermiculite + FYM (1:1) proved to be the best for transplantation survival of plantlets. *Gus* expression varied between 20.2-24.9% in these three *Lilium* cultivars. The surviving explant tissues failed to exhibit the *gus* gene expression four weeks after transfer and all of the explant tissues became brown and died by four weeks after transfer, presumably due to minimal integration of T-DNA into the *Lilium* genome.**

Key words: Bulblet multiplication, *Lilium* spp. micropropagation.

INTRODUCTION

Lilium is one of the 220 genera belonging to the family Liliaceae and comprises about 85 species, including many beautiful ornamental species. Asiatic hybrid lily is one of the important bulbous plants grown worldwide, derived from interspecific hybridization of species of *Lilium* of the section Sinomartagan. Lily, tulip and freesia are the three most important bulbous crops in the commercial market, representing 24% of total flower production (Beattie and White, 2).

Micropropagation of lily is becoming important owing to the advantages of increased multiplication rates and production of plant material free of viruses and other pathogens (Kim *et al*., 7). Tissue culture technique for rapid propagation of *Lilium speciosum* Thunb. var. *glorisoides* Baker has been reported by Cheng *et al*. (3). However, very little work has been done for rapid propagation of Asiatic lily. Taking this into account work has been planned for *in vitro* propagation of Asiatic lily. In this communication, we report the development of efficient protocol for micro propagation and possibility of *Agrobacterium*-mediated genetic transformation in three Asiatic *Lilium* hybrids.

MATERIALS AND METHODS

Bulb scale bases were excised from healthy disease free bulbs. The basal portions of scales were dissected into 4-6 mm size pieces and used as explants. Bulb scale base explants were washed under running tap water for 30 min., agitated in tap water with a few drops of detergent Teepol™ (1%) for 10 min. followed by a treatment with bavistin (0.1%) + dithane M-45 (0.25%) for 10 min. The explants were then rinsed in distilled water and treated with 70% ethanol for 30-60 sec and finally washed with sterile distilled water. The explants were then treated with 0.1% (w/v) aqueous mercuric chloride ($HgCl_2$) solution for 4-9 min. and rinsed 4-5 times with sterilized distilled water. The explants were inoculated on to the culture medium (Murashige and Skoog, 11) containing different concentration of growth regulators and incubated in the culture room at 25 ± 2ºC and 16 h illumination (1400 lux provided by fluorescent tube lights). Observations were recorded on number of days taken to bulblet multiplication, number of bulblets formed per explant, number of roots per bulblet, length of longest root (cm) and percent rooted bulblets. Regenerated bulblets were hardened and transferred to pot.

In the present study, the conditions were optimized for *Agrobacterium*-mediated genetic transformation in three cultivars of Asiatic hybrids of *Lilium*, *i.e.*, Prato, Bunello and Dreamland. The presence of the transgenes was confirmed by *gus* analysis. Vector pTOK233 used in this study, has already been proved ideal for transformation (Suzuki and Nakano, 15). *Agrobacterium tumefaciens* strain LBA 4404 harbouring plasmid T0K233 was used. Plasmid T0K233 contained *Gus* as reporter gene and *Hygr* as selectable marker gene. The *in vitro* raised bulb scales were co-cultivated with *A. tumefaciens*

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LBA 4404 strain harbouring plasmid T0K233. In the present study, a modified MS medium containing acetosyringone, 1 mg/l NAA with acidic pH (5.2) was used for cultivation of bulb scale explants tissues with *Agrobacterium* strain at 28°C. The expression of *Gus* was detected at two stages: (a) three days after co-cultivation and (b) three weeks after co-cultivation.

RESULTS AND DISCUSSION

The cultivar Prato took minimum days (26.6 days) to bulblet multiplication as compared to Brunello (40.0) and Dreamland (42.0) cultivars (Table 1). The MS basal medium supplemented with 1 mg/l NAA or 0.5 mg/l NAA resulted in an earlier response and multiplication of bulblets. This might be due to the fact that NAA promotes formation of adventitious buds in excised organs and tissues. The results are in conformity with the reports by various workers (Aartrijk and Blom Barnhoorn, 1). Significantly, higher (4.42) number of bulblets per explant were recorded in cultivar, Prato, compared to other cultivars, Brunello (3.44) and Dreamland (3.11) (Table 1). The difference in the number of days taken for bulblet multiplication and number of bulblets formed per explant among

Table 1. Effect of medium on number of days taken to bulblet multiplication and number of bulblets formed per bulb in bulb scale base explant of Asiatic *Lilium* hybrids.

Treatment (mg/l)		No. of days taken to bulblet multiplication				No. of bulblets formed per bulb			
NAA	BAP	Cultivar				Cultivar			
		Prato	Brunello	Dreamland	Mean	Prato	Brunello	Dreamland	Mean
-	-	31.00	44.93	48.27	41.40	3.13	2.20	2.20	2.51
0.1	-	25.13	38.67	40.20	34.67	4.00	3.53	3.33	3.62
0.5	-	21.80	35.13	37.07	31.33	5.13	4.33	3.87	4.44
1.0	-	20.87	35.00	35.80	30.56	6.00	4.40	4.00	4.80
1.5	-	24.40	37.33	39.87	33.87	4.07	3.67	3.53	3.76
-	0.05	26.53	39.93	41.00	35.82	4.07	3.00	2.67	3.24
-	0.1	26.33	39.80	40.93	35.69	4.00	3.00	2.53	3.18
-	0.5	26.00	39.27	40.53	35.27	3.93	2.93	2.40	3.09
-	1.0	26.00	39.20	40.40	35.20	3.60	2.73	2.33	2.89
0.1	0.05	29.00	42.20	45.00	38.73	4.73	3.40	3.33	3.82
0.1	0.1	28.67	42.00	44.80	38.49	4.53	3.33	3.00	3.62
0.1	0.5	28.47	41.33	43.27	37.69	4.40	3.27	2.87	3.51
0.1	1.0	28.40	41.20	43.00	37.53	4.07	3.07	2.73	3.29
0.5	0.05	23.27	36.00	38.00	32.42	5.53	4.33	3.80	4.56
0.5	0.1	24.93	37.80	40.00	34.24	5.27	4.27	3.80	4.44
0.5	0.5	27.07	40.13	41.27	36.16	4.60	4.00	3.67	4.09
0.5	1.0	27.33	40.33	41.60	36.42	4.33	4.00	3.60	3.98
1.0	0.05	21.67	35.13	37.20	31.33	6.00	4.40	3.93	4.78
1.0	0.1	24.00	37.00	39.00	33.33	5.87	4.40	3.93	4.73
1.0	0.5	27.80	40.73	42.40	36.98	5.07	4.00	3.73	4.27
1.0	1.0	27.93	40.87	42.67	37.16	4.73	3.87	3.67	4.09
1.5	0.05	29.00	43.27	46.07	39.44	3.53	2.67	2.33	2.84
1.5	0.1	29.40	44.20	46.67	40.09	3.47	2.00	2.27	2.78
1.5	0.5	29.67	44.73	47.87	40.76	3.33	2.40	2.20	2.64
1.5	1.0	30.27	44.80	48.00	41.02	3.20	2.40	2.20	2.60
Mean		26.59	40.04	42.03		4.42	3.44	3.11	
CD at 5%	Medium	: 0.10				Medium	: 0.09		
	Cultivar	: 0.04				Cultivar	: 0.03		
	Medium × Cultivar	: 0.17				Medium × Cultivar	: 0.16		

three cultivars might be due to the difference in their genetic makeup. The maximum numbers of bulblets per explant were observed in MS basal medium supplemented with NAA (1 mg/l) or NAA (1 mg/l) + BAP (0.05 mg/l). Nilmi (12) reported NAA was essential for the formation and growth of bulblets in scale culture and found cytokinins to be less responsive growth regulators for *in vitro* propagation of *Lilium*.

All three cultivars showed 60-70 per cent rooting of bulblets in modified MS medium supplemented with 1 mg/l NAA (Table 2). However, with the decrease in the level of NAA, the per cent rooting of bulblets decreased and varied among the three cultivars. This might be due to the genetic constitution of the cultivars. These results are in conformity with the results obtained by (Maesato *et al*., 8; Dilta *et al*., 4). Modified MS medium supplemented with 1 mg/l NAA earliest (27.73 days) root formation in Asiatic hybrids of *Lilium* (Table 2). The cv. Prato took significantly lowest (28.30) number of days as compared to cv. Brunello (31.48) and cultivar Dreamland (31.84). The difference in the number of days to root formation might be due to genetic factors. The MS medium supplemented with 1 mg/l NAA took minimum number of days to root formation. This might be due to the fact that NAA is a root promoting hormone. Priyadarshi and Sen (13) obtained best rooting in MS medium containing 5.37 mM NAA. Mizuguchi *et at*. (10) obtained early rooting in MS medium supplemented with 1 mg/l NAA in *L. japonicum*.

Maximum (4.25) number of roots was resulted in modified MS medium supplemented with 1 mg/l NAA in Asiatic *Lilium* hybrids (Table 3). The cv. Prato resulted in maximum (3.54) number of roots, which was at par with cv. Brunello (3.50) and both were better than the cv. Dreamland (3.01). The significantly higher number of roots per bulblet was formed in modified MS medium supplemented with 1 mg/l NAA. This might be due to the fact that NAA promotes bud formation and growth promotory role of auxins in *Lilium*. These results are in accordance with the observation made by Nilmi (12) in *L. rubellum* and Maesato *et al*. (8) in *L. japonicum*. The cv. Prato resulted in the maximum root length (2.53 cm) of bulblet, significantly better than the other two cvs Brunello (2.06 cm) and Dreamland (1.98 cm) (Table 3). The significantly higher (2.93 cm) root length was obtained in cv. Prato in modified MS medium supplemented with 0.6 mg/l NAA better than the cv. Dreamland (2.47 cm) and Dreamland (2.40 cm) in modified MS medium supplemented with 0.8 mg/l NAA. This might also be due to the varietal differences in expression of response. The highest (2.58 cm) root length was obtained in bulblets of Asiatic hybrids of *Lilium* in modified MS medium

Table 2. Effect of rooting medium on per cent bulblets forming root and average number of days taken for root formation in Asiatic *Lilium* hybrids.

Medium	Percent bulblets forming root				Average no. of days taken for root formation			
	Cultivar				Cultivar			
	Prato	Brunello	Dreamland	Mean	Prato	Brunello	Dreamland	Mean
MS +90 g/l sucrose + 0.00 mg/l NAA	-	-	-	-	-	-	-	-
MS + 90 g/l sucrose + 0.2 mg/l NAA	66.67 (54.75)	50.00 (45.00)	40.00 (39.23)	52.22 (46.33)	32.47	34.80	35.20	34.16
MS + 90 g/l sucrose + 0.4 mg/l NAA	80.00 (63.44)	68.33 (55.77)	56.67 (48.84)	68.33 (56.02)	29.80	33.00	33.00	31.93
MS + 90 g/l. sucrose + 0.6 mg/l NAA	88.33 (70.11)	76.67 (61.15)	71.67 (57.86)	78.89 (63.04)	27.73	31.07	32.00	30.27
MS + 90 g/l sucrose + 0.8 mg/l NAA	98.33 (85.69)	93.33 (75.24)	90.00 (71.56)	93.88 (77.50)	25.93	29.87	30.07	28.620
MS + 90 g/l sucrose + 1 mg/l NAA	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	25.60	28.67	28.93	27.73
Mean	72.22 (60.67)	64.72 (54.52)	59.72 (51.25)		28.30	31.48	31.84	
CD at 5%: Medium	: 2.12			Medium	: 0.08			
Cultivar	: 1.50			Cultivar	: 0.06			
Medium × cultivar	: 3.49			Medium × cultivar	: 0.14			

Values in parenthesis are Arc sine transformed values

Table 3. Effect of rooting media on number of roots formed per bulblet and average length of longest root (cm) in bulblets of Asiatic *Lilium* hybrids.

Medium	No. of roots formed per bulblet				No. of longest root (cm)			
	Cultivar				Cultivar			
	Prato	Brunello	Dreamland	Mean	Prato	Brunello	Dreamland	Mean
MS + 90 g/l sucrose + 0.00 mg/l NAA	-	-	-	-	-	-	-	-
MS + 90 g/l sucrose + 0.2 mg/l NAA	2.33	2.13	2.00	2.15	1.60	1.20	1.20	1.33
MS + 90 g/l sucrose + 0.4 mg/l NAA	3.13	3.13	2.60	2.95	2.40	1.87	1.80	2.02
MS + 90 g/l sucrose + 0.6 mg/l NAA	3.60	3.60	3.13	3.44	2.93	2.40	2.27	2.53
MS + 90 g/l sucrose + 0.8 mg/l NAA	4.20	4.20	3.53	3.98	2.87	2.47	2.40	2.58
MS + 90 g/l sucrose + 1 mg/l NAA	4.47	4.47	3.80	4.25	2.87	2.40	2.27	2.51
Mean	3.54	3.50	3.01		2.53	2.06	1.98	
CD at 5%: Medium : 0.08				Medium : 0.03				
Cultivar : 0.06				Cultivar : 0.03				
Medium × cultivar : 0.14				Medium × cultivar : 0.06				

- No response

supplemented with 0.8 mg/l NAA. These results are similar to the findings obtained by Kawarabayashi and Asahira (6), who reported the maximum number of roots and root length in MS medium supplemented with NAA in *L. speciosum* cultivar Uchida.

The cv. Prato showed the maximum (80.83%) survival of *in vitro* raised bulblets in pots in greenhouse and was followed by Brunello (74.17%) and Dreamland (71.67%). The significantly higher (91.11%) survival of bulblets was observed in vermiculite + FYM (1:1 v/v) medium due to the retention of optimum moisture with adequate aeration. Maximum (1.51) number of leaves emerged in cv. Prato, which was significantly higher than the cultivar Brunello (1.35) and cultivar Dreamland (1.23). Significantly higher (2.04) number of new leaves emerged in Asiatic hybrids of *Lilium* six weeks after transferring to pots containing vermiculite + FYM (1:1) potting media under greenhouse conditions (Table 4). The difference might be attributed to their genetic constitution. Optimum growth in terms of root, shoot and leaves of *in vitro* raised plantlets of banana cv. Udhyam was observed while cocopeat + vermiculite (1:1) was used as substrate in growth chamber as well as in net house (Saraswathi *et al*., 14). The difference might be due to the varietal character. Similarly, Kawarabayashi

Table 4. Effect of potting media on per cent survival of bulblets and number of new leaves in Asiatic *Lilium* hybrid six weeks after transplanting.

Potting mixture (equal v/v)	Per cent survival of bulblets				No. of new leaves formed per bulblet			
	Cultivar				Cultivar			
	Prato	Brunello	Dreamland	Mean	Prato	Brunello	Dreamland	Mean
Vermicultite + FYM + sand	73.33 (59.01)	66.67 (54.78)	63.33 (52.78)	67.78 (55.52)	1.27	1.13	1.00	1.13
Vermiculite + FYM	93.33 (77.71)	90.00 (71.56)	90.00 (71.56)	91.11 (73.61)	2.27	2.00	1.87	2.04
Vermiculite	90.00 (71.56)	83.33 (66.15)	83.33 (66.15)	85.55 (67.95)	1.80	1.67	1.53	1.67
Vermiculite + FYM + soil	66.67 (54.78)	56.67 (48.85)	50.00 (45.00)	57.78 (49.54)	0.73	0.60	0.53	0.62
Mean	80.83 (65.76)	74.17 (60.33)	71.67 (58.87)		1.51	1.35	1.23	
CD at 5%: Medium : 4.16				Medium : 0.09				
Cultivar : 3.60				Cultivar : 0.08				
Medium × cultivar : NS				Medium × cultivar : NS				

Values in parenthesis are Arc sine transformed values

Table 5. Effect of genotype on transient *Gus* expression Asiatic *Lilium* hybrids using *Agrobactrium* strain LBA 4404 (pTOK233).

Genotype	Plasmid	*Gus* expression after 3 days				*Gus* expression after 3 weeks			
		No. of explant used	Gus*	%	Mean	No. of explant used	Gus*	%	Mean
Prato	TOK 233	16	0	0.0		13	3	23.07	
		12	0	0.0	0.0	23	5	21.73	24.93
		15	0	0.0		10	3	30.00	
Brunello	TOK 233	12	0	0.0		12	3	25.00	
		20	0	0.0	0.0	7	1	14.28	20.23
		18	0	0.0		14	3	21.42	
Dreamland	TOK 233	14	0	0.0		16	4	25.00	
		15	0	0.0	0.0	12	2	16.67	21.58
		12	0	0.0		13	3	23.07	

and Asahira (6) recorded more number of leaves in *L. longiflorum* cv. Georgia and *L. speciosum* cv. Uchida transplanted in soil.

The transient *Gus* expression varied from 14.28 to 30.00% in different genotypes three weeks after co-cultivation. The maximum (24.93%) frequency of *Gus* expression was observed in Asiatic hybrid of *Lilium* cv. Prato followed by Dreamland (21.6%) and Brunello (20.2%) (Table 5). The explant tissues co-cultivated for four weeks were transferred and successfully sub-cultured onto the regeneration or selection medium containing 50 mg/l hygromycin and 250 mg/l cefotaxime. However, the surviving explant tissues failed to exhibit the *gus* gene expression four weeks after transfer and all of the explant tissues became brown and died by four weeks after transfer, presumably due to minimal integration of T-DNA into the *Lilium* genome. Similar results were obtained by Suzuki and Nakano (15). Thus, recalcitrance to plant regeneration might be the major hurdle for transfer of useful genes. It has also been reported that *Agrobacterium* strains differed in their ability to transform a particular plant species as well as in their host range is determined both by genes located in the bacterial chromosome and genes located in the *Vir*-region of the *Ti* plasmid (Hooykaas *et al*., 5). Mutations in these genes might alter their ability to transform certain plant species, yet have no effect on the infection of others (Melchers and Hooykaas, 9).

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