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An Improved Regeneration System of Oriental Lily Hybrid from Ovary-Ovule Culture Using Plant Growth Regulators

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ABSTRACT

The influence of plant growth regulators (PGRs) on bulblet regeneration from oriental hybrid lily 'Casa Blanca' ovary tissues was investigated. Pistils excised from unopened flower buds (5-8 cm long) were sectioned and cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of PGRs singly, or in combination. Bulblets were initiated on MS medium supplemented with 0.5 mg L^{-1} 6-benzyladenine (BA) or with 1.5 and 2 mg L^{-1} zeatin (Zea). The number of bulblets per explant and average fresh weight per bulblet were highest with 2 mg L^{-1} α-naphthaleneacetic acid (NAA) and 1.5 mg L^{-1} BA, whereas bulblet regeneration response varied with PGR treatment. Bulblets rooted with 0.5 mg L^{-1} NAA and were transferred to plastic pots containing cocopeat for hardening. Chromosome observations revealed that all regenerated bulblets tested were diploid (2n = 24).

Keywords: *in vitro* regeneration, oriental Lily 'Casa Blanca', explant-pistil, rooting bulblet, somaclonal uniformity
Abbreviations: **BA**, 6-benzyladenine; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **NAA**, α-naphthaleneacetic acid, **TDZ**, thidiazuron; **Zea**, zeatin

INTRODUCTION

Lilium is an economically important cut flower crop, ranking fourth among the flowers in popularity (Anon 2009). It has a wide applicability in the floral industry as a cut flower and potted plant (Xia *et al*. 2006). Lily comprises more than 80 species belonging to 7 sections. Within the section cultivars bred from Sinomartagon, Archelirion and Leucolirion are the most important in the commercial market (Lim *et al*. 2008). It is propagated vegetatively by above-ground bulbils and underground bulb scales (Kumar *et al*. 2006). The development of new cultivars with different flower colours would potentially increase the marketability of this crop. Numerous studies have been conducted on *in vitro* regeneration of bulblets in lily using different explants (Nhut 1998; Bacchetta *et al*. 2003; Nhut 2003; Kumar *et al*. 2006; Azadi and Khosh-Khui 2007; Xu *et al*. 2008). Lilies are generally propagated by scaling, a technique which produces 3-5 number of bulbs from each bulb scale, depending upon the species or cultivar used and bulb scale size (Stimart and Ascher 1978). Anther cultures have successfully been used for production of haploids in lilies (Han *et al*. 1997; Chu *et al*. 2001; Han *et al*. 2005). Ovary cultures have been used for plant regeneration from *L. formosanum* (Hayashi *et al*. 1986) and for production of interspecific hybrids between *L. longiflorum* and *L.* × *elegance* (Kanoh *et al*. 1988) and *L. concolor* and *L. longiflorum* (Fernández *et al*. 1998). Ovary cultures may also be used to obtain haploid plants which can be induced to homozygous double haploids. Fukai *et al*. (2005) obtained LLO hybrid lilies from *L. formolongi* and chromosome doubled *L. formosonum* var. *Pricei* × oriental hybrid 'Le Reve'. Diploids and mixoploids were reported from ovary culture in Easter lily (Ramsay *et al*. 2003). van Tuyl *et al*. (1991) studied the application of *in vitro* pollination, ovary and ovule culture and embryo rescue for over coming incongruity barriers in interspecific *Lilium* crosses. The objective of this investigation was to study the effect of plant growth regulators (PGRs) on regeneration of oriental hybrid lily cv. 'Casa Blanca' *via* ovary tissue cultures and to determine ploidy levels of regenerated plants.

MATERIALS AND METHODS

Preparation of plant material

Oriental hybrid lily cv. 'Casa Blanca' bulbs obtained from the Department of Floriculture and Landscaping, University of Horticulture and Forestry, Solan, India were cold treated at 2°C for 8 weeks and then planted and grown in 10-cm plastic pots containing a mixture of soil: sand: FYM (farm yard manure) in a glasshouse at 24 ± 2°C and 80% relative humidity. Unopened flower buds 5-8 cm long were excised and dipped in a 5% sodium hypochlorite solution for 10 min under aseptic conditions. Ovaries extracted from surface-sterilized buds were cut into two longitudinal sections.

In vitro culture conditions

Longitudinal sections of the ovary with the cut surface down and slightly embedded were cultured on MS (Murashige and Skoog 1962) medium supplemented with 8 g L^{-1} agar (w/v), 30 g L^{-1} (w/v) sucrose and different concentrations of PGRs (**Table 1**). Cultures without PGRs served as control. The explants were cultured in 100 mL Erlenmeyer flasks (Borosil, Mumbai, India) containing 30 mL of medium. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C at a pressure of 1.1 Kg cm^{-2} for 15 min. All the cultures were maintained in a room with controlled conditions of 24 ± 2°C under a 16-h photoperiod with a photosynthetic photon flux density (PPFD) of 50–60 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps (40W each, Philips). The cultures were transferred to fresh medium at 4-week intervals.

Data were recorded on explants producing bulblets, number of bublets $explant^{-1}$ and average fresh weight after 13 weeks of culture.

The bulblets formed *in vitro* were separated and transferred to rooting medium consisting of MS salts supplemented with 0.5 mg L^{-1} α-naphthalene acetic acid (NAA). After 4 weeks of culture, roots initiated, and these were used for subsequent chromosome analysis.

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Research Note

Hardening and acclimatization

In vitro bulblets regenerated from ovary culture were transferred to cocopeat contained in plastic pots (10 cm). Initially each pot was covered with polythene bags to retain a humid environment and kept in a glasshouse under light (50-60 µmol m^{-2} s^{-1}). Once the plants reached 10 cm in height, they were individually transferred to 10 cm plastic pots containing sand: soil: FYM mixed in a 1: 1: 1 ratio.

Karyotypic analysis

Root tips of regenerated plants were excised and prepared for chromosome observations through a series of steps following the method of Ramsay *et al*. (2003) with modifications. First, root tips were pre-treated with 0.2% colchicine (HiMedia Laboratories Pvt. Ltd., Mumbai, India) for 3 h at 4°C. Next, the root tips were fixed in 1 part glacial acetic acid: 3 parts ethanol for 24 h and rinsed 3-4 times with distilled water. The root tips were hydrolysed in 1 N HCl at 60°C for 10 min and rinsed three times with distilled water. Root tips were placed in Fuelgen's solution (HiMedia Laboratories) for 60 min, rinsed again and stained with acetocarmine (Ranbaxy Fine Chemicals Ltd., Mumbai, India) for microscopic observations of chromosomes. Chromosomes which were easily observed in cells were counted using light microscope (100× magnification). Metaphase chromosomes were counted in four root tip cells of each regenerated plant.

Statistical analysis

Each treatment consisted of 10 explants, and each experiment was repeated in triplicate. Data recorded for different parameters were subjected to completely randomized design (Gomez and Gomez 1984). The statistical analysis based on mean values per treatment was made using analysis of variance (ANOVA). The LSD multiple range test ($P \leq 0.05$) was used to determine differences between treatments.

RESULTS AND DISCUSSION

The explants failed to regenerate bulblets on PGR-free medium and on medium supplemented with 0.5 and 1 mg L^{-1} NAA or with 1 mg L^{-1} indole-3-butyric acid (IBA) alone (**Table 1; Fig. 1A**). Bulblets were initiated when MS medium was supplemented with 0.5 mg L^{-1} 6-benzyladenine (BA) or with 1.5 and 2 mg L^{-1} zeatin (Zea) (**Fig. 1B**). After 3 weeks, ovary explants swelled and bulblets began to form directly in the interior tissue of the ovary. Both concentrations of Zea were equally effective in producing bulblets. The auxins (NAA, IBA) failed to produce bulblets alone, but could form bulblets when used in combination with cytokinins (BA, thidiazuron (TDZ), Zea), indicating that a certain auxin-cytokinin balance is necessary for bulblet regeneration in ovary explant. Ramsay *et al*. (2003) reported plant regeneration in Easter lily ovary tissue with 1 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) and 2 mg L^{-1} BA. In the present study, 1 mg L^{-1} 2,4-D in combination with 1.5 mg L^{-1} BA gave 100% bulblet regeneration. The response decreased with an increase in 2,4-D and BA concentrations. Ramsay *et al*. (2003) observed that 50% of ovary explants formed bulblets in Easter lily on MS medium supplemented with 1 mg L^{-1} 2,4-D and 2 mg L^{-1} BA. Fernández *et al*. (1997) observed that 87.5% explants produced bulblets on N_6 medium supplemented with 2 mg L^{-1} 2,4-D. Gu and Cheng (1983) inoculated unpollinated young lily ovaries of *L. davidii* var. Willmottiae on modified MS medium and regenerated plantlets. Park *et al*. (2000) reportted formation of callus from the cut surface of ovary slices with different concentrations of 2,4-D and BA in lily cultivars 'Casa Blanca', 'Acapulo', 'Star Gazer' and 'Le-Reve'.

The number of bulbets per explant ranged from 1.30 to 4.16 depending upon the treatment. The highest number of bulblets (4.16) was observed with 2 mg L^{-1} NAA and 1.5 mg L^{-1} BA, which was statistically equal with 1.5 mg L^{-1} Zea (**Table 1**). The lowest number of bulblets was observed in a combination of 2 mg L^{-1} 2,4-D and 2 mg L^{-1} BA. Ramsay *et al*. (1994, 1995) obtained maximum shoot differentiation from ovary callus on MS medium supplemented with 5% sucrose, 1 mg L^{-1} 2,4-D and 2 mg L^{-1} BA in *L. longiflorum* 'Ace' and 'Nellie White'. Tribulato *et al*. (1997) regenerated plants from somatic embryos through ovary explants on MS medium supplemented with 2 µM Dicamba or Picloram in *L. longiflorum* 'Snow Queen'. Nhut *et al.* (2006) reported the use of liquid cultures to in-

Table 1 Effect of growth regulators on bulblet regeneration (%), number of bulblets per explant and mean fresh weight per bulblet.

Treatment (mg L^{-1})						Explants producing	Number of bulblets per	Mean fresh weight per
NAA	**IBA**	**2,4-D**	**BA**	**TDZ**	**Zea**	**bulblets (%)**	**explant**	**plant (mg)**
0	0	0	0	0	0	0 (0)	0	0
0.5	0	0	0	0	0	0 (0)	0	0
1	0	0	0	0	0	0 (0)	0	0
0	1	0	0	0	0	0 (0)	0	0
0	0	0	0.5	0	0	50.0 (45.0)	2.66	166.7
0	0	0	0	0	1.5	100.0 (90.0)	4.00	193.7
0	0	0	0	0	2	100.0 (50.0)	3.33	185.4
0.5	0	0.5	0	0	0	50.0 (45.0)	2.83	167.7
0.5	0	0	1	0	0	0 (0)	0	0
1	0	0	0.5	0	0	50.0 (45.0)	3.00	178.7
1	0	0	1	0	0	83.3 (74.9)	2.33	161.5
1	0	0	1.5	0	0	0 (0)	0	0
1	0	0	2	0	0	50.0 (45.0)	1.50	109.3
1.5	0	0	2	0	0	0 (0)	0.	0
2	0	0	1.5	0	0	100.0 (90.0)	4.16	198.8
0	0	0	0	0.5	0	50.0 (45.0)	3.00	182.2
0	0.5	0	0	1	0	100.0 (90.0)	3.66	187.3
0	0.5	0	0	0	0.5	100.0 (90.0)	2.83	172.0
0	0.5	0	0	0	1	0 (0)	0	0
0	0.5	0	1.5	0	0	100.0 (90.0)	2.83	172.0
0	1	0	2	0	0	50.0 (45.0)	2.50	160.7
0	1.5	0	1	0	0	0 (0)	0	0
0	2.0	1	1.5	0	0	100.0 (90.0)	2.33	164.6
0	0	1.5	2	0	0	66.6 (60.0)	2.33	164.2
0	2	2	0	0	0	50.0 (45.0)	1.33	98.4
$LSD_{0.05}$						(4.99)	0.32	5.86

Figures in parentheses are arc sine transformed values

NAA, α-naphthalene acetic acid; **IBA**, indole-3-butyric acid; **2,4-D**, 2,4-dichlorophenoxy acetic acid; **BA**, 6-benzyladenine; **TDZ**, thidiazuron; **Zea**, zeatin

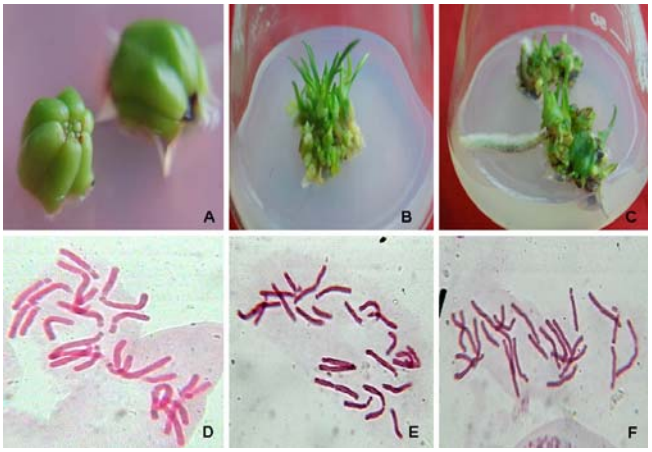

Fig. 1 Regeneration of oriental hybrid lily 'Casa Blanca' through ovary culture. Bulblet regeneration on (**A**) PGR-free MS medium, (**B**) on MS medium supplemented with 2 mg L^{-1} NAA and 1.5 mg L^{-1} BA. (**C**) Rooting of *in vitro*-raised bulblets on MS medium supplemented with 0.5 mg L^{-1} NAA. (**D**) Root tip cell (metaphase) of a diploid plant grown from control bulb (2n = 24). (**E, F**) Root tip cell of regenerated diploid bulblets (2n = 24).

duce and enhance the quality and quantity of somatic embryogenesis in *L. longiflorum* 'Easter Lily'. The percentage of bulblets formed and the number of bulblets per explant from ovary tissue explant were highest with 1 mg L^{-1} indole-3-acetic acid (IAA) or 1 mg L^{-1} IAA and 1 mg L^{-1} kinetin in *L. longiflorum* 'Georgia' (Chung *et al*. 1984).

The average fresh weight per bulblet was highest (198.8 mg) when 2 mg L^{-1} NAA was used in combination with 1.5 mg L^{-1} BA, which was statistically equal with 1.5 mg L^{-1} Zea (**Table 1**). The minimum average fresh weight (98.4 mg) was observed with a combination of 2 mg L^{-1} 2,4-D and 2 mg L^{-1} BA. In *L. longiflorum* 'Ace', Ramsay *et al*. (2003) observed an increase in fresh weight for each explant with 1 mg L^{-1} 2,4-D and 2 mg L^{-1} BA.

The regenerated bulblets rooted with 0.5 mg L^{-1} NAA (**Fig. 1C**) and could be acclimatized under glasshouse conditions with 80% success. All regenerated plants were morphologically indistinguishable from the original cultivar.

To ascertain the ploidy level of regenerated bulblets, chromosome numbers of regenerated bulblets were compared to root tip cells of control plants, which had 24 chromosomes (**Fig. 1D**). A total of 30 bulblets were analyzed and no change was observed in the chromosome numbers (**Fig. 1E, 1F**). Similarly, Uhring (1982) observed unchanged ploidy level in root tips of plantlets regenerated from filament in diploid and amphidiploid *Lilium* 'Black Beauty'. Ramsay *et al*. (2003) reported diploids and mixoploids from ovary culture in Easter lily. Fukai *et al*. (2005) obtained triploids from ovary-ovule culture in *L. formolongi* and *L. formosonum* var. 'Pricei'. The difference in the results may be due the difference in genotype or the regeneration of bulblets directly from the somatic cells of ovary-ovule explants. Fernández *et al*. (1997) reported that all callus-regenerated plantlets from filaments with anthers and tested for chromosome number; all *L. longiflorum* regenerants were diploid (2n = 24). Han *et al*. (2000) developed a haploid callus line from anther culture of Asiatic hybrid lily 'Connecticut' and reported that the survival and growth of the haploid calluses was effected by picloram, NAA and 2,4-D. van Tuyl (1997) and Lim and van Tuyl (2007) produced many new interspecific hybrids in lilies using embryo rescue, ovary slice, ovary and ovule culture techniques and carried out breeding at the polyploidy level to overcome sterility in interspecific hybrids (van Tuyl *et al*. 1990; van Tuyl and Lim 2003) by chromosome doubling through chemicals and by using naturally occurring or induced reduced (2n) gametes (Barba-Gonzalez *et al*. 2008). Zhou *et al*. (2008) also carried out breeding at polyploidy level and obtained intersectional interspecific hybrids of *Longiflorum* × Asiatic (LA) and Oriental × Asiatic (OA) groups of lilies and their polyploidy backcross progenies.

From the present investigation, it may be concluded that the bulblets were regenerated with 0.5 mg L^{-1} BA or with 1.5 and 2 mg L^{-1} Zea directly from ovary explants and that bulblet regeneration was affected by the choice and level of PGRs. The chromosome count of the root tips from regenerated bulblets showed unchanged ploidy level and, therefore, ovary culture in oriental hybrid 'Casa Blanca' may be a useful means of asexual propagation.

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