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In vitro Propagation of *Lilium longiflorum* Var. Ceb-Dazzle Through Direct Somatic Embryogenesis

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Abstract: Experiments were carried out to investigate the effects of various concentrations of Picloram (0, 1, 2, 3, 6 and 9 mg L^{-1}), TDZ (0, 0.5, 1, 1.5 and 2 mg L^{-1}), NAA (1.5 mg L^{-1}) in combination with TDZ (0.08, 0.2 and 0.4 mg L^{-1}), 2,4-D (2.5, 5 and 10 mg L^{-1}) combined with BAP (0.25 mg L^{-1}) and different types of explants (basal, central and distal part of the bulb scale) on direct somatic embryogenesis induction of *Lilium longiflorum* var. Ceb-Dazzle. The explants were surface sterilized and cultured on MS medium supplemented with 3% sucrose, 0.3% Phytagel and various concentrations of mentioned growth regulators. It was found that Picloram at a concentration of 2 mg L^{-1} was the most effective treatment for induction of direct somatic embryogenesis and gave the highest number of embryos (18.6) on each explant. The explants from basal part of the bulb scale showed the best responses (19.9 embryos/explant). TDZ alone or combined with NAA in various concentrations was not able to induce somatic embryogenesis, but gave direct bulblet regeneration. Similar results were obtained for 2, 4-D and BAP combination treatments. Induced somatic embryos were transferred to MS medium without growth regulators for maturation and matured plantlets were successfully acclimatized and transferred to *in vivo* conditions.

Key words: Acclimatization, globular embryo, induction, maturation, picloram

INTRODUCTION

Lilium is one of the 220 genera belonging to *Liliaceae* family and comprises of 85 species, including many beautiful ornamental plants. *Lilium* is one of the most important bulbous plants which is ranked seventh among the cut flowers in the world (Varshney *et al*., 2000). In Iran also, *Lilium* is one of the most popular and highly valuable ornamental plants. Iran is ranked 16th among 42 producers of flower and ornamental plants and its plantations are about 1.3% of the global plantation areas (Bashirazami, 1996; Agricultural Satistics, 2004). To propagate this plant in Iran, substantial amounts of bulbs are imported each year and cause producers encounter much difficulties plus the risk of importing quarantine diseases. Therefore, it is highly desirable if it can be propagated by an efficient, quick, simple and safe method in the country.

Conventionally, *Lilium* is propagated through bulb scaling after breaking dormancy. Scaling method yields a maximum of 3 to 5 bulbs from each scale, depending on the scale size and variety/species. Also reduced vigor of bulbs has been reported with repeated cycles of vegetative propagation (Varshney *et al*., 2000). Considering these problems, developing protocols for its mass propagation using tissue culture techniques either through *in vitro* bulb regeneration or somatic embryogenesis is worthwhile.

Direct somatic embryogenesis is the most promising technique for plant multiplication, because of its high proliferation potential and the ability of minimizing or eliminating the risk of producing chimeric plants (Nuth *et al*., 2001a). So far, indirect somatic embryogenesis (initially through callus formation) has been reported from petal and pistil of Easter lili (Tribulato *et al*., 1997), bulb scales of *Lilium formosamum* Wallace (Nakano *et al*., 2000) and *Lilium longiflorum* hybrids (Kim *et al*., 2003), but to the best of our knowledge, there has been no report on direct somatic embryogenesis induction. In the present work, various concentrations of different plant growth regulators and types of explant were used to induce direct somatic embryogenesis in *Lilium Longiflorum* var. Ceb-Dazzle.

MATERIALS AND METHODS

Plant materials and surface sterilization: Central bulb scales were used as explants after removing outer

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scales of the bulbs. Before excising the explants, bulb scales were surface sterilized using 70% ethanol for 30 sec, followed by 50% sodium hypochlorite solution (5.5% w/v) for 15 min and 2 times rinsing with sterile distilled water. The surface-sterilized scales were then transferred to a water bath at 37°C for 45 min.

Induction of direct somatic embryogenesis: MS basal medium (Murashige and Skoog, 1962), supplemented with 3% (w/v) sucrose, 0.3% (w/v) Phytagel was used and various concentrations of different plant growth regulators including Picloram (0, 1, 2, 3, 6 and 9 mg L^{-1}), TDZ (0, 0.5, 1, 1.5 and 2 mg L^{-1}), NAA (1.5 mg L^{-1}) in combination with TDZ (0.08, 0.2 and 0.4 mg L^{-1}) and 2,4-D (2.5, 5 and 10 mg L^{-1}) combined with BAP (0.25 mg L^{-1}) were added and the pH was adjusted to 5.7 before autoclaving at 121°C and 1.5 atm. for 15 min. Different types of explants (basal, central and distal parts of the bulb scales) were used in Picloram and 2, 4-D treatments. In all experiments, 25 mL of medium was dispensed in to Petri dishes (90×15 mm) and cultures were maintained under 16/8 h photoperiod in a growth chamber at 24±1°C.

Maturation and plantlet regeneration: Effects of four different hormone-free culture media (MS, ½ MS, MS in half strength of micro elements and MS in half strength of macro elements) containing 3% (w/v) sucrose and 0.3% (w/v) Phytagel plus 2 incubation conditions (light and darkness) were investigated on maturation and further development of somatic embryos. Masses of 3-month-old globular embryos were used as explants in these treatments. Three months later, number of bulblets regenerated from each explant was recorded.

Acclimatization: Plantlets were washed with tap water to remove any Phytagel residues and then transplanted in disposable plastic pots containing Peatmos soil, caped with small transparent plastic cups. After 2 weeks a few small holes were made at the top of the caps for aeration, and they were removed completely after 1 month.

Statistical analysis: One month after induction, number of somatic embryos induced on each explant was counted under a stereo microscope. Number of regenerated bulblets/explant was also counted in maturation and plantlet regeneration treatments after 3 months. All treatments had at least 3 replications, each containing 5 explants. Data were analyzed based on simple or factorial complete randomized designs
using GLM method of SAS system (Release 6.12, SAS Institute Inc., Cary, NC, USA). Means were separated by Duncan's multiple range test (Duncan, 1995).

RESULT AND DISCUSSION

Induction of direct somatic embryogenesis: Among tested plant growth regulators (TDZ, BAP, 2,4-D, Picloram) only Picloram was able to induce direct somatic embryogenesis. The primary sign of somatic embryogenesis induction was swelling of the explant tissue one month after culture. Globular embryos were appeared directly from the surface of the explant tissues in all Picloram treatments after 2 months (Fig. 1). Significant differences were observed among various concentrations of Picloram (α = 1%) and the highest number of induced somatic embryos (18.6 embryos/explant) was obtained using 2 mg L^{-1} Picloram (Table 1).

Different types of explant had significant effects on the number of induced somatic embryos and the highest number of somatic embryos (19.9 embryos/explant) was obtained from basal explants
(Table 2). Combination effects of Picloram and

Table 1: Effect of various concentrations of Picloram on number of somatic embryos/explant

	Picloram (mg L^{-1})					
	0	1	2	3	6	9
Embryos/Explant	0.0c	11.4b	18.6a	16ab	15.6ab	15.3ab

Different letter (s) within a row indicate significant differences at p= 0.01 by Duncan's multiple range test

Table 2: Effect of explant types treated with Picloram on number of somatic embryos/explant

	Explant type		
	Basal	Central	Distal
Embryos/Explant	19.9a	14.1a	4.4b

Different letter(s) within a row indicate significant differences at p=0.01 by Duncan's multiple range test

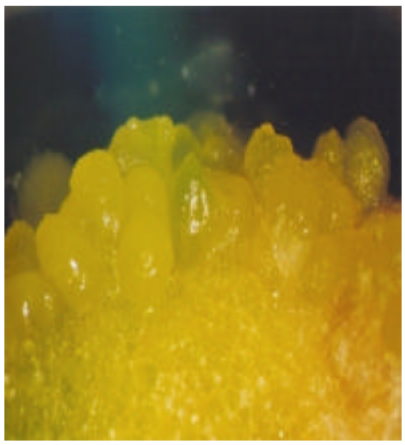

Fig. 1: Developing somatic embryos (globular stage), 2 months after culture

Table 3: Combination effects of Picloram and explant types on number of somatic embryos/explant

	Picloram (mg^{-1})					
Explant type	0	1	2	3	6	9
Basal	0c	24.5ab	22ab	25ab	22.6ab	20.5ab
Central	0c	9abc	30.6a	18.1ab	17abc	16.7abc
Distal	0c	0c	10bc	4.8c	5.1bc	10.1abc

Different letter (s) indicate significant differences at p = 0.01 by Duncan's multiple range test

explant types were also significant and the highest number of somatic embryos (30.6 embryos/explant) was achieved using 2 mg L^{-1} Picloram on central explants (Table 3). Other growth regulators used in this research were not able to induce somatic embryogenesis but they often caused bulblet regeneration.

Effect of explant types on regeneration of several Korean native lilies was studied by Jeong (1996), who showed that explants taken from the basal part of bulb scales were more suitable for regeneration than other parts (central and distal). Results obtained from Picloram treatments are similar to Kim *et al.* (2003). They reported that using various concentrations of Picloram on explants from Oriental and Easter lilies caused embryogenic callus formation. In this project using Picloram induced direct somatic embryogenesis in *Lilium longiflorum* var. Ceb-Dazzle, which would be more desirable due to a lower risk of producing chimeric plants and also somaclonal variations. There are also some reports on the application of Picloram for somatic embryogenesis induction in *Lilium longiflorum* Wallace (Nakano *et al.*, 2000) and *Lilium formasanum* (Suzuki *et al.*, 1998).

Picloram has been referred as one of the most effective plant growth regulators for inducing somatic embryogenesis in many plants (Karun *et al.*, 2004; Gude and Dijkema, 1997; Bach 1992). According to Nuth *et al.* (2002), TDZ and NAA could induce somatic embryogenesis on *in vitro-pseudo* bulblet explants of *Lilium longiflorum*, but in the present study, similar treatments did not induce somatic embryogenesis. The different response seen here might be attributed to the types of explant or genotype used. It has been reported that beside the explant type and growth regulators, several other factors can affect somatic embryogenesis, from which perhaps plant genotype is one of the most important one. Individual genotypes within a given species could vary greatly in their embryogenesis capacity. In addition, each individual genotype may have unique requirements for its optimal regeneration capacity (Merkel *et al.*, 1995).

Maturation and plantlet regeneration: Results showed that both culture media and incubation conditions had significant effects on maturation of somatic embryos. Maximum number of regenerated bulblets per each explant (7.25) was achieved on MS medium with half strength of macro and micro elements without growth regulators (Table 4) and more somatic embryos were converted to whole plantlets (7.56 bulblets/explant) when cultures incubated under light conditions compared to darkness (Table 5). Roots began to form after 2 weeks (Fig. 2a) and shoots were developed after 4 weeks (Fig. 2b). It took about 3 months for a somatic embryo to develop a whole plantlet with roots, bulblet and leaves (Fig. 2c). Basal medium without growth regulators has been recommended as one of the best treatment for maturation of lilies somatic embryos (Nakano *et al.*, 2000; Nuth *et al.*, 2001b, 2002). There are also some reports that bulblet regeneration of *Lilium* explants is

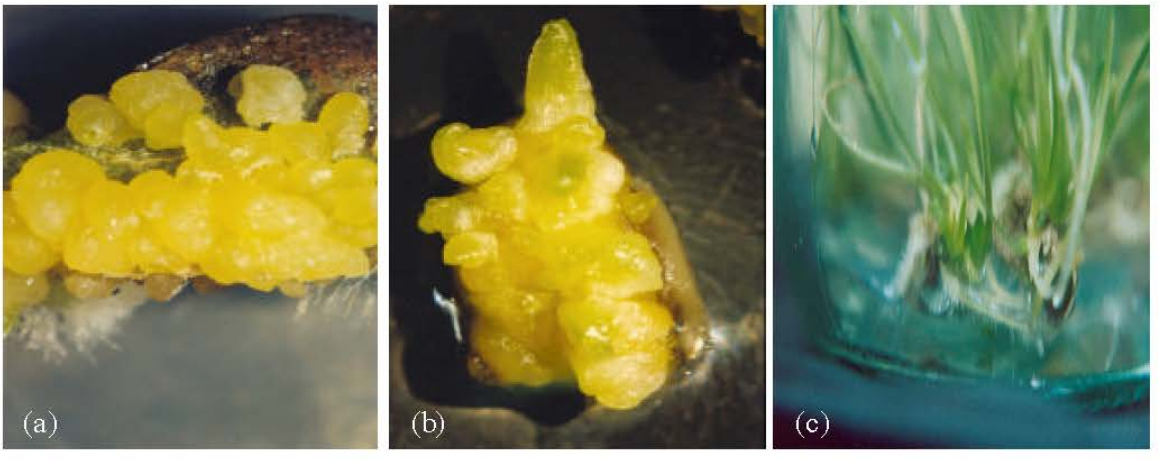

Fig. 2: Maturation of somatic embryos on hormone-free half strength MS medium: Root formation, 2 weeks after transfer (a); Appearance of leaf-like organs on globular embryos, one month after transfer (b) and; Whole plantlet development, after 3 months (c).

Table 4: Effects of different culture media on number of bulblets/explant

	Media			
	MS	1/2MS	½ Micro	½ Macro
Bulblet/explant	3.37ab	7.25a	5.87a	2.25b

Different letter (s) indicate significant differences at p = 0.05 Duncan's multiple range test, respectively

Table 5: Effects of different culture media on number of bulblets/explant

	Incubation conditions	
	Light	Darkness
Bulblet/explant	7.56a	1.81b

Different letter (s) indicate significant differences at p = 0.01 Duncan's multiple range test, respectively

Fig. 3: Transferring plantlets to Peatmos-containing pots for acclimatization

much better under light than dark incubation conditions (Maesato *et al*., 1994, Jeong 1996). Results from the present study seem to be in consistence with these reports.

Acclimatization: When whole plantlets regenerated, they were transferred to greenhouse for acclimatization. More than 250 plantlets were transferred, all of them survived after 2 months (Fig. 3).

In conclusion, the best treatment to induce direct somatic embryogenesis in *Lilium longiflorum* var. Ceb-Dazzle bulb scales appeared to be MS medium supplemented with 2 mg L^{-1} Picloram. Induced somatic embryos were maturated and germinated on hormone-free half strength MS medium, incubated in light conditions and produced normal roots and shoots.

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