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DEVELOPMENT OF PLANT REGENERATION SYSTEM VIA SOMATIC EMBRYOGENESIS FROM ROOTS OF *LILIUM* HYBRID CULTIVARS

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Abstract

A simple and efficient protocol for *in vitro* plant regeneration through somatic embryogenesis was developed in four *Lilium* cultivars. Roots from *in vitro* seedlings of four lily cultivars were cultured on Murashige and Skoog (MS) medium (1962) supplemented with the auxin picloram (PIC) used alone (0.5, 1.0, and 2.0 mg l^{-1}) or in combination with the cytokinins BAP or TDZ at 0.5 mg l^{-1}. Picloram with the combination of cytokinins was found to be positive to improve the callus formation. Generally speaking MS medium supplemented with 1.0 mg l^{-1} PIC and 0.5 mg l^{-1} BAP gave the highest percentage of explants forming callus in three *Lilium* cultivars ('Kankado' = 95.7%; 'Manissa' = 98%; 'Siberia' = 80%); for 'Sorbonne' the best performance for callus formation was scored on MS medium + 1.0 mg l^{-1} PIC and 0.5 mg l^{-1} TDZ (88.7%). Embryoids regeneration occurred after a subsequent transfer onto fresh medium and a further one month of culture. The number of embryoids per 0.1 g of callus ranged from 11.7 ('Siberia' on MS medium + 1.0 mg l^{-1} PIC and 0.5 mg l^{-1} TDZ) to 70.3 ('Manissa' on MS medium + 1.0 mg l^{-1} PIC and 0.5 mg l^{-1} BAP). The callus induction efficiency, callus growth index, callus growth intensity, and numbers of embryoids induced from 0.1 g of callus of root were higher than that of bulb scales and leaves. Embryoids cultured on MS medium with 0.1 mg l^{-1} CPPU developed into complete plants.

Key words: cut flower, embryoids, embryogenic calluses, monocotyledonous, picloram, root system

INTRODUCTION

The monocot genus *Lilium* (Liliaceae) includes one of the most important cut-flower plants because of their large and attractive flowers (Bacchetta et al. 2003, Azadi et al. 2010). The demand of lily flowers has increased considerably during the last few decades, and thus new varieties are needed to meet the market demand. Establishment of somatic embryogenesis system provides a good alternative to the conventional methods in order to have at disposal an efficient propagation method and a system to perform genetic improvement through genetic transformation approaches.

Many studies have been already conducted on *in vitro* plant regeneration of *Lilium*. Regeneration of lily plantlets has been achieved through different morphogenic pathways: direct or indirect adventitious shoot induction from *in vitro* cultured leaves (Wickremesinhe et al. 1994, Bacchetta et al. 2003) and bulb scales (Simmonds and Cumming 1976, Priyadarshi and Sen 1992, Kanchanapoom et al. 2012); callus culture from anther and filament (Arzate-Fernandez et al. 1997); embryogenic callus derived from styles and flower pedicels (Tribulato et al. 1997), leaf (Suzuki et al. 1998) and bulb scale tissues (Nakano et al. 2000); suspension cultures composed of meristematic nodular cell clump and protoplast cultures (Mii et al. 1994, Godo et al. 1996, 1998, Komai et al. 2006). The biggest drawback of somatic embryogenesis is the low regeneration potential which hampers the development of further scientific and industrial applications. To our knowledge only a few reports are available on the tissue culture of *Lilium* roots (Pan et al. 2011), focused to organogenesis and not embryogenesis.

In this context, four *lilium* oriental hybrid cultivars, which account for 90% of the lily market in China were selected for their large flowers, beautiful color and pleasant aroma. The aim of our study was to investigate the effect of different concentrations and combinations of plant growth regulators (PGRs) for induction of somatic embryogenesis from root explants.

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MATERIALS AND METHODS

Plant material

Roots (2-3 cm from shoot base) were excised from *in vitro* grown seedlings of four *Lilium* oriental cultivars of hybrid origin, bred especially for ornamental purposes, and having official cultivar names ('Sorbonne', 'Kankado', 'Manissa', and 'Siberia'). They were cultured on MS (Murashige and Skoog 1962) medium supplemented with 0.5 mg l^{-1} α-naphthaleneacetic acid (NAA) for three months. Root segments (1.0-1.2 cm) were cut and inoculated on callus induction medium.

In order to assess the efficiency of *in vitro* regeneration from callus derived from root explants compared with *in vitro* regeneration from other tissues, the bulb scales and leaves were also used in 'Manissa'.

Culture medium and conditions

The medium used during the callus induction, embryoids culture and the regeneration phase was MS supplemented with 30.0 g l^{-1} sucrose and 5.0 g l^{-1} agar (Shanghai Yuhan Bio-tech Co., Ltd). The pH of the medium was adjusted to 5.8 ± 0.1 with 0.1 N NaOH before autoclaving at 121°C for 20 min. The callus induction, embryoids culture, and regeneration phases were performed in a 200-ml glass bottle containing 50 ml of medium with gas permeable membrane tissue culture plastic cover. Diameter of the transparent cover is 6.0 cm and diameter of the gas permeable membrane is 1.0 cm.

Callus initiation

For callus induction and embryoid culture, MS medium supplemented with the auxin 4-amino-3,5,6-trichloropicolinic acid (picloram; PIC) used alone (0.5, 1.0, and 2.0 mg l^{-1}) or in combination with the cytokinins 6-benzylaminopurine (BAP) or (1-Phenyl-3-(1,2,3,-thiadiazol-5-yl)urea (Thidiazuron; TDZ) at 0.5 mg l^{-1} were investigated to determine the optimal medium. The cultures were incubated at 25 ± 2°C in the dark. Two months after culture initiation, the frequency of callus induction, callus growth index and callus formation intensity of the four *Lilium* oriental cultivars ('Sorbonne', 'Kankado', 'Manissa', 'Siberia') were recorded. For each treatment, ten explants were cultured in each glass bottle containing 50 ml medium with three repetitions. Callus type was determined at the same time.

Embryoids induction

Calluses formed on callus induction medium were subcultured onto the same callus initiation medium to induce embryoids. The cultures were incubated at 25 ± 2°C in the dark. One hundred mg fresh weight of calluses were plated in each glass bottle and four replicates were carried out for each treatment. After one month of culture the numbers of embryoids induced from 0.1 g of calluses was determined.

Bulb scales, leaves and roots of 'Manissa' were cultured on MS medium supplemented with 1.0 mg l^{-1} picloram and 0.5 mg l^{-1} BAP to induce callus. The cultures were incubated at 25 ± 2°C in the dark. Three replicates were carried out for each explant. Two months later, the frequency of callus induction, callus growth index, callus formation intensity, and callus color were determined. Four replicates were carried out for each explant. After one month of culture the numbers of embryoids induced from 0.1 g of calluses were determined.

Regeneration of plantlets

Plant regeneration was induced by transferring cotyledonary shaped embryoids to MS medium with 0.1 mg l^{-1} N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU). The culture was incubated at 25 ± 2°C with a 16 h photoperiod at 50 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Phillips). Four weeks later, plant regeneration rates (number of survival plants / number of total cotyledonary shaped embryoids × 100%) were recorded.

Statistical analysis

The frequency of callus induction, the callus growth index, and intensity of nodular callus formation were determined after two months of culture. The numbers of embryoids induced from 0.1 g of calluses were determined after one month of subculture.

The callus induction frequency (in %) was determined by dividing the number of explants forming callus to the total number of explants × 100%. The callus growth index including the mass increase in the explant and the forming callus was determined using the formula: (final fresh weight – initial fresh weight) / initial fresh weight.

To describe the intensity of nodular callus formation a four-degree scale was adopted, where 1 = callus covering 0-25% of the explant surface, 2 = callus covering 25%-50% of the explant surface, 3 = callus covering 50%-75% of the explant surface, 4 = callus covering 75%-100% of the explant surface.

All the results were evaluated by one-way analysis of variance (ANOVA). The data were analyzed using SPSS 16.0 and the statistically significant differences were identified by Duncan's multiple range tests at significance level of $p \leq 0.05$.

RESULTS

Embryogenic callus initiation

After 2 months of culture, primary calluses grew on root segments of all four cultivars and on all tested

media (Fig. 1B). All the calluses exhibited a nodular-like structure. 'Manissa' and 'Siberia' calluses were deep yellow, while 'Kankado' and 'Sorbonne' calluses were pale yellow (Table 1). However, the induction frequency of the calluses varied among the cultivars (Table 2). The effect of picloram and other cytokinins on callus induction of roots from *Lilium* was helpful, with highest frequency of calluses induction observed in the presence of 1.0 mg l^{-1} picloram and 0.5 mg l^{-1} BAP in 'Kankado' (95.67%), 'Manissa' (98.00%) and 'Siberia' (80.00%), while 1.0 mg l^{-1} picloram and 0.5 mg l^{-1} TDZ in 'Sorbonne' (88.67%) (Table 2). There were no statistically significant differences among 'Kankado' and 'Manissa' on callus induction frequency, but there were statistically significant differences between 'Siberia' and 'Kankado', 'Siberia', and 'Manissa' for the frequency of callus induction. Calluses were induced when picloram was added either alone or in combination with other cytokinins (Table 2), indicating that picloram was a key PGR, which could effectively induce root to produce callus. Picloram with the combination of BAP ('Kankado', 'Manissa', 'Siberia') or TDZ ('Sorbonne') could significantly promote the callus induction frequency (Table 2). Callus growth index of the four varieties was significantly different on the medium of 1.0 mg l^{-1} picloram and 0.5 mg l^{-1} BAP. The highest callus growth index was noted in 'Manissa' (6.7) while the lowest was 'Siberia' (2.8).

Table 1. Callus growth conditions through somatic embryogenesis of different lily cultiva

Cultivars	Callus growth index	Callus formation intensity	Callus color and type
'Sorbonne'	6.72 ± 0.19 c	4	YN
'Siberia'	2.85 ± 0.09 d	2	DYN
'Manissa'	9.12 ± 0.23 a	4	DYN
'Kankado'	7.68 ± 0.21 b	3	YN

Note: Data were determined 2 months after culture initiation. Data are mean ± standard error of three independent experiments. Different letters within a column indicate significant differences at $p \leq 0.05$ by Duncan's multiple range test.
Callus color and type: YN - yellowish nodular; DYN - deep yellowish nodular.

Four cultivars had different callus formation intensity (Table 1), but 'Manissa' having the highest one of more than 90% (Fig. B).

The average embryoids number induced from 0.1 g of root-derived calluses on different variants of the medium ranged from 11.7 to 70.3 (Table 2). The best medium for 'Siberia', 'Kankado' and 'Manissa' was MS supplemented with 1.0 mg l^{-1} picloram and 0.5 mg l^{-1} BAP, while the best one for 'Sorbonne' was MS supplemented with 1.0 mg l^{-1} picloram and 0.5 mg l^{-1} TDZ (Table 2). Statistical analysis showed that picloram

Table 2. Effects of PGRs on calluses and embryoids induction from lily roots.

Cultivars	Picloram (mg l^{-1})	Callus induction (%)	Numbers of embryoids induced from 0.1 g of callus
'Sorbonne'	PIC 0.5	64.67 ± 2.33 bc	42.67 ± 0.67 cd
	PIC 1.0	66.67 ± 2.03 b	44.33 ± 0.88 bc
	PIC 2.0	58.00 ± 1.00 c	38.33 ± 0.88 d
	PIC 1.0 + BAP 0.5	84.33 ± 2.96 a	47.67 ± 1.20 ab
	PIC 1.0 + TDZ 0.5	88.67 ± 3.30 a	49.67 ± 2.60 a
'Siberia'	PIC 0.5	61.33 ± 2.96 bc	17.33 ± 0.88 bc
	PIC 1.0	64.67 ± 2.33 b	19.67 ± 0.67 b
	PIC 2.0	53.33 ± 3.76 cd	15.00 ± 1.16 c
	PIC 1.0 + BAP 0.5	80.00 ± 1.73 a	23.67 ± 1.20 a
	PIC 1.0 + TDZ 0.5	51.00 ± 1.00 d	11.67 ± 0.33 d
'Manissa'	PIC 0.5	74.67 ± 3.93 bc	59.00 ± 1.00 b
	PIC 1.0	68.67 ± 2.96 c	58.00 ± 1.16 bc
	PIC 2.0	65.67 ± 4.67 c	54.33 ± 1.45 c
	PIC 1.0 + BAP 0.5	98.00 ± 1.00 a	70.33 ± 1.45 a
	PIC 1.0 + TDZ 0.5	83.30 ± 2.03 b	61.00 ± 1.73 b
Kankado'	PIC 0.5	84.67 ± 2.33 bc	36.00 ± 0.00 bc
	PIC 1.0	87.78 ± 2.81 ab	38.00 ± 1.16 ab
	PIC 2.0	76.53 ± 5.04 cd	34.00 ± 1.73 c
	PIC 1.0 + BAP 0.5	95.67 ± 1.33 a	41.33 ± 0.88 a
	PIC 1.0 + TDZ 0.5	73.11 ± 0.11 d	27.33 ± 1.45 d

Data are mean ± standard error. Means of embryogenic callus induction and embryoids number for each cultivar followed by the same letter are not significantly different ($p \leq 0.05$).

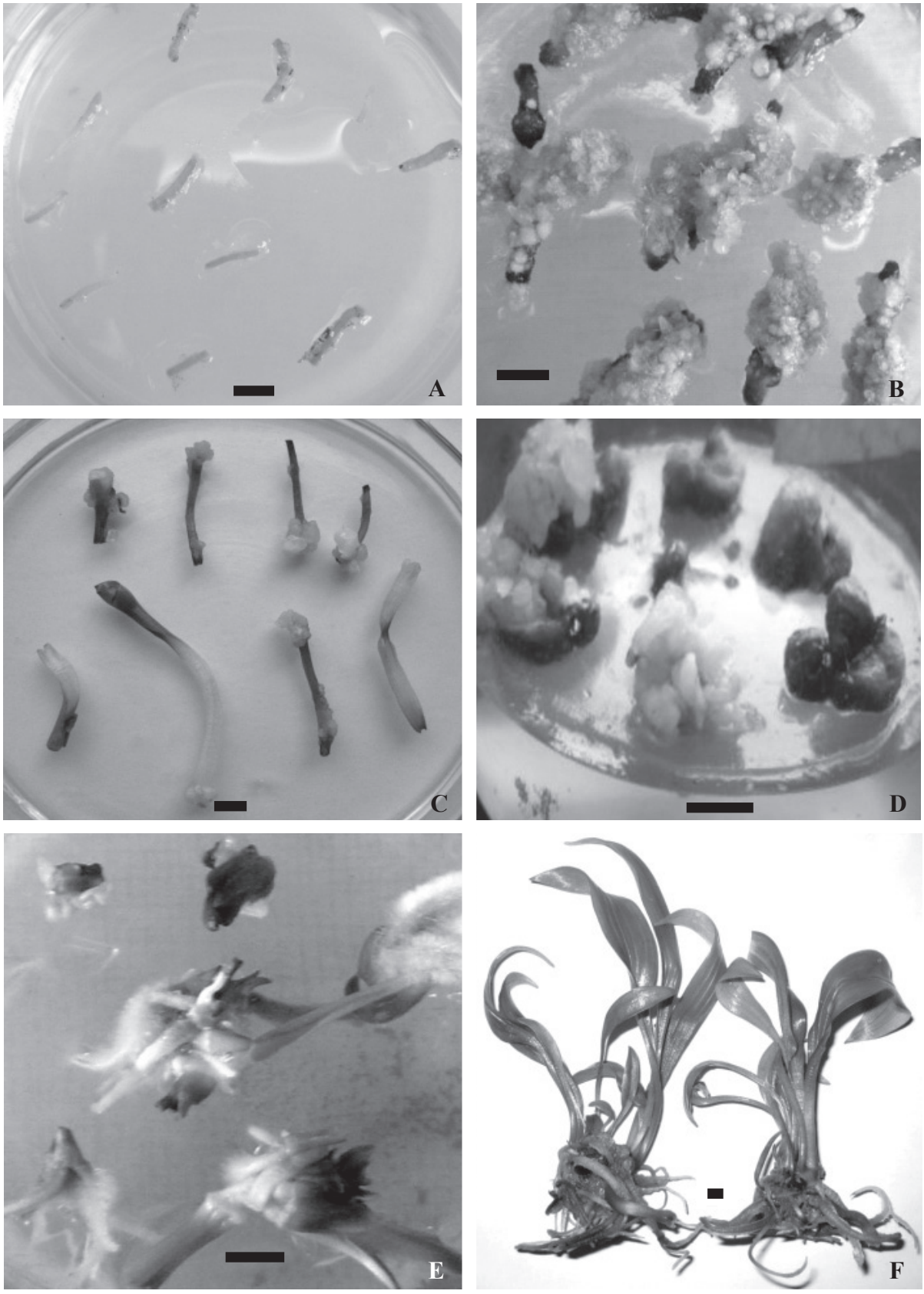

Fig. 1. Callus formation and plant regeneration of 'Kankado'. A) Roots cultured on the callus induction medium, B) Calluses formed on callus induction medium, C) Calluses induced from leaves, D) Bulb scales cultured on the callus induction medium, E) Regenerated plants cultured on medium containing 0.1 mg l^{-1} CPPU in light, F) Whole plants originated from root explants. Bar = 5 mm.

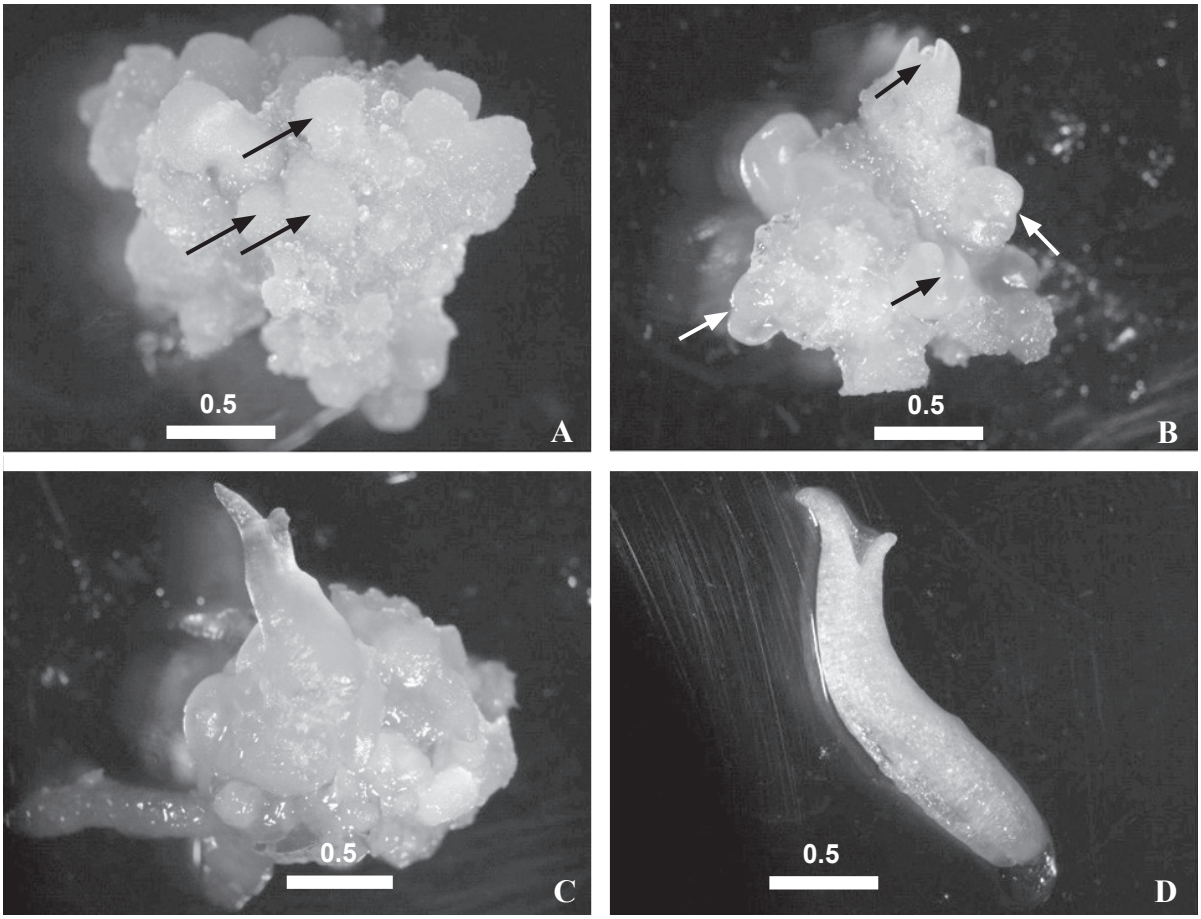

Fig. 2. Somatic embryogenesis of *Lilium*. A) Somatic embryos at globular stage cultured in dark, B) Shield-shaped embryoids cultured in dark, C) A cotyledonary embryoids cultured in dark, D) Mature embryoids with shoot and root poles. Bar = 0.5 mm.

with the combination of BAP ('Kankado', 'Manissa', 'Siberia') or TDZ ('Sorbonne') could significantly promote the average embryoid number induced from 0.1 g of root-derived calluses.

In the early phase of the process, the calluses looked soft, compact and would remain in this state for one month before new proliferation or differentiation of yellowish protuberances appeared. After two-month culture, calluses were transferred to the same medium to induce embryoids. Upon transferring onto fresh media, the nodular calluses became glossy, and eventually various structures with globular or rounded shapes were observed (Fig. 2A). The embryos underwent through stages of ontogeny i.e. globular, shield-shaped (Fig. 2B), and cotyledonary shaped embryos (Fig. 2C) and developed into mature embryos with shoot and root poles (Fig. 2D).

After two months of culture, roots, bulb scales, and leaves cultured on MS medium supplemented with 1.0 mg l^{-1} picloram and 0.5 mg l^{-1} BAP produced callus with different callus induction efficiency. The highest callus induction efficiency was 93.3% in roots whereas the lowest was 64.4% in leaves. Compared with leaves and bulb scales, roots had the highest callus induction efficiency (93.3%), callus growth index (7.8), callus formation intensity (covering 75%-100% of the explant surface), and numbers of embryoids induced from 0.1 g of callus (66.3). Moreover, they showed different callus color. Callus color from bulb scales, leaves, and roots were creamy white, yellow and deep yellow, respectively (Table 3).

Regeneration of plantlets

After transferring to the plant regeneration medium, embryoids gradually turned green and developed into seedlings with shoot and root (Fig. 1E). After one month they elongated and formed vigorous roots at which stage they were ready for acclimatization to outdoor conditions. On MS medium with 0.1 mg l^{-1} CPPU, regeneration efficiency of different cultivars and different explants was more than 90%.

DISCUSSION

Phytohormones can influence many developmental processes in plants, ranging from seed germination to

Table 3. Callus induction efficiency, callus growth index, callus formation intensity, and numbers of embryoids induced from 0.1 g of callus as affected by different explants in 'Manissa'.

	Root	Bulb scale	Leaf
Callus induction (%)	93.33 ± 2.22 a	81.11 ± 2.96 b	64.45 ± 2.96 c
Callus growth index	7.79 ± 0.09 a	1.04 ± 0.04 c	2.31 ± 0.03 b
Callus formation intensity	4	3	1
Numbers of embryoids induced from 0.1 g of callus	66.33 ± 1.45 a	19.67 ± 0.88 c	30.67 ± 1.20 b
Callus color	DY	CW	Y

Data are mean ± standard error of three independent experiments. Different letters within a row indicate significant differences at $p \leq 0.05$ by Duncan's multiple range test.
Callus color: Y - yellowish; DY - deep yellow; CW - creamy white.

shoot, root, and flower formation. *In vitro* callus induction and development require the presence of auxins and cytokinins. Picloram, a herbicide with auxin-like properties similar to 2,4-Dichlorophenoxyacetic acid (2,4-D) (Kefford and Caso 1966), had been successfully utilized for induction of totipotent callus lines in tissue cultures of lily (Mori et al. 2005), and other plants such as *Gasteria* and *Haworthia* (Beyl and Sharma 1983), *Typha* (Zimmermann and Read 1986) and *Allium* (Phillips and Hubstenberger 1987, Phillips and Luteyn 1983). Moreover, picloram was shown to be more effective than 2,4-D and NAA in the initiation of callus and induction of embryoids in *Lilium longiflorum* (Tribulato et al. 1997) and other type of bulbous plants, such as *Hyacinthus orientalis* (Lu et al. 1988). The present study showed that picloram had beneficial effects on callus induction and embryoids germination of *Lilium* using root explants.

Calluses were effectively induced when picloram was added either alone or in combination with other cytokinins. In addition, the callus induction frequency and the average embryoids number induced from 0.1 g calluses were significantly promoted when picloram was used in combination with BAP ('Kankado', 'Manissa', 'Siberia') or TDZ ('Sorbonne'). Similar results were also observed in other *Lilium* species (Mori et al. 2005), *Tulipa gesneriana* (Ptak and Bach 2007) and *Rudgea jasminoides* somatic embryogenesis (Stella and Braga 2002). But in garlic, it has been reported that there was a higher frequency of callus induction on a medium with picloram alone compared with picloram combined with a cytokinin (Zheng et al. 2003), contrary to the results obtained in this study. It may be due to the fact that different plants had different responses to exogenous PGRs.

To date, many explants of *Lilium* have been used as explants for callus induction in lilies. In these explants, higher induction efficiency has been reported in explants such as pseudo-bulblet transverse thin cell layer (the fresh weight of embryo-like structure clusters was 2.4 g after 45 days in culture) (Nhut et al. 2001, 2002), but it was more difficult to operate and succeed. Our results showed that the callus induction efficiency, callus growth index, callus formation intensity, and numbers of embryoids induced from 0.1 g of callus from roots was significantly higher than from leaves and bulb scale (Table 3, Fig. 1 C,D), and this indicated that roots were more efficient than other explants for callus induction and were easy to culture. Endogenous PGRs level in different explants or different parts of the same explants was different, which led to differences in the ability of somatic embryogenesis. This ability in different parts of *Solanum melongena* leaves was positively correlated with the level of polyamines (Yadav and Rajam 1997). A correlation between endogenous PGRs level and somatic embryogenesis had also been reported in wheat (Jiménez and Bangerth 2001). Thus we hypothesize that the reason for higher induction and callus growth index of roots in our study may be due to the endogenous PGRs level, which was higher in roots than in bulb scales and leaves, making it easier to induce callus on the same PGR conditions.

Although embryoids can grow on MS medium without PGRs, in our study, 0.1 mg l^{-1} CPPU was used to make the embryoids grew shoots and roots spontaneously on MS medium. Watanabe (1989) reported that CPPU can significantly promote the lily stem cell number, enhance the mechanical strength of the stem and accelerate the lily bulbs growth. In our experiment, the embryoids of lily, irrespective of their origin, were able to convert into well-developed plants on media containing 0.1 mg l^{-1} CPPU.

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