DEVELOPMENT OF AN EFFICIENT REGENERATION SYSTEM VIA SOMATIC EMBRYOGENESIS FROM ROOTS OF *LILIUM* HYBRIDS

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Abstract

A simple and efficient protocol for *in vitro* plant regeneration through somatic embryogenesis was developed in *Lilium* hybrids. Roots of four lily cultivars were cultured on Murashige and Skoog medium (1962) supplemented with different plant growth regulators to induce callus. After two months of culture, nodular callus was obtained at a frequency up to 98.00 % on MS medium supplemented with 1.0 mg l^{-1} picloram and 0.5 mg l^{-1} 6-Benzylaminopurine (6-BAP). These calli were transferred onto proliferation medium to induce somatic embryoids. The highest number of somatic embryos induced from 0.10 g fresh weight of calli was 70.33 in 'Manissa'. The callus induction efficiency of root was higher than that of bulb scale and leaves. The somatic embryoids were cultured on MS medium with 0.100 mg l^{-1} N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU) to regenerate plants. Picloram had played a key role in somatic embryogenesis using roots as explants.

Key words

embryogenic calli, histological observation, *Lilium* hybrid, picloram, root system, somatic embryogenesis

Running title: *Yin Zhou et al. Somatic embryogenesis from roots of Lilium hybrids*

INTRODUCTION

Lilium, a monocot belonging to the family *Liliaceae* (Azadi et al. 2010), is one of the most important cut-flower species because of its large and attractive flowers (Bacchetta et al. 2003). 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.

~~To date, there were~~ many studies 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 (Bacchetta et al. 2003, Wickremesinhe et al. 1994) and bulb scale (Priyadarshi and Sen 1992, Simmonds and Cumming 1976); callus culture of filament ~~with anther~~ (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 (Godo et al. 1996, Godo et al. 1998, Mii et al. 1994, Komai et al. 2006). The ~~most important~~ drawback ~~faced in~~ the embryogenetic ~~pathway~~ is the low regeneration potentiality and this hampers to develop further scientific and industrial applications. To our knowledge ~~relatively~~ few reports are available on the embryogenic potential of *Lilium* roots. The establishment of callus cultures from roots can be easily performed without destroying the entire plantlets. In this ~~backdrop~~, efforts were made to explore the feasibility of using root to induce embryogenic callus and to regenerate plantlets ~~in this paper~~. Four *lilium* oriental hybrids which accounted for 90% of the lily market in China were selected in our study for their large flowers, beautiful color and pleasant aroma. Different concentrations and combinations of plant growth regulators on root derived callus and the embryo induction frequency have been evaluated.

MATERIALS AND METHODS

Plant material

Roots (2-3 cm from shoot base) used to initiate callus were excised from *in vitro* seedlings of four *lilium* oriental hybrids cvs ('Sorbonne', 'Kankado', 'Manissa', 'Siberia') cultured on MS (Murashige & Skoog, 1962) medium supplemented with 0.5 mg l^{-1} NAA over 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 to *in vitro* regeneration from other tissues, for cv 'Manissa' bulb scale and leaves were used too.

Culture medium and conditions

The basal medium (BM) 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 phase were performed in a 200-ml glass bottle containing 50 ml of medium with breathable membrane tissue culture plastic cover.

Embryogenic callus initiation

For callus induction and embryoid culture, five medium formulations with different combinations of auxin and cytokinin were investigated to determine the optimal medium, the BM was supplemented with varying concentrations of different growth regulators as indicated in Table 1. The culture was incubated at 25±2°C in the dark. Two months after culture initiation, the frequency of callus induction was recorded (number of explants forming callus / number of total explants × 100%) (Table 2). For each treatment, ten explants were cultured on each glass bottle containing 50 ml medium with three repetitions (3 glass bottles per replication). Callus type and callus growth were determined at the same time.

Calli formed on callus induction medium were subcultured onto the same medium (Table 1) to induce somatic embryoids. Hundred mg FW of calli were plated on each glass bottle and four replicates were carried out for each treatment. After one month of

culture the numbers of embryos induced from 0.1g of calli were calculated (Table 2).

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} 6-BAP in the dark to induce callus. Two months later, callus induction efficiency and callus color were calculated.

Regeneration of plantlets

Plant regeneration was induced by transferring embryoids to MS medium with 0.1 mg l^{-1} CPPU. The culture was incubated at 25±2°C with a 16/8h light and dark photoperiod at 50 μmol m^{-2} s^{-1} providing by cool white fluorescent lamps (Phillips).

Histological observation

The callus was sampled every 10 day intervals to histological observation according to Hu (2005). After fixing in FAA (70% ethanol: formalin: acetic acid, 90:5:5 v/v/v), they were stained with Ehrlich's hematoxylin, dehydrated with an ethanol series (50%, 70%, 85%, 95% and 100%), and then infiltrated with xylene and embedded in paraffin wax. Samples were sectioned into 10-12 μm thin sections using a rotary microtome and observed under Olympus CX31 microscope (Olympus, Japan).

Statistical analysis

The observation results were evaluated by analysis of variance. 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 $\alpha \leq 0.05$ and the mean and standard error (SE) were determined.

RESULTS

Embryogenic callus initiation

Root segments excised from *in vitro* plantlets of four lily cultivars were subjected to five callus induction treatments with different concentrations of auxin and cytokinin in the first series of experiments (Table 1). After 2 months of culture, primary calli were induced on root segments of all four cultivars and in all five culture media. All the calli exhibited a nodular-like structure. 'Manissa' and 'Siberia' calli were deep yellowish while 'Kankado' and 'Sorbonne' calli were yellowish. Callus

from roots presented different induction efficiencies in the four cultivars (Table 2). The effect of picloram and other cytokinins on callus induction of roots from *Lilium* was helpful, with highest frequency of callus induction observed in the presence of 1.00 mg l^{-1} picloram and 0.50 mg l^{-1} 6-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%). 'Sorbonne' and 'Manissa' generated high degree of callus while 'Siberia' generated low degree calli (Table 2). There were no statistically significant differences among 'Kankado', 'Manissa' and 'Sorbonne' on callus induction rate. But there were statistically significant differences between 'Siberia' and 'Kankado', 'Siberia' and 'Manissa' for the percentage of callus induction rate of roots (data not shown). Calli were effectively induced when picloram was added either alone or in combination with other cytokinins, but the callus induction frequency was significantly promoted when 6-BAP ('Kankado', 'Manissa', 'Siberia') or TDZ ('Sorbonne') was added to the induction medium compared to picloram alone (Table 2).

The effect of various combinations of picloram, 6-BAP and TDZ on proliferation of embryoids was determined after onee subculture. The average embryoids number induced from 0.100 g of root-derived calli on different media ranged from 11.67 to 70.33. The best medium for 'Siberia', 'Kankado' and 'Manissa' was MS medium supplemented with 1.00 mg l^{-1} picloram and 0.50 mg l^{-1} 6-BAP, the best one for 'Sorbonne' was MS medium supplemented with 1.0 mg l^{-1} picloram and 0.5 mg l^{-1} TDZ (Table 2). Statistical analysis showed that the embryoids were significantly promoted when 6-BAP ('Kankado', 'Manissa', 'Siberia') or TDZ ('Sorbonne') was added to the induction medium compared to picloram alone (Table 2).

In the early phase of the process, these calli looked soft, compact and would remain in this state for one month before new proliferation or differentiation of yellowish protuberances could appear. After two months culture, calli were transferred to the same medium and continued proliferating. Upon transferred onto fresh media, a progressive proliferation occurred. The nodular calli became glossy, and eventually various structures with globular or rounded shapes were observed (Fig.1-A). The

embryos ~~traversed all the known~~ stages of ontogeny viz. globular, shield-shaped (Fig.1-B), ~~a~~ cotyledonary shaped embryos, and developed into ~~a~~ whole plant (Fig.1-C, D).

After two months of culture, roots, bulb scales and leaves cultured on MS medium supplemented with 1.00 mg l^{-1} picloram and 0.50 mg l^{-1} 6-BAP produced callus, but they showed different callus induction efficiency. The highest callus induction efficiency was 93.33 % in roots whereas the lowest was 64.46 % in leaves (Table 3). Moreover, they showed different callus color. Callus color from bulb scales, leaves and roots were creamy white, yellow and deep yellow, respectively.

Regeneration of plantlets

After transferring to the plant regeneration medium, embryoids gradually turned green and developed into somatic ~~embings~~ with shoot and root poles (Fig.1-E). After one month they elongated and formed vigorous roots for acclimatization to outdoor conditions (Fig. 1-F). On MS medium with 0.1 mg l^{-1} CPPU, ~~better regeneration system was observed~~ (regeneration efficiency of 98.3%) and the acclimatized plantlets grew well without showing morphological variations.

Histological observation

Histological observation of callus cultures has been conducted, and an oval somatic embryo was shown in Fig. 1-G. Embryogenic cells divided actively to form clusters (Fig. 1-H) when transferred onto somatic embryo induction media. Histological observation demonstrated that the callus derived from roots was embryogenic and the process of plantlet regeneration from calli was via somatic embryogenesis.

DISCUSSION

Phytohormones can influence many developmental processes of plants, ranging from seed germination to shoot, root and flower formation. *In vitro* callus induction and development require the presence of auxins and cytokinins. Picloram, (4-amino-3,5,6-trichloropicolinic acid) a herbicide with auxin-like properties similar to 2,4-D (Kefford and Caso 1966), had been successfully utilized for totipotent callus lines in tissue cultures of lily (Mori et al. 2005), several grasses and other plants such as *Gasteria* and *Haworthia* (Beyl and Sharma 1983), *Typhus* (Zimmermann and Read

1986) and *Allium* (Phillips and Hubstenberger 1987). Picloram was shown to be more effective than 2,4-D and NAA in the initiation of callus and induction of somatic embryos in *Lilium longiflorum* (Tribulato et al. 1997) and other type of bulbous plants, such as *Hyacinthus orientalis* (Lu et al. 1988). Effect of picloram on callus induction ability of roots has been confirmed in the present study. ~~Individual effect of picloram and combined effect of picloram and 6-BAP, or TDZ were tested for the induction of embryonic calli and embryoids~~. The results showed that picloram in combination with 6-BAP (in 'Kankado', 'Manissa' and 'Siberia') or TDZ (in 'Sorbonne') elicited callus formation at the highest percent of explants than picloram alone except for 'Kankado' on the medium containing 1.0 mg l^{-1} picloram with and without 6-BAP (Table 2). 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). It has been reported that there was a higher frequency of callus induction on ~~appropriate level of~~ picloram compared to picloram combined with ~~other~~ cytokinin ~~in garlic~~ (Zheng et al. 2003), contrary results were obtained in this study. It ~~may~~ be due to the fact that different plants had different responses to exogenous hormone.

It has already been reported that picloram gave better results than 2,4-D for both callus induction and subsequent morphogenesis in several liliaceous species: somatic embryogenesis from leaf sections of *Gasteria* and *Haworthia* (Beyl and Sharma 1983) and callus formation as well as plant regeneration from shoot tips of *Allium cepa* (Phillips and Luteyn 1983). The present study showed for the first time that picloram had beneficial effects on callus induction of *Lilium* species using root explants.

To date, bulb scales (Mori et al. 2005, Suzuki et al. 1998, Nakano et al. 2000), immature leaves (Wickremesinhe et al. 1994), seeds (Mii et al. 1994), shoot tips (Godo et al. 1996, Godo et al. 1998), filaments with anthers (Arzate-Fernandez et al. 1997), styles and flower pedicels (Tribulato et al. 1997) have been used as explants for callus induction in lilies. However, there were relatively few reports on callus induction and plant regeneration from roots. Higher induction efficiency has been reported in explants such as filaments and thin cell layer (Arzate-Fernandez et al.

1997, Nhut et al. 2001, Nhut et al. 2002), but they were more difficult to obtain. Here we found that the average callus induction efficiency from roots was higher than leaves and bulb scale (Table 3), indicated that roots had higher regeneration rates than other explants and was easy to be cultured. The reason for higher induction and regeneration efficiency of roots may be due to the fact that the root apical had strong meristematic cells, or the endogenous hormones level in roots was higher than bulb scales and leaves thus made it easier to induce callus on the same hormone conditions. In our study, embryoids grew shoots and roots spontaneously on MS medium with 0.1 mg l^{-1} CPPU and regenerated plants were robust when transplanted to field. ~~It was concluded that roots were better explants to induce embryos and regenerate plants.~~

Although embryoids can grow on MS medium without PGRs, CPPU was used in order to make the plant grow robust and fast. 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. Watanabe (1989) has reported that CPPU can significantly promote the lily stem cell number, enhance the mechanical strength of the stem and to accelerate the lily bulbs growth. CPPU has been used to regenerate seedlings from embryoids in our study which was proved to be suitable for lily embryo regeneration.

Table 1 PGR concentrations in callus induction, somatic embryos initiation and plant regeneration

Table 2 Effects of plant growth regulators on callus and embryoid induction from lily roots

Note: Data are mean ± SE. Percentage of EC induction and number of embryos with the same letter is not significantly different ($\alpha \leq 0.05$).

[X]: Data were recorded 2 mo. after culture initiation. Values represent the mean ± SE of three independent experiments, each of which consisted of 30 explants.

[Y]: Data was determined after the first subculture. Values represent the mean ± SE of four independent experiments.

[V]: Callus color and type were determined 2 mo. after culture initiation. YN: yellowish nodular; DYN: deep yellowish nodular.

Table 3 Callus induction efficiency of different explants in 'Manissa'

[x]: Data were recorded 2 mo. after culture initiation. Values represent the mean ± SE of three independent experiments, each of which consisted of 30 explants.

[y]: Callus color and type were determined 2 mo. after culture initiation. Y: yellowish; DY: deep yellowish; CW: creamy white.

Fig. 1 Formation of callus cultures and plant regeneration of 'Kankado'.

A: Yellowish callus at globular stage cultured in the dark. Bar= 0.5mm. B: Shield-shaped embryos cultured in the dark. Bar= 0.5mm. C: A cotyledonary shaped embryo cultured in the dark. Bar= 0.5mm. D: Mature somatic embryo. Bar= 0.5mm. E: Regenerated plants containing roots and shoots cultured in the light. Bar= 5mm. F: Embryogenic tissue originated from root explants containing 0.1 mg l^{-1} CPPU. Bar= 5mm. G: Shield-shaped somatic embryo by histological observation. Bar= 0.5mm. H: Embryogenic cells dividing actively to form clusters (arrows). Bar= 0.05mm.

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