

Enhancing *in vitro* Growth of Bulbs for Mass Propagation of Lily Germplasm

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Abstract - Plants regenerated from *in vitro* cultures carry chromosomal variations, especially in long-term culture. Reducing the duration of plant tissue culture is one of the ways to reduce genetic and epigenetic changes. In this study, we reduced the duration of long-term culture and repeat subculture using small bulblets derived from bulb scales in two lily cultivars. The adventitious bulblets derived from bulb-scale tissue were cultured on three different media containing Murashige and Skoog (MS) basal medium supplemented with 1 g/L Charcoal, MS medium containing 0.3 mg/L IAA and 0.4 mg/L BA hormone with or without Charcoal, respectively. About seven weeks later, the number of newly propagated multiple shoots in the two media, A and B media, showed little differentiation. Compared to both media, the number of propagated multiple shoots increased 5-fold in MS medium containing 0.3 mg/L IAA and 0.4 mg/L BA hormone without Charcoal (C medium). The number of propagated multiple shoots ranged from 5 to 6 and 4 to 6 with an average of 5 in TropicalPink and GreenStar cultivars, respectively. The flow cytometric measurements indicated no variation in the ploidy level between control and *in vitro* propagated plants.

Key words – Activated charcoal, Bulb, *Lilium*, Ploidy, Propagation

Introduction

Lily, a member belonging to the genus *Lilium*, is one of the most economically important and popular ornamental crops cultivated worldwide as cut flowers and outdoor garden plants in many countries (Xia *et al.*, 2006). Consumer demand in the international flower market is often changing rapidly, and when new varieties are introduced to the market, old and unpopular varieties often disappear rapidly, despite their importance as genetic resources in the future. Also, the genus *Lilium* is facing a serious loss or genetic erosion due to weak immune system against pathogens, diseases, pests, adverse weather and other factors. Therefore, the development and supplementation of cryopreservation method, which is regarded as the only long-term method for the preservation of vegetative genetic resources, is an important strategy to respond rapidly to changing market demand and climate conditions (Halmagyi *et al.*, 2004; Wang and Perl, 2006). After developing high-quality varieties of *Lilium*, since it is difficult to obtain disease-

free bulbs, various *in vitro* tissue culture techniques have been used to establish mass propagation systems using bulb-scales (Joshi and Dhar, 2009) and apical meristems (Liu *et al.*, 2011). *In vitro* tissue culture conservation is susceptible to contamination and somaclonal variation (Chen *et al.*, 2011). Depending on the duration *in vitro* tissue culture in *Lilium*, challenges such as genetic variation, browning phenomenon, and reduced regeneration efficiency need to be addressed. *In vitro* cultures are associated with chromosomal variations, which are generally found in plants regenerated from long-term *in vitro* culture (Larkin and Scowcroft, 1981). Repeated subculture may reduce the regenerative efficiency (Wang *et al.*, 2002), and increase the amounts of somatic variation (Scowcroft, 1984). Genetic changes can be reduced by decreasing the age of plant tissue by reducing the duration of the sub-culture period.

The effects of activated charcoal (AC) on morphogenesis have been reported in *in vitro* tissue cultures of previous studies (Fridborg and Erikson, 1975; Fridborg *et al.*, 1978). It also facilitates generation of medium similar to natural soil environment following the addition of AC (Wang and Huang,

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1976). However, Ahuja (1985) reported that although addition of AC to the medium increased shoot elongation and leaf size, the addition of AC alleviated the inhibition of shoot buds and reduced the number of shoot formations in *Eucalyptus*. Also, Yang *et al.* (1992) reported that the addition of AC to the medium increased shoot elongation and inhibited the number of shoots differentiated in *Nicotiana tabacum*. Based on the results of previous studies, this study investigated the positive or negative effects of AC on the growth and differentiation of bulblets in lily cultivars and increased the number of shoots from bulblets for use in cryopreservation.

Materials and Methods

Plant material

The lily accessions used in this study are TropicalPink (*Lilium* FA hybrid ‘FA97-2’ × L. Asiatic hybrid ‘Kokarde’) and GreenStar [(SilkyWhite × Sunray) × Bomi] cultivars of *Lilium* belonging to *Liliaceae*. The lily accessions were obtained

from National Institute of Horticultural and Herbal Science (NIHHS) of the Rural Development Administration (RDA).

Surface sterilization

The plant materials used TropicalPink and GreenStar accessions to obtain disease-free bulblets from scales for cryopreservation in *Lilium*. Bulbs were washed to remove bacterial clump and residues under running tap water. Inner scales of the bulbs in these two cultivars were sterilized to the modified surface-sterilization as described previously by Askari *et al.* (2014) and Song *et al.* (2019). Scales detached from the bulbs were surface-sterilized for 20 min in 1.6% (w/v) sodium hypochlorite (NaClO). These scales were then rinsed for 3 min with sterile distilled water and 3 min with 0.03% NaClO on a clean bench. During the final step, we submerged the scales in 0.03% NaClO for 2 h instead of sterile distilled water. The inner scales were dried on the sterilized paper for 3 min.

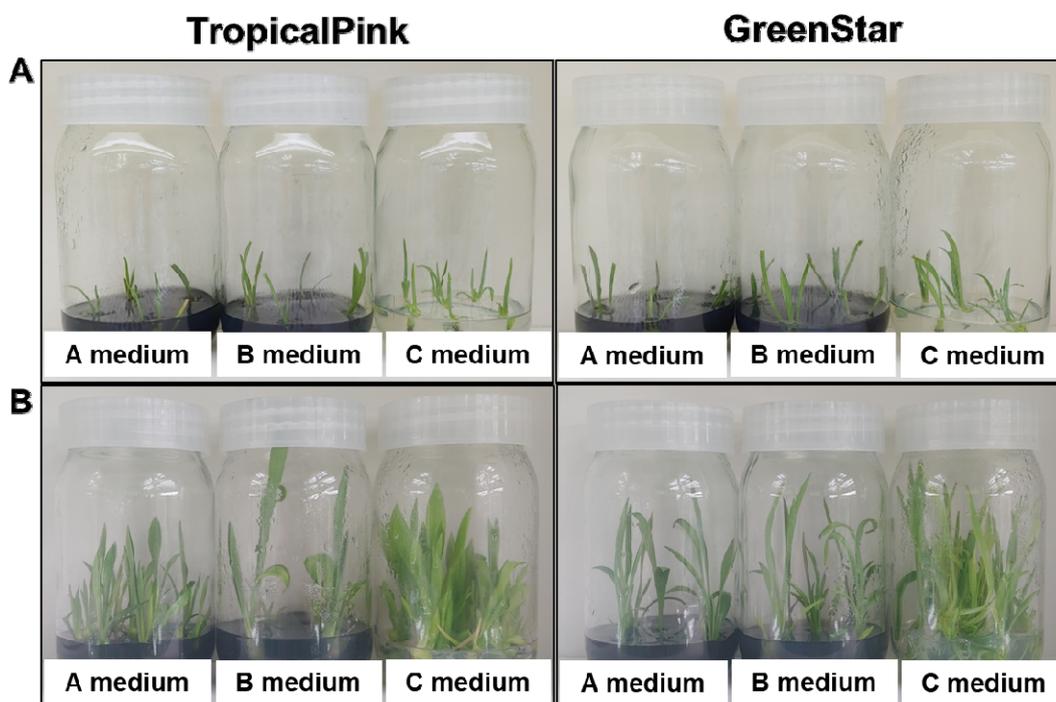


Fig. 1. Comparison of growth and propagation on three different media in two *Lilium* spp. The newly generated bulblets from inner scales were transferred to different media for 1 week (A) and the plants were propagated after 7 weeks of cultivation (B). Murashige and Skoog (MS) basal medium supplemented with 1 g/L Charcoal (A medium), MS medium containing 0.3 mg/L IAA and 0.4 mg/L BA hormone with (B Medium) or without Charcoal (C Medium).

***In vitro* propagation conditions**

Plants during *in vitro* micropropagation were stored in the incubation room at $24 \pm 1^\circ\text{C}$ under 16 h of photoperiod. The inner bulb scales were cultured and inverted on Murashige and Skoog (MS) medium containing 0.1% Plant Preservative Mixture (PPM, Apollo Scientific Limited, UK), which is a robust broad-spectrum biocide designed for use in plant tissue culture (Guri and Patel, 1998). The adventitious bulblets formed on the surface of inner scales were separated and cultured in MS medium, immediately. After bacterial examination on the Brain Heart Infusion (BHI, Difco, agar Becton Dickinson) agar medium plates (Song *et al.*, 2019), non-contaminated bulblets were transferred to following media: MS basal medium supplemented with 1 g/L Charcoal (A medium), MS medium containing 0.3 mg/L indole-3-acetic acid (IAA) and 0.4 mg/L benzyl adenine (BA) hormone with (B Medium) or without Charcoal (C Medium) (Fig. 1).

Flow cytometry

The ploidy variations were analyzed by flow cytometry according to the procedure described by Naing *et al.* (2019). The plant sample, comprising about 20 mg of leaf tissue, in a petridish containing 0.5 mL nuclei extraction buffer (Sysmex Partec GmbH, Germany) was chopped using a sharp razor blade. The chopped sample was filtered through 50 μm nylon filter (CellTrics filter, Sysmex Partec GmbH, Germany) and the filtered solution was mixed with 2.0 mL of the staining buffer (Sysmex Partec GmbH, Germany). The prepared sample from each plant was analyzed to determine the ploidy variation using a CyFlow Ploidy Analyzer (Sysmex Partec GmbH, Germany).

Results and Discussion

The conventional and existing method of bulb-proliferation using *in vitro* tissue culture technology produces small bulbs directly by cultivating tissues such as scales for bulblet formation during *in vitro* micropropagation. Several bulbs can be produced via division of the new bulblet and recultivation. *In vitro* micropropagation is used to rapidly propagate cultivars, and results in the production of pathogen-free propagation sources (Hartmann *et al.*, 2011). Although

this method is a secure way for the vegetative propagation of lily, it is time-consuming and may induce increasing levels of somatic variation (Scowcroft, 1984), since the separation and regrowth of the bulblets may take months to reproduce via repeated subculture. Various studies focus on the factors contributing to efficient *in vitro* micropropagation, including the use of explants (Joshi *et al.*, 2009), media conditions (Ozel *et al.*, 2015), and callus induction (Youssef *et al.*, 2019). Although some studies investigated the effects of AC on *in vitro* plant differentiation (Paek *et al.*, 1998), additional studies are needed to confirm the effects of AC on the propagation of lilies. Therefore, in this study, we checked the effects of AC on *in vitro* plant propagation in lily cultivars and facilitated plantlets propagation by excluding of AC from the propagation medium for cryopreservation.

Proliferation of adventitious bulblets under controlled media conditions

Appropriate methods for *in vitro* propagation are important to increase the production rate and plant quality rapidly. This experiment was conducted to determine the most appropriate media conditions for propagation of adventitious bulblets of lily cultivars within the short period of time for cryopreservation. The multiplication method in this experiment is based on new bulblets formed from bulb-scales in two lily cultivars, TropicalPink and GreenStar. The adventitious bulblets formed from the inner scales were cultured on three different media containing MS basal medium supplemented with 1 g/L AC (A medium), MS medium containing 0.3 mg/L IAA and 0.4 mg/L BA hormone with AC (B medium) or without AC (C medium), respectively. After nearly seven weeks, the number of new plantlets propagated to small bulblets in the two media, A and B media, showed little variation (Fig. 1). However, the propagated plantlets belonging to TropicalPink and GreenStar cultivars in the C medium increased 1.5- and 1.8-fold, respectively. The number of multiple shoots increased about 5-fold in C medium compared to both A and B media. The number of propagated plantlets ranged from 5 to 6 and 4 to 6 with an average of 5 in TropicalPink and GreenStar cultivars, respectively (Table 1). However, the propagated plantlets were not rooted on C medium in both cultivars. New shoots formation and roots inhibition rather than plant growth was promoted in

Table 1. Propagation of small bulbs induced from inner scales after transfer to the different media in two lily cultivars

Cultivars of lily	Medium ^z	No. of bulblets tested	No. of plantlets after 7 weeks	No. of plantlets propagated	Root status
TropicalPink	A	8	9	1.3 ± 1.2	O
	B	4	4	0.3 ± 0.6	O
	C	9	14	5.3 ± 0.6	X
GreenStar	A	6	7	0.7 ± 0.6	O
	B	6	7	0.7 ± 0.6	O
	C	6	11	5 ± 1	X

^zMurashige and Skoog (MS) basal medium supplemented with 1 g/L Charcoal (A medium), MS medium containing 0.3 mg/L IAA and 0.4 mg/L BA hormone with (B Medium) or without Charcoal (C Medium).

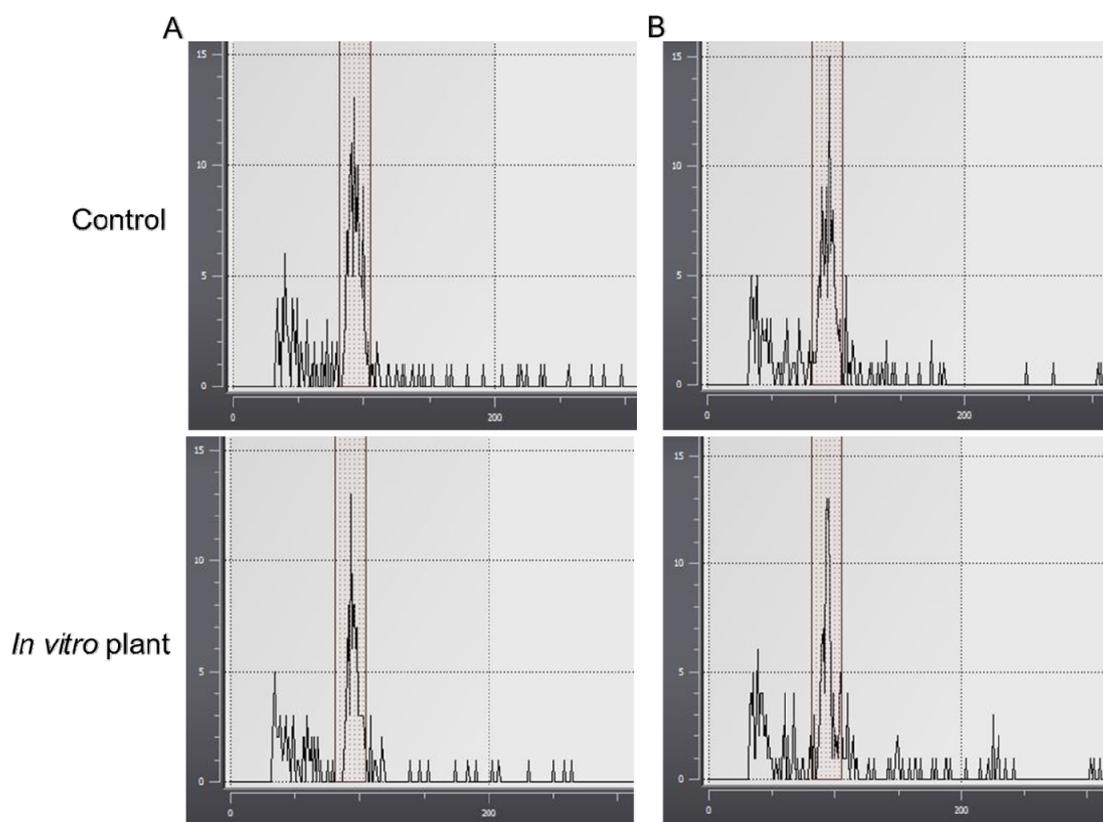


Fig. 2. Comparison of the ploidy levels between plants in the greenhouse (control) and plants generated *in vitro* from different lily cultivars, A: TropicalPink and B: GreenStar.

the medium including IAA 0.3+BA 0.4 without AC (C medium), while plant growth and development were induced by absorbing hormones, especially BA, in the B medium supplemented with AC (Fig. 1). Thus, the formation of multiple shoots was induced by the accumulation of plant hormones in the bulblets on C medium containing IAA and BA by excluding AC. The most crucial feature of adding AC to the culture media is decline in concentration of the added

hormones, naphthaleneacetic acid (NAA) and BA, in the medium due to the absorption of the growth regulating substance by AC (Steimitz and Yahel, 1982). Similar results were reported that new shoots are induced from plantlets laterally due to excessive levels of plant hormones such as auxin and cytokinin (Amasino and Miller, 1982) and by another study in *Nicotiana tabacum* (Yang *et al.*, 1992). Most of the reports confirmed the positive effects of AC in medium

promoting growth and development of plant tissues (Ebert *et al.*, 1993; Fridborg *et al.*, 1978). The addition of AC to the plant tissue culture medium induces absorption of growth inhibitors secreted from culture tissues, inhibits the growth of calli, and root formation of somatic embryogenesis (George and Sherrington, 1984). However, AC induced negative results were also reported in some micropropagation. The addition of AC to overcome phenolic exudation in the medium reduced the number of shoots due to the adsorption of hormones needed for plant growth in *Gymnema sylvestre* micropropagation (Komalavalli and Rao, 2000). Further, the addition of AC to the medium increased shoot elongation and leaf size, but suppressed the shoot formation and differentiation in *Eucalyptus* (Ahuja, 1985), *N. tabacum* (Yang *et al.*, 1992), and cashew (*Anacardium occidentale*) (Boggetti *et al.*, 1999).

Based on various aspects of the effects of AC on *in vitro* micropropagation, we induced multiple shoots of the adventitious bulblets on the C medium containing the plant hormones by excluding AC. The proliferation technique used in this study reduced the long-term culture of plant by reducing repeated subculture, and provided basic information for multiple shoot formation from adventitious bulblets in lily cultivars for use in cryopreservation.

Examination of ploidy levels by flow cytometry

The ploidy levels of plants grown generally in the greenhouse (control) and *in vitro* propagated plants of two different lily cultivars, TropicalPink and GreenStar, were measured by flow cytometry. As a result of the ploidy test, the ploidy levels between the control and *in vitro* plant indicated no difference in the number of chromosomes or genetic variation (Fig. 2). Therefore, the *in vitro* propagated plants of the two cultivars are genetically similar to the control plants grown in the greenhouse by reducing the duration of *in vitro* propagation and the repeat subculture in this study.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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