

Potential utilization of filaments and ovules as explant sources in *in vitro* propagation of *Lilium* sp.

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Abstract

Lilies are important cut and pot flowers locally and globally; however, qualified planting materials from *in vitro* culture are still limited. The culture generally uses scales and leaves, while filaments and ovules are rarely utilized. Filaments and ovules of EXO CF/E, RDF CG/E, and DZ CG/E genotypes of *Lilium* sp. were explored for their potential to establish *in vitro* propagation protocol using different Chu N6 medium and their modifications. The 9.9 shoots/explant from 1.5 regenerative explants/replication of RDF CG/E filaments was proven on Chu N6 medium containing 6.79 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 2.27 μM thidiazuron (TDZ), and 90 g L⁻¹ sucrose; 3.3 regenerative explants/replication and 8.4 shoots/explant of EXO CF/E ovules were determined on Chu N6 medium with 2.26 μM 2,4-D, 6.81 μM TDZ and 90 g L⁻¹ sucrose; and slightly improved on Chu N6 macro elements, full vitamins of MS basal medium with 3.39 μM 2,4-D, 3.41 μM TDZ, 150 ml L⁻¹ coconut water (CW) and 60 g L⁻¹ sucrose. The shoots were proliferated on the established media. The shoots of EXO CF/E, RDF CG/E and DZ CG/E genotypes produced varied leaves/shoot, leaf length, leaf width, roots/shoot, root length and bulb diameter on Chu N6 medium hormone free with 1.5% AC, followed by hardening them in a glass house for one month. The hardened-plantlets were acclimatized in plastic pots containing charcoal husk and bamboo compost (1:1) with 39.8-63.6% survivability. The protocol has the potential to be applied to other genotypes or lilies by specific improvements at the acclimatization stage.

Keywords: adventive shoots; filament; *in vitro*; *Lilium* sp.; ovule

Introduction

Lilies are the most important, beautiful and economically viable blossoming plants, widely used as cut flowers and potted plants in the commercial market locally and globally (Ruffoni *et al.*, 2011; Saetiew and

Received: 31 Jul 2023. Received in revised form: 02 Nov 2023. Accepted: 29 Jan 2024. Published online: 12 Feb 2024.

From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

Umamanit, 2015; Kumar and Kumar, 2021; Rafiq *et al.*, 2021). In the Netherlands, *Lilium* sp. and their hybrids are ranked in the second-largest flower bulb crop (El Sawy *et al.*, 2021) and an important bulbous flower crop in India (Singh, 2017; Singh *et al.*, 2019; Sharma and Sharma, 2021). In Indonesia, the plant is generally cultivated in Cugenang-Cianjur and Cisarua-Bogor, Jawa Barat; Bandungan-Semarang, Jawa Tengah; and Batu-Malang, Jawa Timur (Tokobungahias, 2012). The flowers have generally been planted by medium to big growers and rarely carried out by small farmers due to high production costs, especially for buying qualified planting materials. The *Lilium* cut flower is sold for IDR 10,000 to IDR 99,000 per stem (Shabur, 2022), while the planting material of the plants in bulb shape is sold IDR 4,000 to IDR 45,000 per bulb depending on *Lilium* types (Shabur, 2022). The benefit-cost ratios per 540 squares meters were 21.20 in the first season and 21.02 in the second season in Egypt under the greenhouse, ascorbic acid and artificial light treatment (El Sawy *et al.*, 2021). Though the *Lilium* flower has high economic value, developing the plants in commercial scale for small growers and farmers is limited by the availability and sustainability of cheaper and better qualified planting materials.

Traditionally, *Lilium* sp. is generally propagated vegetatively with stem bulbils, stem base bublets, using scales from the main bulb and generatively by using seeds from the pods (Ruffoni *et al.*, 2011; Bakhshaie *et al.*, 2016; Deswiniyanti and Lestari, 2020; Rafiq *et al.*, 2021). Though vegetative methods were easier carried out, maintained desirable characters and produced identical quality as the parent (Askari *et al.*, 2018), the methods were not suitable for commercial purposes of the plant (Lestari *et al.*, 2020; Yan *et al.*, 2020) due to the restricted the number of offspring (Bakhshaie *et al.*, 2010; Askari *et al.*, 2018). While generative method that is generally used to produce new cultivars, produce undesirable variation of offspring, take more time to produce seeds/bulbs and have a long juvenile period (Bakhshaie *et al.*, 2016; Rafiq *et al.*, 2021). The method is also not suitable for scaling up the plant commercially. Therefore, producing high quality and productivity with a lower price of *Lilium* planting materials using tissue culture as a very useful approach for rapid propagation is importantly addressed (Tokgoz and Altan, 2020), especially in developing and establishing an *in vitro* mass propagation protocol (Joshi and Dhar, 2009; Naeem *et al.*, 2013; Rafiq *et al.*, 2021)

Developing and establishing *in vitro* mass propagation protocols for *Lilium* sp. with varied results was reported previously. Saetiew and Umamanit (2015) successfully established micropropagation of *Lilium formolongo* using leaves as an explant source regenerated on MS medium augmented with 5.37 μM α -naphthalene acetic acid (NAA) and 2.27 μM TDZ via callogenesis under dark incubation producing a 2.93 cm callus and after 14 weeks of incubation the callus produced 5.05 shoots and 36.5 leaves under light incubation. Bulb scales planted on MS medium containing 5.37 μM NAA and 4.44 μM N-6 benzylaminopurine (BAP) stimulated a 100% growth percentage with 4.3 shoots per explant and 1.4 microbulbs of *Lilium longiflorum* (Deswiniyanti and Lestari, 2020). A single scale of *Lilium candidum* planted on MS medium with 0.23 μM TDZ and 1.07 μM NAA resulted in 6.5 bublets per explant and 26 bublets per baby food culture jar were regenerated on the similar medium, rooted $\frac{1}{2}$ MS medium fortified by 3.2 μM IBA and successfully transferred *ex vitro* with high survivability (Royandazagh, 2019). Culturing leaf explants of *Lilium orientalis* 'Starfigther' on MS medium added by 2.27 μM TDZ and 45.24 μM 2,4-D regenerated 5.7 shoots after 1 month and significantly proliferated with 8.3 shoots/explant, 5.4 leaves/shoot and 8.8 scales/explant (Youssef *et al.*, 2019). Leaves and scales of the Asiatic *Lilium* cv. 'Strawberry' and 'Cream', Oriental *Lilium* cv. 'Double Surprise', and *Lilium longiflorum* Thunb. cv. 'White Heaven' regenerated on MS medium containing 6.21-10.35 μM Picloram and 1.1 μM NAA were suitable combination for somatic embryogenesis with an induction efficiency of 88-96% (Yan *et al.*, 2020). Basal scale segments of oriental hybrid *Lilium* 'Ravena' planted on MS medium with 2.69 NAA and 8.88 μM BAP produced 76.2% culture establishment, 5.5 bublets/explant, 2.2 cm shoot length, and 3.4 leaves. The shoots were rooted in MS medium with 7.38 μM IBA and successfully acclimatized with 98.9% survivability in a mixture of perlite and vermiculite (1:1) after hardening (Rafiq *et al.*, 2021). This research result above confirmed that *in vitro* mass propagation of *Lilium* sp. was generally established using

leaves and scales as explant sources using MS medium with varied combinations and concentrations of hormones, however, establishing the new *in vitro* mass propagation protocol using flower parts such as the filament and ovule as explant source and Chu N6 medium after 2011 until today has not been published yet.

The current research was conducted to explore potentially production of *Lilium* sp. plantlets from the filament and ovule explants. The objective of the research was to establish potential utilization of filaments and ovules as explant sources in micropropagation of *Lilium* sp.

Materials and Methods

Planting materials, disinfection, explant preparation and culture incubation

Planting materials used in the experiment were young and unopened flower buds in 5-6 cm in length derived from *Lilium* sp. received from Melrimba Ltd. Jl. Raya Puncak, Cisarua, Bogor, Jawa Barat-Indonesia that kept in three different codes, i.e., EXO CF/E, RDF CG/E and DZ CG/E due to confidential company, were then called as genotypes. The materials were then prepared for disinfection purpose by cutting the young and unopened flower buds, collecting, and putting them in the plastic container for pre-disinfection.

Placing the prepared explants under tap water for ± 30 minutes was used as pretreatment. After the pretreatment, the explants were immersed in a 1% sunlight liquid solution for ± 30 minutes and shaken manually, followed by rinsing them using clean water 4-6 times to remove all the sunlight liquid solution. The young buds were then brought into the laminar for further disinfection. The explants were treated in 0.05% mercury chloride for three minutes with manual shaking, followed by rinsing with sterile distilled water for three minutes, dipped in 0.01% mercury chloride for seven minutes and shaken manually, and rinsed them six times in sterile water. Each time was two minutes.

Explant sources investigated in the experiment were filaments and ovules. The filaments and ovules were isolated by removing all petals covering them under binocular microscopes with $10 \times$ magnification. Using tissue culture blade, the filaments were sliced in the basal part and their distal part removed, then cultured horizontally on the culture media tested. While ovules were cut transversally using a tissue culture blade in the basal and distal part, then cultured on the culture medium horizontally.

Cultured explants both filaments and ovules were incubated under dark incubation for one month. The explants were then moved to incubation under 16 h photoperiod of cool fluorescent lamps with $13 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 23 ± 1 °C until explant regeneration clearly observed.

The basal media used in the experiment were Chu N6 and Murashige-Skoog (MS) medium with vitamins. 3.99 g of Chu N6 and 4.43 g of MS basal medium were weighted on analytical balance, then poured into the 1-liter plastic beaker glass containing 950 ml distilled-water, added by 90 or 60 g L⁻¹ sucrose, and mixed uniformly using a wood mixer manually. The medium solution was then added with hormones as treatments researched, measured and adjusted to a pH medium of 5.8. All hormones used in the research were Phytotechnology Laboratory products. The solution media were added by the Pure it water until 1000 ml, 4.9 g L⁻¹ agar, homogenized manually using a wood mixer, cooked on a gas stove until boiling status, and then poured the boiled medium solution into jam bottles (9 cm in height and 6 cm in diameter) as culture vessels with 25-30 ml for each jam bottle. The bottles with different composition types of media were autoclaved in a 1941X of All American autoclave at 121 °C with 15 kPa for 20 minutes. The sterile media were then placed on the rack and ready to be used 48 h after sterilization.

Adventitious shoot initiation derived from different Lilium sp. genotypes and initiation culture media

Three different genotypes of *Lilium* sp used in the first experiment and given different codes were EXO CF/E, RDF CG/E, and DZ CG/E. Two different explant types tested in the experiment were filaments and ovules. While initiation culture media (IM) applied in the experiment were Chu N6 medium with 9.05 μM

2,4-D, 0 μM TDZ and 90 g L^{-1} sucrose (IM-1), Chu N6 medium with 6.78 μM 2,4-D, 2.27 μM TDZ and sucrose in 90 g L^{-1} (IM-2), Chu N6 medium with 4.52 μM 2,4-D, 4.54 μM TDZ and 90 g L^{-1} sucrose (IM-3), Chu N6 medium with 2.26 μM 2,4-D, 6.81 μM TDZ and 90 g L^{-1} sucrose (IM-4), Chu N6 medium added by 0 μM 2,4-D, 9.08 μM TDZ and 90 g L^{-1} sucrose (IM-5), MS medium with 8.28 μM picloram, 9.08 μM TDZ and sucrose in 60 g L^{-1} (IM-6), MS medium with 6.21 μM picloram, 2.27 μM TDZ and sucrose in 60 g L^{-1} (IM-7), MS medium added by 4.14 picloram, 4.54 μM TDZ and sucrose in 60 g L^{-1} (IM-8), and medium of MS augmented with 2.07 μM picloram, 6.81 μM TDZ and 60 g L^{-1} sucrose (IM-9). In the initiation stage, there were two different experiments carried out separately based on explant types. The experiment was arranged in a completely randomized design (CRD) factorial pattern with three replications. Three different genotypes were the first factor and IM was the second factor. Three bottles were for each treatment. Four explants were cultured in each bottle.

Variables measured in the experiment were: (1) percentage regeneration of explant (%); (2) percentage browning of the explant (%); (3) number of regenerative explants/replications; and (4) number of shoots/explants. Periodical observation from initial culture until the end of the experiment was carried out to know each alteration of the explant in response to culture medium. Measuring and taking data based on the variables observed was conducted at ± 70 days after culture.

Adventitious shoot initiation improvement of EXO CF/E ovules in different improved initiation culture media

The promising initiation culture medium determined from the previous experiment was improved by adding indole acetic acid (IAA), NAA and CW. The ovules of EXO CF/E were used in the experiment due to the positive response proven in the previous experiment and the sufficient material availability to carry out the experiment. Improved-initiation culture media (IPM) investigated in the stage were Chu N6 medium with 2.26 μM 2,4-D, 6.81 μM TDZ and sucrose in 90 g L^{-1} (IPM-1, as control), Chu N6 medium containing 2.26 μM 2,4-D, 6.81 μM TDZ, 2.87 μM IAA and sucrose 90 g L^{-1} (IPM-2), Chu N6 medium containing 2.26 μM 2,4-D, 6.81 μM TDZ, 2.68 μM NAA and sucrose in 90 g L^{-1} (IPM-3), Chu N6 medium containing 2.26 μM 2,4-D, 6.81 μM TDZ, CW in 150 ml L^{-1} and sucrose in 90 g L^{-1} (IPM-4), Chu N6 macro elements, full vitamins of MS basal medium augmented with 3.39 μM 2,4-D, 3.41 μM TDZ, 2.87 μM IAA and sucrose in 60 g L^{-1} (IPM-5), Chu N6 macro elements, full vitamins of MS basal medium augmented with 3.39 μM 2,4-D, 3.41 μM TDZ, 2.68 μM NAA and sucrose in 60 g L^{-1} (IPM-6), Chu N6 macro elements, full vitamins of MS basal medium augmented with 3.39 μM 2,4-D, 3.41 μM TDZ, CW in 150 ml L^{-1} and sucrose in 60 g L^{-1} (IPM-7), and Chu N6 macro elements, full vitamins of MS basal medium augmented with 3.39 μM 2,4-D, 3.41 μM TDZ, glutamine in 500 mg L^{-1} and sucrose in 60 g L^{-1} (IPM-8). CRD with four replications was applied to the experiment with three bottles in each treatment and four ovule explants cultured in each bottle. Variables observed, data taken and measured were similar to those previous experiments.

Preparation of plantlets and their acclimatization

Regenerated adventitious shoots that resulted from the previous experiment were proliferated by subculturing the shoots periodically in established initiation culture medium both suitable for filaments and ovules, i.e., ICM 2 and IPM-7 (Figure 1G). The single proliferated shoots were rooted on Chu N6 medium hormone free supplemented with 1% activated charcoal (Figure 1H). In the plantlet preparation step, there were $\pm 7-8$ bottles for each genotype used as a replication and repeated nine times. In this step, proliferated shoots from three genotypes were evaluated under CRD with nine replications with 7-8 bottles per treatment and four shoots cultured per bottle. The rooted shoots were hardened by placing them in a glass house for one month (Figure 1I). After one month of plantlet hardening, the plantlets were harvested by gently taking plantlets from culture vessels, removing the remains of agar from the root surface under tap water followed by immersing the bottom part of plantlets in a 1% systemic pesticide solution for fungi and bacteria for 3 minutes (Figure 1J), then air-dried for a while on magazine paper (Figure 1K). Before planting well-rooted shoots in

pots (diameter 15 cm) filled with charcoal husk and bamboo compost (1:1, v/v), three different performances of plantlets were measured: leaf number per shoot, length of leaves (cm), leaf width (mm), root number per shoot, length of roots (cm), and bulb diameter (mm).

After measuring their performances, the plantlets were acclimatized in pots containing acclimatization media that were watered sufficiently. Each pot was planted with 20-25 plantlets (Figure 1L). Pots containing plantlets were placed in a glass house in a reduced light area. Water spraying to maintain and support the growth of plantlets was carried out periodically, following condition of media. In the acclimatization stage, plantlets derived from three genotypes were investigated under CRD with nine replications with one pot per treatment and 20-25 plantlets per pot. Variables in the experiment were: (1) number acclimatized plantlets per pot; (2) survival plantlets per pot; (3) death plantlets per pot; and (4) percentage of survivability. The data were observed and measured 1.5 months after acclimatization.

Data analysis

Analysis of variance (ANOVA) was used to analyse all quantitative data using SAS Release Windows 9.1 resulted from the initiation to acclimatization stage were analysed. Significant differences between means were further assessed by the Tukey test, $P = 0.05$ (Mattjik and Sumertajaya, 2006).

Results

It was clearly known based on periodical observation that swelling explants both from filaments and ovules were occurred in slicing surface area in the basal parts of explants 10-15 days after culture. Though browning explants were observed in the experiment, the browning explants did not inhibit the regeneration capacity of explants. Furthermore, 1.2-1.5 months after culture, callus initiation took place, with the volume of the callus compared to the origin size of the explant varying from + to +++ (Figures 1A and 1D). In the next step, the initial adventitious shoot was obviously formed 7-15 days after the callus initiation (Figure 1B and 1E). The adventitious shoots were clearly observed and recorded 2.5 months after culture initiation (Figure 1C and 1F). The adventitious shoots were varied from 1-9 shoots derived from filaments (Figure 1C) and 1-14 shoots derived from ovules in improved medium (Figure 1F) with different leaf length and width. Based on the number of regenerative explants per replication (NRER) and number of shoots per explant (NSE), utilization of filaments as explant sources was better than ovules; based on NSE derived from the filament explant, RDF CG/E gave better results than DZ CG/E and EXO CF/E and IM-3 was the most potential initiation culture medium compared to others (Supplementary data: Tables 1 and 2). While derived from ovules explants, EXO CF/E were better than DZ CG/E and RDF CG/E, and IM-4 were a promising medium for adventitious shoot formation (Supplementary data: Tables 5 and 6).

Combination treatments based on *Lilium* sp. genotypes and initiation culture media both for filaments and ovules as explant sources that were carried out in two different experiments indicated significant interaction effect in all variables noted. For filaments as explant sources, The RDF CG/E filaments cultured on IM-2 (Chu N6 medium with 6.78 μ M 2,4-D, 2.27 μ M TDZ and 90 g/l sucrose) were the optimal treatment, resulting in 37.5% explant regeneration, no browning explants, 1.5 NRER and 9.9 NSE (Supplementary data: Tables 3 and 4; Tables 1 and 2; Figures 1B and 1C). The second-best combination treatment was noted on DZ CG/E filaments cultured on IM-1, while other combination treatments had lower results compared to the two best combination treatments. In the second experiment, ovules of EXO CF/E cultured on IM-4 were the high potential combination treatment in producing the highest adventitious shoots with 81.3% regeneration explants, 18.8% browning explants, 3.3 NRER and 8.4 NSE (Supplement data: Tables 7 and 8; Tables 3 and 4). DZ CG/E ovules cultured on IM-1 was recorded as the second-best combination treatment. Other combinations dominantly did not have positive responses to support the research purpose.

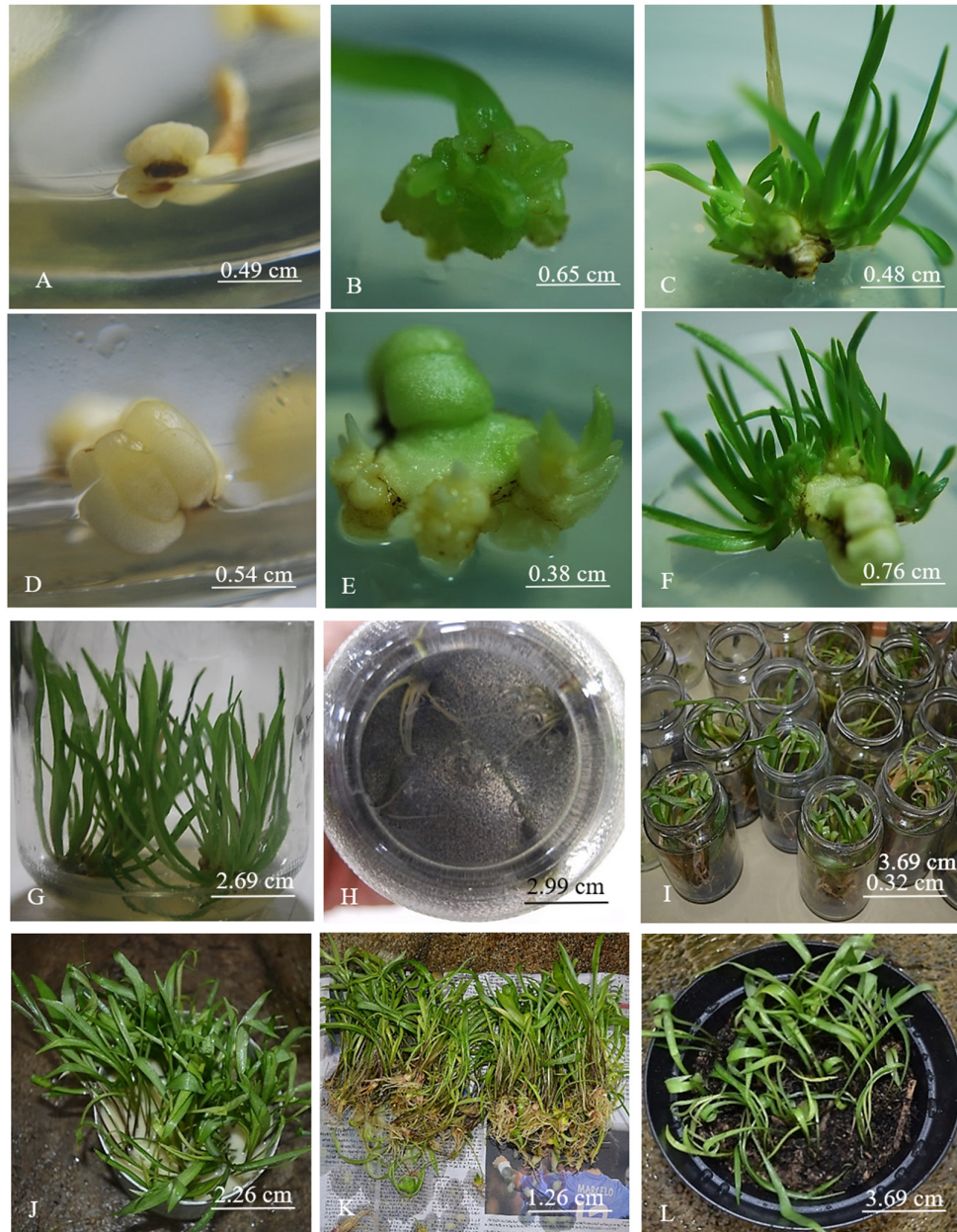


Figure 1. *In vitro* mass propagation of *Lilium* sp. via adventitious shoot regeneration derived from the filament and ovules as explant sources from initiation to acclimatization

A. Callus initiation derived from the filament of RDF CG/E on Chu N6 medium with 6.78 μM 2,4-D, 2.27 μM TDZ and sucrose in g L^{-1} (IM-2) 1.1 months after culture, B. Initial adventitious shoots derived from filaments on IM-2 at 1.4 months after culture under light incubation, C. Adventitious shoots derived from filaments on IM-2 at 2.5 months after culture, D. Callus derived from ovules of EXO CF/E on Chu N6 macro elements, full vitamins of MS basal medium augmented with 3.39 μM 2,4-D, 3.41 μM TDZ, CW in 150 ml L^{-1} and sucrose in 60 g L^{-1} (IPM-7) 1.15 months after culture, E. Initial adventitious shoots derived from ovules on IPM-7 at 1.5 months after culture under light incubation, F. Adventitious shoots derived from ovules on IPM-7 at 2.5 months after culture, G. Adventitious shoot proliferation on IPM-7 at 1.5 months after subculture, H. Rooted shoots on Chu N6 hormone free with 1.5% AC 1.4 after culture, I. RDF CG/E plantlets one month after hardening under glass house condition, J. Immersing roots of The RDF CG/E plantlets in 1% pesticide solution for ± 3 minutes, K. Short drying of plantlets after pesticide treatments on magazine paper, L. Growth of Acclimatized-plantlets 2 days after acclimatization in charcoal husk and bamboo compost (1:1).

Table 1. Effect of interaction between initiation culture media and *Lilium* sp. filament genotypes on number of regenerative explants/replication

<i>Lilium</i> sp. genotype /initiation culture medium	IM-1	IM-2	IM-3	IM-4	IM-5	IM-6	IM-7	IM-8	IM-9
EXO CF/E	0.0 ± 0.00 b	0.0 ± 0.00 b	1.8 ± 0.29 a	1.0 ± 0.65 a	1.5 ± 0.58 a	1.5 ± 0.41 a	1.0 ± 0.41 a	1.8 ± 0.29 a	1.8 ± 0.87 a
RDF CG/E	1.3 ± 0.63 a	1.5 ± 0.41 a	1.3 ± 0.58 a	1.5 ± 0.41 a	0.0 ± 0.00 b	1.5 ± 0.41 a	1.0 ± 0.48 a	0.0 ± 0.00 b	1.5 ± 0.41 a
DZ CG/E	1.5 ± 0.41 a	2.3 ± 0.96 a	2.3 ± 0.29 a	1.3 ± 0.65 a	1.3 ± 0.61 a	0.0 ± 0.00 b	1.5 ± 0.41 a	0.0 ± 0.00 b	1.5 ± 0.71 a
Tukey value	13.00	21.00	5.14	1.80	10.33	27.00	2.00	47.41	0.23
Probability	*	*	*	ns	*	*	ns	**	ns

Note: ns-no significant different; * - significant different; ** - very significant different. IM-1, Chu N6 medium with 9.05 μM 2,4-D, 0 μM TDZ and 90 g L^{-1} sucrose, IM-2, Chu N6 medium with 6.78 μM 2,4-D, 2.27 μM TDZ and sucrose in g L^{-1} , IM-3, Chu N6 medium with 4.52 μM 2,4-D, 4.54 μM TDZ and 90 g L^{-1} sucrose, IM-4, Chu N6 medium with 2.26 μM 2,4-D, 6.81 μM TDZ and 90 g L^{-1} sucrose, IM-5, Chu N6 medium added by 0 μM 2,4-D, 9.08 μM TDZ and 90 g L^{-1} sucrose, IM-6, MS medium with 8.28 μM picloram, 9.08 μM TDZ and sucrose in 60 g L^{-1} , IM-7, MS medium with 6.21 μM picloram, 2.27 μM TDZ and sucrose in 60 g L^{-1} , IM-8, MS medium added by 4.14 picloram, 4.54 μM TDZ and sucrose in 60 g L^{-1} , and IM-9, medium of MS augmented with 2.07 μM picloram, 6.81 μM TDZ and 60 g L^{-1} sucrose. Means followed by the different letters in the same column indicated differences under Tukey test ($p < 0.05$).

Table 2. Interaction effect of initiation culture media and *Lilium* sp genotype filaments on number of adventitious shoots/explant

<i>Lilium</i> sp. genotype /initiation culture medium	IM-1	IM-2	IM-3	IM-4	IM-5	IM-6	IM-7	IM-8	IM-9
EXO CF/E	0.0 ± 0.00 b	0.0 ± 0.00 c	6.5 ± 1.08 a	4.8 ± 1.50 a	4.0 ± 1.22 a	2.3 ± 0.65 b	4.8 ± 0.96 b	4.0 ± 0.82 a	4.5 ± 1.25 a
RDF CG/E	2.3 ± 0.96 b	9.9 ± 0.85 a	4.5 ± 1.08 b	6.0 ± 0.81 a	0.0 ± 0.00 b	6.3 ± 1.71 a	6.8 ± 1.26 a	0.0 ± 0.00 b	6.3 ± 0.96 a
DZ CG/E	8.5 ± 1.29 a	5.5 ± 0.91 b	5.3 ± 0.65 ab	5.3 ± 0.96 a	4.8 ± 0.29 a	0.0 ± 0.00 b	2.8 ± 0.87 c	0.0 ± 0.00 b	2.3 ± 0.29 b
Tukey value	40.10	38.04	4.45	1.50	32.38	36.08	19.86	36.00	18.09
Probability	**	**	*	ns	**	**	*	**	*

Means followed by the different letters in the same column indicated differences under Tukey test ($p < 0.05$).

To improve regeneration capacity on adventitious shoot formation of ovule explants, an improvement culture medium was carried out based on Chu N6 medium with 2.26 μM 2,4-D, 6.81 μM TDZ and 90 g/l sucrose as control. From the seven-improvement media tested, it was revealed that IPM-7, Chu N6 macroelements, and full vitamins of MS basal medium added by 3.39 μM 2,4-D, 3.41 μM TDZ, 150 ml/l CW and 60 g/l sucrose was the best improved medium for adventitious shoot formation. The medium slightly improved the number of adventitious shoots/explant from 8.4 shoots in the previous experiment to 11.5 shoots in the experiment, though the number of regenerative explants/replication were lower than in the previous experiment (Table 3, 4 and 5). The second-best improvement was noted at IPM-6 and the lowest result was at IPM-8.

Table 3. Interaction effect of initiation culture media and *Lilium sp* genotype ovules on number of regenerative explants/replications

Initiation culture medium / <i>Lilium sp.</i> genotype	EXO CF/E	RDF CG/E	DZ CG/E
IM-1	0.0 ± 0.00 c	1.3 ± 0.29 a	1.8 ± 0.50 a
IM-2	0.5 ± 0.41 bc	0.0 ± 0.00 b	0.0 ± 0.00 b
IM-3	1.3 ± 0.65 b	0.0 ± 0.00 b	0.0 ± 0.00 b
IM-4	3.3 ± 0.96 a	0.0 ± 0.00 b	0.0 ± 0.00 b
IM-5	0.0 ± 0.00 c	0.0 ± 0.00 b	0.0 ± 0.00 b
IM-6	0.0 ± 0.00 c	0.0 ± 0.00 b	0.0 ± 0.00 b
IM-7	0.8 ± 0.29 bc	0.0 ± 0.00 b	0.0 ± 0.00 b
IM-8	0.0 ± 0.00 c	0.0 ± 0.00 b	0.0 ± 0.00 b
IM-9	0.0 ± 0.00 c	0.0 ± 0.00 b	0.0 ± 0.00 b
Tukey value	26.33	35.00	47.00
Probability	**	**	**

Means followed by the different letters in the same column indicated differences under Tukey test ($p < 0.05$).

Table 4. Effect of interaction between initiation culture media and *Lilium sp* genotype ovules on adventitious shoots produced/explant

Initiation culture medium / <i>Lilium sp.</i> genotype	EXO CF/E	RDF CG/E	DZ CG/E
IM-1	0.0 ± 0.00 c	4.8 ± 1.26 a	5.9 ± 0.85 a
IM-2	3.5 ± 2.65 bc	0.0 ± 0.00 b	0.0 ± 0.00 b
IM-3	3.0 ± 0.91 bc	0.0 ± 0.00 b	0.0 ± 0.00 b
IM-4	8.4 ± 0.63 a	0.0 ± 0.00 b	0.0 ± 0.00 b
IM-5	0.0 ± 0.00 c	0.0 ± 0.00 b	0.0 ± 0.00 b
IM-6	0.0 ± 0.00 c	0.0 ± 0.00 b	0.0 ± 0.00 b
IM-7	4.8 ± 1.71 ab	0.0 ± 0.00 b	0.0 ± 0.00 b
IM-8	2.0 ± 0.00 bc	0.0 ± 0.00 b	0.0 ± 0.00 b
IM-9	0.0 ± 0.00 c	0.0 ± 0.00 b	0.0 ± 0.00 b
Tukey value	10.61	37.00	39.00
Probability	**	**	**

Means followed by the different letters in the same column indicated differences under Tukey test ($p < 0.05$).

Table 5. Improvement media for adventitious shoot regeneration derived from EXO CF/E ovules as explant sources

Initiation culture medium	Percentage of explant regeneration (%)	Percentage of browning explants (%)	Number of regenerative explants/bottles	Number of shoots/explants
IPM-1	25.0 ± 12.50 bc	87.5 ± 14.43 a	1.0 ± 0.58 bc	3.8 ± 1.71 bc
IPM-2	25.0 ± 12.50 bc	56.3 ± 12.50 ab	1.0 ± 0.50 bc	3.8 ± 1.26 bc
IPM-3	12.3 ± 14.43 bc	37.5 ± 14.43 b	0.5 ± 0.58 bc	2.8 ± 0.96 c
IPM-4	18.8 ± 12.50 bc	50.0 ± 20.41 b	0.8 ± 0.50 bc	3.0 ± 1.83 bc
IPM-5	31.3 ± 12.50 abc	37.5 ± 14.43 b	1.3 ± 0.50 abc	5.3 ± 1.71 abc
IPM-6	50.0 ± 20.41 ab	31.3 ± 12.50 b	2.0 ± 0.82 ab	9.3 ± 2.50 ab
IPM-7	68.8 ± 31.46 a	31.3 ± 12.50 b	2.8 ± 1.26 a	11.5 ± 2.52 a
IPM-8	6.3 ± 14.43 c	31.3 ± 12.50 b	0.3 ± 0.57 c	0.8 ± 0.96 c
Tukey value	5.88	6.43	5.51	18.16
Probability	*	*	*	**

Note: IPM-1, Chu N6 medium with 2.26 μM 2,4-D, 6.81 μM TDZ and sucrose in 90 g L⁻¹ (control); IPM-2, Chu N6 medium containing 2.26 μM 2,4-D, 6.81 μM TDZ, 2.87 μM IAA and sucrose 90 g L⁻¹; IPM-3, Chu N6 medium containing 2.26 μM 2,4-D, 6.81 μM TDZ, 2.68 μM NAA and sucrose in 90 g L⁻¹; IPM-4, Chu N6 medium containing 2.26 μM 2,4-D, 6.81 μM TDZ, CW in 150 ml L⁻¹ and sucrose in 90 g L⁻¹; IPM-5, Chu N6 macro elements, full vitamins of MS basal medium augmented with 3.39 μM 2,4-D, 3.41 μM TDZ, 2.87 μM IAA and sucrose in 60 g L⁻¹; IPM-6, Chu N6 macro elements, full vitamins of MS basal medium augmented with 3.39 μM 2,4-D, 3.41 μM TDZ, 2.68 μM NAA and sucrose in 60 g L⁻¹; IPM-7, Chu N6 macro elements, full vitamins of MS basal medium augmented with 3.39 μM 2,4-D, 3.41 μM TDZ, CW in 150 ml L⁻¹ and sucrose in 60 g L⁻¹; and IPM-8, Chu N6 macro elements, full vitamins of MS basal medium augmented with 3.39 μM 2,4-D, 3.41 μM TDZ, glutamine in 500 mg L⁻¹ and sucrose in 60 g L⁻¹. Means followed by the different letters in the same column indicated differences under Tukey test ($p < 0.05$).

Hormone free medium with 1.5% AC successfully produced plantlets of *Lilium sp* with different performances. The plantlets derived from EXO CF/E and RDF CG/E generally almost had similar performances with 5.2-5.5 number of leaves/shoots, 8.9-10.1cm in leaf length, 4.5-4.9 leaf width, 5.5-5.9 roots/shoot, 17.4-18.3 mm in root length with 7.5-7.6 mm bulb diameter (Table 6). While the DZ CG/E plantlets had a lower number of leaves/shoots of 1.8 leaves, 2.9 roots/shoot, 7.4 mm roots length, and 3.2 mm bulb diameter with wider leaves up to 11.1 mm (Table 6). Furthermore, the different performances of the three *Lilium sp.* plantlets also indicated different capabilities during their acclimatization. Based on the acclimatization stage, it was revealed that high survivability up to 63.6% was exhibited by RDF CG/E plantlets, followed by the EXO CF/E plantlets and the lowest results were determined on DZ CG/E plantlets (Table 7).

Table 6. The plantlet performances of *Lilium sp.* genotypes

<i>Lilium sp.</i> genotype	Number of leaves/shoots	Length of leaves (cm)	Width of leaves(mm)	Number of roots/shoots	Root length (mm)	Bulb diameter (mm)
EXO CF/E	5.2 ± 0.62 a	8.9 ± 1.62 ab	4.5 ± 0.50 b	5.8 ± 1.23 a	17.4 ± 2.56 a	7.5 ± 0.56 a
RDF CG/E	5.5 ± 0.52 a	10.1 ± 0.91 a	4.9 ± 1.22 b	5.5 ± 1.15 a	18.3 ± 3.88 a	7.6 ± 0.57 a
DZ CG/E	1.8 ± 0.28 b	7.4 ± 1.59 b	11.1 ± 2.78 a	2.9 ± 0.53 b	7.4 ± 2.48 b	3.2 ± 0.63 b
Tukey value	28.93	7.66	15.84	24.25	31.34	32.20
Probability	**	*	**	**	**	**

Means followed by the different letters in the same column indicated differences under Tukey test ($p < 0.05$).

Table 7. The plantlet acclimatization data of the three *Lilium sp.* genotypes

<i>Lilium sp.</i> genotype	Number of acclimatized plantlets	Survival plantlets/pot	Death plantlets/pot	Percentage of survivability
EXO CF/E	23.5 ± 2.74 a	10.9 ± 3.08 b	12.8 ± 3.36 a	45.0 ± 14.09 b
RDF CG/E	23.5 ± 5.06 a	14.6 ± 4.27 a	8.8 ± 4.70 b	63.6 ± 21.36 a
DZ CG/E	20.7 ± 5.79 a	8.9 ± 4.62 b	12.6 ± 4.80 a	39.8 ± 21.49 b
Tukey value	2.89	12.75	4.89	9.88
Probability	ns	*	*	*

Means followed by the different letters in the same column indicated differences under Tukey test ($p < 0.05$).

Discussion

From the research, it was known that several interesting results had been successfully revealed. In this research, filament explants had a higher regenerative capacity on adventitious shoot formation than ovule explants. The filament explants of RDF CG/E genotypes gave better results than DZ CG/E and EXO CF/E genotypes, while in ovule explants, EXO CF/E genotype was better than DZ CG/E and RDF CG/E genotypes. In other studies, scale explants derived from *in vitro* cultures showed greater ability to induce callus than petioles and leaves of *Lilium leucanthum* (Tang *et al.*, 2010); bulb scale explants produced a high percentage of shoot regeneration compared to leaf segments (El-Naggar *et al.*, 2012); basal part of shoots induced better results on shoot formation than the tip part of shoots (Deswiniyanti *et al.*, 2020); basal scale segments stimulated better results in the highest culture establishment compared to tip scale segments (Rafiq *et al.*, 2021). Furthermore, fruity pink cultivar was more responsive induction callus media than cultivars of sorbon and purple diamond (Kurniati *et al.*, 2011); lily hybrids cv. 'White Heaven' induced higher rates of SE induction from both leaves and scales than cv. 'Eyeliner', 'Strawberry' and 'Cream', 'Double surprise' and 'Triumphator' in dark incubation (Yan *et al.*, 2020); 'Clone K' induced better responses on *in vitro* mutation induction compared to 'Formolongi' and 'Oriental 05' clones (Putri *et al.*, 2021); *Lilium* 'Batavus' and 'Hyde Park' varieties gave better responses in production of microbulbs than *Lilium* cv. 'Champion Diamond' and

'Yellow Diamond' (Lapiz-Culqui *et al.*, 2022). These results confirmed that each *Lilium* sp. variety or genotype or explant had different responses to *in vitro* regeneration, either via callogenesis, adventitious formation, axillary proliferation, and somatic embryogenesis.

IM-2, Chu N6 medium with 6.78 μM 2,4-D, 2.26 μM TDZ, and sucrose in 90 g L⁻¹ for the filament explants; and IM-4, Chu N6 medium added 0.5 mg/l 2,4-D, 1.5 mg/l TDZ and 90 g/l sucrose for the ovule explants, were the appropriate combinations of adventitious shoot formation of RDF CG/E filaments and EXO CF/E ovules. The IM-4 was modified by varying macro and micro elements, hormones and sucrose concentration and determined IPM-7, Chu N6 macro elements, full vitamins of MS basal medium augmented with 3.39 μM 2,4-D, 3.41 μM TDZ, CW in 150 ml L⁻¹ and sucrose in 60 g L⁻¹ for slight improvement of the adventitious shoot formation, producing 2.8 NRER and 11.5 NSE. Another research using generative organs, Pramanik and Rachmawati (2010) proved high shoot initiation frequency up to 53.3% by culturing petal explants of 'Oriental' lily planted on MS medium with 4.54 μM TDZ, 4.65 μM kinetin, and 6.78 μM 2,4-D, basal parts of young petals of 'Oriental' *Lilium* planted on MS medium added by 0.91 μM TDZ and sucrose in 30 g L⁻¹ regenerated 4.5 microbulbs per explant (Kurniati *et al.*, 2011).

Application of IM-2, IM-4 and IPM-7 for filament and ovule explants for the adventitious shoot formation, was also comparable to other research results (Tables 1-5) using leaf and bulb scales in an *in vitro* culture of *Lilium*. Bulb scales of *Lilium longiflorum* cv Easter lily planted on MS medium fortified by 18.09 μM 2,4-D stimulated 70% shoot organogenesis with 14 shoots/explant (Kanchanapoom *et al.*, 2011), *Lilium* 'Prato' explants regenerated on MS medium fortified by 8.87 μM N-6 benzyladenine (BA) and 2.69 μM NAA produced 6 shoots/explant with 5.1 cm shoot height (El-Naggar *et al.*, 2012), leaves cultured on the MS medium with 5.37 μM NAA and 2.27 μM TDZ regenerated 5.05 shoots and 36.5 leaves after 14 weeks incubation (Saetiew and Umamanit, 2015), leaf explants of *Lilium orientalis* 'Starfigther' on MS culture medium with 2.27 μM TDZ and 45.24 μM 2,4-D produced 8.3 shoots/explant, 5.4 leaves/shoot (Youssef *et al.*, 2019), bulb scales planted on MS medium added by 5.37 μM NAA and 4.44 μM BAP stimulated 100% growing percentage with 4.3 shoots/explant and 1.4 microbulbs of *Lilium longiflorum* (Deswiniyanti and Lestari, 2020), basal scale segments of 'Oriental' hybrid *Lilium* 'Ravena' planted on MS medium 2.69 μM NAA and 8.88 μM BAP produced maximum culture establishment up to 76.2%, 5.5 bublets/explant, 2.2 cm shoot length and 3.4 leaves (Rafiq *et al.*, 2021). These above results gave evident that each explant had optimal regeneration results in a specific culture medium.

From previous research, it was reported that 16.7 roots/shoot were established on MS medium with 2.69 μM NAA (Hoesen, 2009), the ½ MS medium with 0.27 μM NAA was more suitable for rooting; 19 roots/shoot and 5.2 cm root length of *Lilium longiflorum* were resulted in MS medium with NAA in 2 mg/l (Mir *et al.*, 2012), the individual shoots were successfully planted in MS medium augmented with 2.46 μM IBA for root formation (Saetiew and Umamanit, 2015), 6.5 roots/shoot were established in MS medium with 12.08 μM NAA (Fauziah *et al.*, 2019), and 13.3% root formation was determined on MS medium added by 5.37 μM NAA (Deswiniyanti *et al.*, 2020). In the experiment, Chu N6 medium hormone free, supplemented with 1% activated charcoal, was successfully induced 2.9 to 5.8 roots/shoot with 7.4 to 18.3 mm root length.

Acclimatization of hardened plantlets for one month derived from three genotypes of *Lilium* sp. in charcoal husk and bamboo compost (1:1) with water spraying periodically was successfully established with 39.8-63.6% survivability. In other studies, Hoesen (2009) was significantly proved high survivability up to 85% of *Lilium* sp. plantlets in combination media of compost, sand, and soil (2:1:1), 66.7% survivability of bublets were established in combination of sand, soil, perlite and vermiculite (1:1:2:2) and fertilized with half-strength MS solution (Joshi and Dhar, 2009), plantlets were effectively acclimatized in autoclaved soil (Tang *et al.*, 2010), 98% survivability of *L. formolongo* plantlets was planted in the soil substrate and covered by clear plastic for 5 weeks (Saetiew and Umamanit, 2015), 100% survival rate of plantlets was established in peat moss combined with regular watering (100 ml/plant) and pacing to a shady place (Royandazagh, 2019), rooted shoot

clusters of *Lilium candidum* were optimally transferred *ex vitro* in nitrogen rich peat (Tokgoz and Altan, 2020). The lower survivability of the plantlets in the research was presumably due to no clear plastic covering them during the initial acclimatization at least for 7 days.

Conclusions

Based on experiments carried out serially, a new micropropagation protocol for *Lilium* sp. of three genotypes using filaments and ovules as explant sources from initiation to acclimatization was successfully established. High initiation of adventitious shoot formation of RDF CG/E filaments was proved on Chu N6 medium fortified by 6.79 μM 2,4-D, 2.27 μM TDZ, and 90 g L^{-1} sucrose (IM-2) and Chu N6 macro elements, full vitamins of MS basal medium added by 3.39 μM 2,4-D, 3.41 μM TDZ, CW in 150 ml L^{-1} and sucrose in 60 g L^{-1} (IPM-7) suitable for EXO CF/E ovules. The adventive shoots were proliferated on the IM-2 and IPM-7 under subcultures periodically. The proliferated shoots were rooted in Chu N6 medium hormone-free with 1.5 % AC, followed by hardening them in a glass house for one month, then successfully acclimatized in plastic pots containing a combination of charcoal husk and bamboo compost (1:1) under water spraying periodically resulted in 39.8-63.6% survivability. These results confirmed that establishing a new *in vitro* culture protocol of *Lilium* sp. via adventitious shoot formation using filaments and ovules as explant sources has the potential to develop for other genotypes by specific improvements in their acclimatization stage.

Authors' Contributions

Conceptualization-BW, FR, SK; Data curation-FR, DP, HS, MW; Formal analysis-BW, FR, HS; Investigation; Methodology-BW, FR, SK, DP, HS; Resources- SK, DP, MW; Supervision and Validation-BW, SK, DP; Writing - original draft-BW, FR; Writing - review and editing-BW, FR, SK, DP, HS, MW. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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