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REGULAR ARTICLE

Leaf-derived organogenesis *in vitro* for mass propagation of lisianthus (*Eustoma grandiflorum* (Raf.) Shinn)

Budi Winarto[1]*, Fitri Rachmawati[1], Anggraeni Santi Setyawati[2], Jaime A. Teixeira da Silva[3]

[1]Tissue Culture Department, Indonesian Ornamental Crops Research Institute, Jl. Raya Ciherang, Pacet-Cianjur 43253, West Java, Indonesia; [2]Pasar Minggu Experimental Garden, Indonesian Ornamental Research Station, Jl. Ragunan No. 29A, Pasar Minggu, South Jakarta 12640, Jakarta, Indonesia; [3]P. O. Box 7, Miki-cho Post Office, Ikenobe 3011-2, Kagawa-ken 761-0799, Japan

ABSTRACT

Lisianthus (*Eustoma grandiflorum* (Raf.) Shinn) is an important ornamental commodity in South-East Asia. However, mass propagation of the plant at a commercial scale to satisfy market demands is faced by limited availability of high quality and uniform seedlings as planting material. Using different regeneration media and leaf explants for callus induction, regeneration, proliferation, root formation and acclimatization were studied. High callus induction and adventitious shoot formation were possible from leaf explants of *E. grandiflorum* 'White Lavender' cultured on Murashige and Skoog (MS) medium supplemented with 3.0 mg/l thidiazuron (TDZ) and 0.3 mg/l α-naphthalene acetic acid (NAA), but high quality shoots (8.0) was established on MS medium containing 0.5 mg/l N^6-benzyladenine (BA) and 0.002 mg/l NAA. In the same medium, adventitious shoots could be multiplied up to the fourth subculture at a rate of 1.74 which decreased to a 1.57 multiplication rate in subsequent subcultures. Shoots rooted easily on MS medium containing 0.1 mg/l BA and 0.02 mg/l NAA with 3.9 roots per shoot. The plantlets, which were successfully acclimatized in a mixture of burned-rice husk and organic manure (1:1, v/v) with 90% survival, grew well after repotting.

Keywords: Adventitious shoots; *Eustoma grandiflorum*; Leaf explant; Regeneration medium; Tissue culture

INTRODUCTION

Lisianthus (*Eustoma grandiflorum* (Raf.) Shinn), a member of the *Gentianaceae* family (Mousavi et al., 2012a), is an important ornamental plant commodity in South East Asia, including Indonesia (Demas et al., 2009). In Indonesia, lisianthus is widely cultivated in Cipanas-Cianjur and Cihideung-Bandung, West Java; Batu-Malang, East Java; and Baturiti-Tabanan, Bali. It is commonly used as a cut and pot flower with a rose-like flower, varying in size and shape, colorful and with a long vase-life (up to 6 weeks) (Shimizu and Ichimura, 2005; Yamada et al., 2008; Mousavi et al., 2012ab). Cut flowers, which are sold as bundles, are expensive, and are sold for as much as 350,000 rupiahs (USD 28.8) per inflorescence.

Conventionally, *E. grandiflorum* is propagated vegetatively by cuttings and sexually by seeds (Mousavi et al., 2012ab; Rezaee et al., 2012). However the vegetative method is laborious and time consuming (Mousavi et al., 2012a) and the seed technique is hampered by cross pollination and is not efficient due to a low seed germination rate of 34-39% (Arpana et al., 2012; Mousavi et al., 2012b; Rezaee et al., 2012). The seeds are extremely small and progeny is not uniform and inconsistent, displaying rosette (Furukawa et al., 1990; Mousavi et al., 2012a). Seed-derived plants, in some cultivars, show wide variation due to their heterozygous character and take at least 4.5 months or more to reach the flowering stage (Furukawa et al., 1990). Tissue culture is one way to circumvent these limitations.

Micropropagation is a powerful tool for the large-scale propagation of ornamental plants and is extensively used for many plant species and cultivars (Esizad et al., 2012; Rezaee et al., 2012; Kaviani, 2014). The success of the micropropagation method depends on several factors such as genotype, explant type, source and its physiological condition, media, plant growth regulators, culture

***Corresponding author:**
Budi Winarto, Tissue Culture Department, Indonesian Ornamental Crops Research Institute, Jl. Raya Ciherang, Pacet-Cianjur 43253, West Java, Indonesia. E-mail: budi.winarto67@yahoo.co.id

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conditions, etc. (George et al., 2007). In *E. grandiflorum*, *in vitro* propagation studies with varied results were also reported previously. Multiple shoots were easily induced from shoot tips and internodal stem sections on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3 mg/l N^6-benzyladenine (BA) and 0.2 mg/l α-naphthalene acetic acid (NAA), proliferated on MS medium fortified with 3 mg/l BA and rooted on MS medium supplemented with 2 mg/l indole-3-acetic acid (IAA) (Semeniuk and Griesbach, 1987). The highest percentage of regeneration and maximum number of glaucous shoots (15 shoots/explant) were obtained 4 weeks after culturing shoot tips on MS medium supplemented with 1 mg/l BA and 0.25-0.86 mg/l IAA and indole-3-butyric acid (IBA) (Paek and Hahn, 2000). Shoot tips cultured on MS medium augmented with 0.5-1.0 mg/l Kinetin (KIN) were suitable for producing many shoots (Esizad et al., 2012; Kaviani, 2014). Axillary buds derived from two-month-old seedling explants cultured on B5 medium (Gamborg et al., 1968) supplemented with 1.5 mg/l NAA were the best for callus induction (Mousavi et al., 2012a), while axillary buds on B5 medium containing 0.5 mg/l gibberellic acid (GA_3) and 1.5 mg/l BA produced high shoot regeneration, as many as 7.6 shoots/explant (Mousavi et al., 2012b). Most studies using shoot tips and axillary buds on different media were applied to produce axillary shoots, but producing adventitious shoots from leaf explants is limited.

Adventitious shoot proliferation studies for *E. grandiflorum* were reported previously by Semeniuk and Griesbach (1987) and Rezaee et al. (2012). However, complete information was not recorded in both studies. In the present study, leaf-derived organogenesis *in vitro* for the mass propagation of *E. grandiflorum* 'White Lavender' using young leaves as the explant source was successfully established. The protocols consisted of callus initiation, adventitious shoot regeneration and proliferation, root formation and acclimatization.

MATERIALS AND METHODS

Plant materials and preparation

This experiment was conducted at the Tissue Culture Laboratory of the Indonesian Ornamental Crop Research Institute. Lateral shoots (± 8 cm in length with 4-5 leaves) of *E. grandiflorum* 'White Lavender' from Ciputri Molek grower, Pasir-Sarongge, Pacet-Cianjur, West Java, Indonesia were used as the explant source in the experiment. Explants (± 8 cm) were initially surface sterilized with a 1% pesticide solution (50% benomyl + 20% streptomycin sulphate) for 30 min and rinsed with distilled water five times (5 min each rinse). Subsequently, explants were immersed in a 1% solution of sodium hypochloride (5.25% NaOCl) containing a few drops of Tween-20 for 10 min, followed by 2% NaOCl for 5 min, rinsed six times with sterile distilled water (SDW) (5 min each) and blotted onto sterile tissue paper. After the sterilization, young first and second leaves (counted from the shoot tip position) were isolated carefully using forceps and tissue culture blades. The explants were then cultured on culture media.

Callus initiation and adventitious shoot regeneration

In this experiment, isolated leaves were cultured in an abaxial-side down position on media (Fig. 1a). In the first experiment, MS medium containing different concentrations of plant growth regulators were used as initiation and regeneration media (IRM): (1) 0.25 mg/l TDZ and 0.015 mg/l NAA (IRM-1), (2) 0.08 mg/l TDZ (IRM-2), (3) 3 mg/l TDZ and 0.3 mg/l NAA (IRM-3), (4) 3 mg/l TDZ and 0.01 mg/l NAA (IRM-4), (5) 0.5 mg/l BA and 0.002 mg/l NAA (IRM-5), (6) 0.1 mg/l BA and 0.002 mg/l NAA (IRM-6) and (7) 0.1 mg/l BA and 0.1 mg/l NAA (IRM-7). All media were supplemented with 30 g/l sucrose, solidified with 7 g/l Swallow agar and sterilized for 20 min at 121°C and 15 psi.

A factorial experiment was arranged in a completely randomized design with four replications. Each treatment consisted of five Petri dishes (9 cm in diameter), each with four explants. Cultures were then incubated under light at 25±2°C and a 16-h photoperiod under white florescent lamps with a photosynthetic photon flux density of 13 μmol/m^2/s.

Parameters observed in the experiment were: (1) initial callus formation (days), (2) callus formation score (– to +++, where – = no callus formation, + = little callus formed (< 25% of total explant surface area), ++ = moderate amount of callus formed (25-50% of total explant surface area), +++ = abundant callus formation (> 50% of total explant surface area), (3) percentage of explant regeneration (PER, %), (4) number of adventitious shoots produced per explant (NSE). Routine observations were performed to observe all changes to cultures during incubation. Parameters 2-4 were recorded about two months after culture initiation.

Multiplication of adventitious shoots

Adventitious shoots were multiplied by subculturing them at two adventitious shoots/cluster on MS medium supplemented with 0.5 mg/l BA, 0.002 mg/l NAA, 30 g/l sucrose and 7 g/l Swallow agar. The medium was sterilized as indicated above. Shoots were subcultured periodically every two months until a reduction in shoot quantity and quality was clearly observed. There were 10 bottles and three replicates per treatment. Each bottle consisted of 4

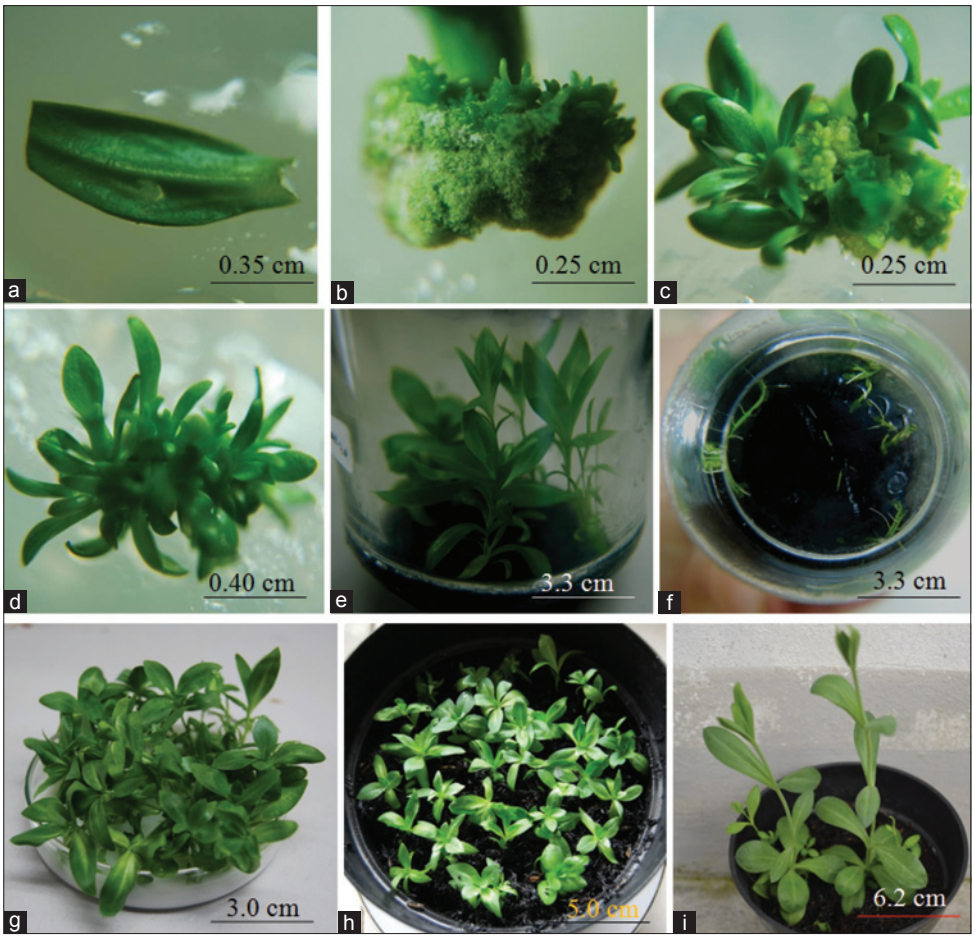

Fig 1. Leaf-derived organogenesis *in vitro* of lisianthus 'White Lavender'. a. Condition of leaf explant in initial culture. b. Callus formed and initial adventitious shoots regenerated on MS medium containing 3 mg/l TDZ and 0.01 mg/l NAA two months after culture. c. Regenerated adventitious shoots on MS medium supplemented with 0.5 mg/l BA and 0.002 mg/l NAA two months after culture. d. Multiplication of adventitious shoots derived from the first explant on MS medium fortified with 0.5 mg/l BA and 0.002 mg/l NAA two months after culture. e-f. Root formation on shoots cultured on MS medium containing 0.1 mg/l BA and 0.1 mg/l NAA two months after culture. g. Well-rooted shoots immersed in 1% pesticide solution for 3 min. h. High survival of plantlets in a mixture of burned-rice husk and organic manure (1:1, v/v) 1 month after acclimatization. i. Development of acclimatized plantlets two months after repotting in the same substrate.

explants. Data consisted of the average number of shoots per initial shoot cultured and the multiplication rate of shoots per subculture period.

Adventitious root formation

To induce adventitious roots, shoots (± 2 cm in height with 4 leaves) were cultured in different MS medium formulations. The adventitious shoots derived from the first and second leaf explants were tested. MS medium contained: (1) 0.25 mg/l TDZ and 0.015 mg/l NAA (ARM-1; ARM = adventitious root medium), (2) 0.08 mg/l TDZ (ARM-2), (3) 0.3 mg/l TDZ and 0.3 mg/l NAA (ARM-3), (4) 0.3 mg/l TDZ and 0.01 mg/l NAA (ARM-4), (5) 0.5 mg/l BA and 0.002 mg/l NAA (ARM-5), (6) 0.1 mg/l BA and 0.02 mg/l NAA (ARM-6) and (7) 0.1 mg/l BA and 0.1 mg/l NAA (ARM-7). All media were supplemented with 30 g/l sucrose, solidified with 7 g/l Swallow agar and sterilized for 20 min at 121°C and 15 psi.

The factorial experiment was arranged in a completely randomized design with four replications. Each treatment consisted of 5 jam bottles (7 cm in diameter and 13 cm in height). Each bottle contained 5 shoots. Cultures were then incubated in light conditions described above.

Parameters observed two months after culture initiation were: (1) initial period of root formation (days), (2) percentage of root formation (PRF, %), (3) number of roots per shoot (NRS), and (4) root length (cm). Routine observations were performed to observe all changes to cultures during incubation.

Plantlet acclimatization

For acclimatization, well rooted-shoots (± 6 cm in height with 4-6 leaves and two months old) were carefully removed from culture vessels and remaining agar was washed off gently with distilled water. Plantlets were then immersed in a

1% pesticide solution (50% benomyl and 20% streptomycin sulphate) for 3 min. Small plants (4–6 cm in height) were cultivated in plastic pots (30 cm in diameter) containing a mixture of burned-rice husk and organic manure (1:1, v/v). Approximately 30 plantlets were cultivated in each plastic pot, replicated five times, then covered with transparent plastic for 15 days and placed in a glasshouse under reduced light intensity (100–120 μmol/m^2/s). The total number of plantlets that survived or died was recorded after two months of acclimatization.

Statistical analysis

Quantitative data were analyzed by analysis of variance (ANOVA). In cases where significant differences were obtained ($p=0.05$), Duncan's Multiple Range Test (DMRT) was used to compare means. Data given in percentages were arcsine ($\sqrt{x}$) transformed before statistical analysis to ensure a normal distribution of data with a coefficient of variation less than 20% (Mattjik and Sumertajaya, 2006).

RESULTS

Callus initiation and adventitious shoot regeneration

Callus formation was initially observed 2-3 weeks after culture on wounded areas of leaves. The callus grew continuously, enlarging its volume and increasing in size. After 5 weeks of incubation, each explant produced callus on all media with the highest callus formation on leaf explants cultured on IRM-3 (MS medium containing 3 mg/l TDZ and 0.3 mg/l NAA) (++/+++). The callus produced initial adventitious shoots 8-9 weeks after incubation (Fig. 1b) but was clearly observed 11 weeks after culture. The number of shoots varied (4-13) with 1-3 leaves easily observed 4 months after culture.

Different types of leaf explants and regeneration media tested in this experiment significantly influenced callus and adventitious shoot formation, but there was no interaction effect in both treatments. IRM-4 (MS medium fortified with 3 mg/l TDZ and 0.01 mg/l NAA) was the most appropriate regeneration medium to induce high adventitious shoot formation with 11.7 days after the callus formation period, and 73% of explants regenerated and 8.4 adventitious shoots were produced per explant (Table 1). However the highest quality adventitious shoots with green and vigorous shoots was established on IRM-5 (MS medium containing 0.5 mg/l BA and 0.002 mg/l NAA) (Fig. 1c). Other media tended to reduce adventitious shoot induction and regeneration. Although the first leaf explants showed better results for all parameters observed, there was no significant difference compared to the second leaf explants (Table 2).

Multiplication of adventitious shoots

Four months of periodic subculture (2 months each) of adventitious shoots cultured on selected medium increased the number of shoots in each subculture (Fig. 1d). The shoots derived from the first and second leaves that were cultured on MS medium containing 0.5 mg/l BA and 0.02 mg/l NAA proliferated successfully, gradually forming shoots. From 8 shoots in the first culture, they produced 61.6 shoots in the fifth subculture (Fig. 2a). The shoot multiplication rate was between 1.52 and 1.74 (Fig. 2b). Adventitious shoot production increased from the first until the fourth subculture and then decreased. Although there was no significant difference between the first and the second leaf explants in terms of proliferation capacity, the first leaf explants tended to give better results in terms of shoot proliferation.

Adventitious root formation

Initial adventitious roots were clearly observed 13-20 days after culture in the wounded area and on the basal part of the stem. One to six roots, 0.3-2.5 cm in length, were easily recorded 2.5 months after culture. Different types of rooting media and shoots induced a significantly different response on adventitious root formation, but there was no interaction effect between both treatments. ARM-6 medium was the most suitable medium to initiate adventitious roots. This

Table 1: Effect of regeneration media on adventitious shoot formation

Initiation and regeneration medium (RM)	Callus initiation period (days)	Callus score	Explant regeneration (%)	Number of adventitious shoots produced/ explant
IRM-1	13.2d	++	50.0d	7.9bc
IRM-2	13.9d	-/+	55.0cd	6.9d
IRM-3	11.5e	++/+++	53.9d	6.9d
IRM-4	11.7e	+/++	73.0abc	8.4a
IRM-5	17.5c	+	77.8ab	8.0ab
IRM-6	20.7b	+	66.7bcd	7.5c
IRM-7	23.5a	-/+	89.7a	7.6cd

Note: Average data is derived from observations of 20 explants. MS medium containing different concentrations of plant growth regulators: (1) 0.25 mg/l TDZ+0.015 mg/l NAA (IRM-1); (2) 0.08 mg/l TDZ (IRM-2); (3) 3 mg/l TDZ+0.3 mg/l NAA (IRM-3); (4) 3 mg/l TDZ+0.01 mg/l NAA (IRM-4); (5) 0.5 mg/l BA+0.002 mg/l NAA (IRM-5); (6) 0.1 mg/l BA+0.002 mg/l NAA (IRM-6); (7) 0.1 mg/l BA+0.1 mg/l NAA (IRM-7). Mean values followed by the same letter in the same column are not significantly different based on DMRT at p=0.05

Table 2: Effect of explant types on adventitious shoot formation

Initiation and regeneration medium (RM)	Callus initiation period (days)	Callus score	Explant regeneration (%)	Number of adventitious shoots produced/ explant
First leaves	16.0a	+/++	68.5a	8.7a
Second leaves	17.8a	+/++	64.7a	8.5a

Average data is derived from observations of 20 explants. Mean values followed by the same letter in the same column are not significantly different based on DMRT at p=0.05

medium was able to stimulate root initiation in the shortest period (as few as 13.6 days after culture) with 92% root formation, 3.9 roots produced per shoot and roots 2.1 cm long (Table 3; Fig. 1e and 1f). Other media gave lower results in terms of root formation. There were also no significant differences in root formation between both types of shoots both from the first and the second leaves (Table 4).

Plantlet acclimatization

Preparing well-rooted shoots in combination with a gradual process of acclimatization by immersing plantlets in 1% pesticide solution (Fig. 1g), transferring plantlets into a mixture of burned-rice husk and organic manure (1:1, v/v), covering transparent plastic for 15 days and placing pots under reduced light intensity area (100–120 μmol/m^2/s) resulted in the high survival of plantlets (81-100%; 90% average) after one month of acclimatization (Fig. 1h). After two months of acclimatization and repotting, the acclimatized plantlets grew well and developed well, increasing in size and height (Fig. 1i). Thus, the acclimatization of *in vitro* plantlets derived from first and second leaf explants is possible in lisianthus.

Table 3: Effect of rooting media on adventitious root formation

Adventitious root medium (ARM)	Root initiation period (days)	Root formation (%)	Number of roots produced/ shoot	Root length (cm)
ARM-1	15.1c	77.8bc	2.8b	1.1cd
ARM-2	18.0a	10.8e	0.2d	1.4bc
ARM-3	18.5a	100.0a	2.7b	0.6e
ARM-4	17.8ab	66.7c	2.3b	0.7de
ARM-5	17.4ab	27.8d	0.8c	1.7b
ARM-6	13.6d	91.7ab	3.9a	2.1a
ARM-7	16.3bc	29.4d	2.9b	2.3a

Average data is derived from observations of 25 plantlets. MS medium containing (1) 0.25 mg/l TDZ+0.015 mg/l NAA (ARM-1); (2) 0.08 mg/l TDZ (ARM-2); (3) 0.3 mg/l TDZ+0.3 mg/l NAA (ARM-3); (4) 0.3 mg/l TDZ+0.01 mg/l NAA (ARM-4); (5) 0.5 mg/l BA+0.002 mg/l NAA (ARM-5); (6) 0.1 mg/l BA+0.02 mg/l NAA (ARM-6); (7) 0.1 mg/l BA+0.1 mg/l NAA (ARM-7). Mean values followed by the same letter in the same column are not significantly different based on DMRT at p=0.05

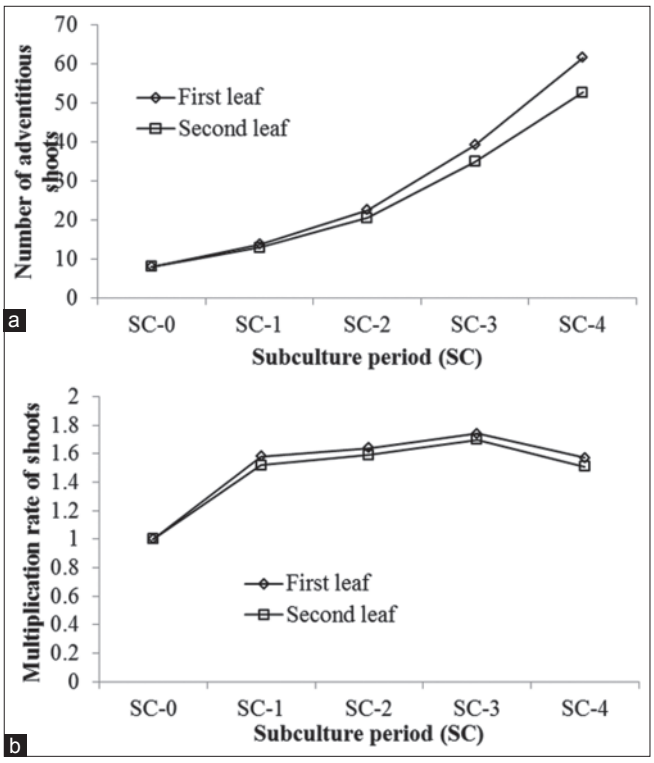

Fig 2. Proliferation of adventitious shoots. a. Increasing number of adventitious shoots forming gradually after periodic subculture. b. Multiplication rate of adventitious shoots after periodic subculture.

DISCUSSION

The suitability of medium and explant type in the *in vitro* culture of plants plays an important role in obtaining high morphogenic results in culture (George et al., 2007). The application of different IRM had a significant effect on callus initiation. MS medium with 3 mg/l TDZ and 0.3 mg/l NAA and the first leaf explant was the most suitable medium and explant to obtaining high callus formation. Nhut et al. (2006) reported high callogenesis on MS medium containing 0.0–1.0 mg/l BA in combination with 0.2 mg/l NAA, 0.5 mg/l BA for segments of leaf explant and 0.2 mg/l IBA (Hecht et al., 1994), or 2 mg/l NAA and shoot tips (Esizad et al., 2012). B5 medium supplemented with 0-1.5 mg/l NAA, 25 g/l (w/v) of sucrose and 7 g/l (w/v) agar and axillary buds was also optimal medium for callus formation of lisianthus (Mousavi et al., 2012a). Basal Linsmaier and Skoog medium (Linsmaier and Skoog, 1965) containing 3 mg/l IAA, 3 mg/l NAA, 0.1 mg/l Kin, and B5 medium containing 0.225 mg/l BA and 1.86 mg/l NAA and segments of leaf explant were established as the best media and explant source for the induction of callus (Rezaee et al., 2012).

Profuse callus induction, in some cases, was not associated with high regeneration capacity of the callus to produce well-developed shoots. Callus formation established on IRM-3 (MS medium augmented with 3 mg/l TDZ and 0.3 mg/l NAA) produced 6.9 shoots/explant. High number of shoots/explant was reported on IRM-4 (MS medium fortified with 3 mg/l TDZ and 0.01 mg/l NAA), but well-developed adventitious shoots (as many as 8.0 shoots/explant) were induced on leaf

Table 4: Effect of explant types on root formation

Adventitious root medium (ARM)	Root initiation period (days)	Root formation (%)	Number of roots produced/ shoot	Root length (cm)
First leaves	16.8b	63.9a	2.1a	1.2a
Second leaves	17.9a	51.6b	2.1a	1.5a

Average data is derived from observations of 25 plantlets. Mean values followed by the same letter in the same column are not significantly different based on DMRT at p=0.05

explants cultured on IRM-5 (MS medium supplemented with 0.1 mg/l BA and 0.002 mg/l NAA). Almost similar results were reported by Paek and Hahn (2000) in which high concentrations of BA (3.0-5.0 mg/l) and Kin (3.0-5.0 mg/l) resulted in hyperhydric shoots. Well-developed shoots (15 shoots/explant) formed on MS medium augmented with 1.0 mg/l BA and 0.25-0.86 mg/l IAA and IBA. Esizad et al. (2012) and Kaviani (2014) noted a high number of shoots per explant derived from node explants on MS medium containing 0.5 mg/l Kin (8.86 and 8.75 shoots/explant, respectively). Fewer adventitious shoots regenerated in leaf explants on MS medium supplemented with 0.5 mg/l GA_3 and 1.5 mg/l BA with 7.6 shoots/explant (Mousavi et al., 2012b) and 1.0 mg/l BA and 1.0 mg/l GA_3 with 1.5 shoots/explant (Mousavi et al., 2012a).

Proliferation of adventitious shoots via periodic subcultures was successful in this research. Shoot multiplication rate increased gradually up to the fourth subculture and decreased thereafter. A high multiplication rate of 1.74 was recorded on the first leaf explants on MS medium fortified with 0.1 mg/l BA and 0.002 mg/l NAA. In another study, high shoot proliferation was possible when shoots were subcultured on MS medium supplemented with 0.5 mg/l Kin (Esizad et al., 2012). However, Semeniuk and Griesbach (1987) achieved high shoot proliferation on MS medium containing 3 mg/l BA.

Root formation on shoots derived from *in vitro* culture of lisianthus is an important means of obtaining high plantlet survival at the acclimatization stage. Acclimatization is a critical stage that is often associated with slow growth and significant plant loss (Uzun et al., 2014). In this study, many roots (3.9) formed on MS medium with 0.1 mg/l BA and 0.02 mg/l NAA (ARM-6), while 2.40 roots/shoot formed on MS medium supplemented with 2 mg/l Kin and 0.5 mg/l NAA (Esizad et al., 2012), 2.55 roots on MS medium fortified with 1 mg/l Kin and 0.5 mg/l NAA (Kaviani, 2014), and 62.0 roots on B5 medium with 1.5 mg/l NAA (Mousavi et al., 2012a). The well-rooted shoots in this study were successfully transferred to *ex vitro* conditions into a mixture of burned-rice husk and organic manure (1:1, v/v) with 90% survival. Survival of plantlets as much as 100% was recorded for plantlets acclimatized in a mixture of peat and perlite (1:1, v/v) (Esizad et al., 2012; Kaviani, 2014). High survival during the acclimatization stage can be affected by the drainage of the mixture of burned-rice husk and organic manure, also proved in the two other studies employing a mixture of peat and perlite (Esizad et al., 2012; Kaviani, 2014).

CONCLUSION

Leaf-derived organogenesis *in vitro* for the mass propagation of *E. grandiflorum* was successfully studied and resulted in an *in vitro* regeneration protocol for cultivar 'White Lavender' using leaf explants, followed by high frequency shoot proliferation, rooting and acclimatization. These results will be useful for the propagation of *E. grandiflorum* for preparing high quality planting commercial materials.

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Author contributions

B. W. contributed to research planning and all intermittent steps until manuscript preparation and revisions. F. R. and A. S. S. were involved in research planning, carrying out experiments and collecting data. J. A. T. d S. was involved with advice regarding data analysis, experimental design, and manuscript writing and editing. All four authors approved the manuscript for publication, take public responsibility for the content, and declare no conflicts of interest associated with any aspect of this manuscript (financial or other).

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