In Vitro Organogenesis of Lisianthus [*Eustoma grandiflorum* (Raf.) Shinn] Derived from Leaf Explant

H. Shintiavira, D. Pramanik* and B. Winarto

Indonesian Ornamental Crops Research Institute (IOCRI), Jln. Raya CiherangPacet-Cianjur 43253, West Java, Indonesia

**Corresponding author, Email: pramanik_dewi53@yahoo.com*

Abstract

In vitro organogenesis of Lisianthus [*Eustoma grandiflorum* (Raf.) Shinn] derived from leaf explant as explant source was successfully studied. High callus formation up to 50% in leaf explants was established on Gamborg B5 medium supplemented with 0.1 mg L^{-1} α-naphatelene acetic acid (NAA) and 0.5 mg L^{-1} N6-benzylamino purine (BAP). The callus was initiated on the Gamborg B5 medium fortified by 0.1 mg L^{-1} 2,4-dichloropenoxy acetic acid (2,4-D) and 0.5 mg L^{-1} BAP and successfully produced 18.7 shoots per explant in 28 days under light incubation. The best growth performance of plantlet without rosette problem was recorded on Murashige and Skoog (MS) medium augmented with 10 mg L^{-1} giberellic acid (GA3) with 3 shoots per planlet and 9.3 cm shoot height after 4 week in culture. The shoots were easily rooted on MS medium containing 2.0 mg L^{-1} indole-3-acetic acid (IAA) and 1 mg L^{-1}activated charcoal (AC) with 1.9 number of roots per shoot and 1.8 cm root length after 4 weeks in culture. Plantlets derived from the study were successfully acclimatized during 6 weeks on a mixture of burned rice husk and cocopeat (1:1, v/v) with 80% survivability. Results of the study give high chance in producing well planting materials for growing *E. grandiflorum* cultivation in Indonesia.

Keywords: *Eustoma grandiflorum*, young leaves, tissue culture, clonal propagation and seedlings

Introduction

Lisianthus [*Eustoma grandiflorum* (Raf.) Shinn] has high potential as one of economical ornamental plant in Indonesia (Demas et al., 2009). The lisianthus is generally sold as a cut and pot flower. As cut flower, it is sold for US$ 28.00 per bunch. The rose like flower of the lisinthus has various size, colour, and long vase life up to 6 weeks and increased by application of salicylic acid (Shimizu and Ichimura, 2005; Yamada et al., 2008; Kawabata et al., 2009; Mousavi et al., 2012a,b; Selvakumari et al., 2012; Bahrami et al., 2013) and is now widely cultivated in Cipanas-Cianjur and Cihideung-Bandung, West Java; Batu-Malang, East Java; and Baturiti-Tabanan, Bali. Unfourtenly, development of the lisianthus in Indonesia is constrained by the availability of qualified-seeds and seedlings for commercial purposes .

Traditionally, the lisianthus are propagated by cuttings vegetatively and seeds generatively (Mousavi et al., 2012a,b; Rezaee et al., 2012). The vegetative method is generally laborious and time consuming with less regenerants produced, therefore it is not suitable for commercial purpose (Mousavi et al., 2012a). Whereas utilization of seeds are not efficient and result in uneven plants with varied plant height, flowering time and flower number (Paek and Hahn, 2000). The extremely small seeds of the lisianthus have low germination rate (34-39%) (Arpana et al., 2012; Mousavi et al., 2012b; Rezaee et al., 2012) and in some cases the heterozygous characters of seed-derived plants lead to wide variation of plants with prolonged flowering stage of more than 4.5 months (Furukawa et al., 1990). To circumvent these limitations, application and utilization of tissue culture method

as a powerful tool for the large-scale propagation of the lisianthus can be addressed.

Application of tissue culture works especially to prepare high-quality seedling for mass propagation protocol on Lishianthus was successfully established previously. Semeniuk and Griesbach (1987) used MS medium (Murashige and Skoog, 1962) containing 3.0 mg L^{-1} N^6-benzyladenine (BA) and 0.2 mg L^{-1} NAA to initiate multiple shoots derived from shoot tips and internodal stem sections. MS medium fortified with 3 mg L^{-1} BA was applied for proliferation of shoots and MS medium supplemented with 2 mg L^{-1} IAA was used for their rooting. Moreover, Paek and Hahn (2000) reported that the highest percentage of regeneration and maximum number of shoots (15 shoots/explant) were determined on MS medium supplemented with 1 mg L^{-1} BA and 0.25-0.86 mg L^{-1} IAA and indole-3-butyric acid (IBA) in 4 weeks after culture. MS medium augmented with 0.5-1.0 mg L^{-1} Kinetin (KIN) was applied for the same purpose (Esizad et al., 2012; Kaviani, 2014), while B5 medium (Gamborg et al., 1968) supplemented with 1.5 mg L^{-1} NAA was applied for callus induction (Mousavi et al., 2012a). Thus, the application of B5 medium containing 0.5 mg L^{-1} gibberellic acid (GA_3) and 1.5 mg L^{-1} BA produced high shoot regeneration, as high as 7.6 shoots/explant (Mousavi et al., 2012b). However studies on adventitious shoot induction and proliferation derived from leaf explants are a few as reported by Nhut et al. (2006), Razaee et al. (2012) and Winarto et al. (2015). Therefore a new route in *in vitro* mass propagation of the lisianthus shall be addressed to increase alternative protocol choices in preparing seedlings for commercial scale.

The new *in vitro* mass propagation protocol of the lisianthus using young leaf explants as explant source was successfully established in this research. New findings of callus formation, shoot regeneration and proliferation, root formation and acclimatization stages were described in detail in this paper.

Materials and Methods

Plant Materials and Explants Preparation

Eustoma grandiflorum (Raf.) Shinn cv. Voyage 2 Green (young green flower) and Reina White (white flower) were used as donor plants. The donor plants were grown in polybags in mixture of cocopeat and burned rice husk (1:1, v/v), and then placed in glass house. The plants were watered every day and fertilized with 2 g L^{-1} Growmore (20N:20P:20K) once week. The young leaf explants (4-month old) were harvested from the donor plants and used as explant sources.

The young leaves were pretreated by immersing in 2% solution of detergent for 5 min., soaking in 10% alcohol for 10 min, then in 2.5% bactericide (20% Kanamycin Sulphate) and fungicide (50% Benomyl) for 30 min and rinsing with distilled water 3 times (5 min each), respectively. After pretreatment, the explants were sterilized by dipping in 1% sodium hypochloride (NaOCl) for 3 min, 2% NaOCl for 3 min, followed by 3 times rinsing explants with sterile distilled water (5 min each).

After sterilization, the explants were prepared by cutting of them with 0.5×0.5cm . The explants were cultured on the treatment media. The cultures were then incubated under light conditions with 16-h photoperiode under cool fluorescent lamp with ~13 µmol m^{-2} s^{-1} light intensity at 24±1°C.

Basal media used in the study were LS medium (Linsmaiere and Skoog, 1965), B5 medium (Gamborg et al., 1968), and MS medium. All components of media were pro analysis components from Merck, Damstadt-Germany. Plant growth regulators utilized in the research were BAP, NAA, IAA, 2,4-D, GA_3 (Phytotech, US). Sucrose (Himedia, Mumbai, India) in concentration of 30 g L^{-1} and 2 g L^{-1} gerlite (Duchefa, Haarlem, Netherlands), sugar (Gulaku, Lampung, Indonesia) in concentration 30 g L^{-1}, 7 g L^{-1} agar (Double Swallow Sunrise, Jakarta, Indonesia), and pH media adjusted in 5.8 were applied in all *in vitro* propagation stages. All media were poured in jam bottles (7 cm in diameters, 12 cm in height, 30 ml media in each) and autoclaved at 121°C and 15 kPa for 20 min for sterilization (All American autoclave, Wisconsin, US).

Callus Initiation and Shoot Formation

Young leaf explants prepared previously from *E. grandiflorum* 'Voyage 2 Green' and 'Reina White' were used in the experiment. For initiation stage, three different basalt media i.e. Linsmaier and Skoog (LS; 19), B5 and MS were tested. Each medium

augmented with 0.5 mg L^{-1}BAP and 0.1 mg $L^{-1}$of different types of auxin (2,4 D; IAA; NAA) (Table 1).

The experiment was arranged in a randomized complete block design (RCBD) with three replications per treatment. Each treatment consisted of 3 bottles. Each bottle contained 3 pieces of young leaf explants. Variables observed in the study were (1) Callus score (- to ++++), where - no callus formed, + 1-25% of total explant surface produced callus , ++ 26-50% of total explant surface produced callus, +++ 51-75% of total explant surface produced callus and ++++ 76-100% of total explant surface produced callus; (2) Number of shoots per explant. Periodical observation was carried out to know the growth of explant during incubation. Callus score was recorded 4 weeks after inoculation, while number of initiated shoots from callus was noted after 8 week in culture.

Multiplication Rate of Shoots Derived

Multiplication rate of shoots from two varieties was studied by culturing single shoot on MS medium PGR free (Medium 1) and MS fortified by 0.02 mg L^{-1} NAA and 0.2 mg L^{-1} BAP (Medium 2) under 3 periodical subcultures monthly. The experiment was arranged in RCBD with 6 replications per treatment. Each treatment consisted of 3 bottles and each bottles contained 3 shoots. The multiplication rate was calculated by counting number of shoots in the end of each culture divided by number shoots in initial culture.

Application of GA_3 to Improve the Shoot Growth

Improvement of the shoots growth was carried out by adding GA_3 in different concentration of 0, 2, 5, 7 and 10 mg L^{-1}in selected MS medium. The experiment was arranged in a completely randomized design (CRD) with 3 replications. Each treatment consisted of 3 bottles. Each bottle contained 3 shoots. Periodical observation was carried out to know the response of shoots growth under GA_3 treatments. Parameters observed in the study were shoots height (cm) and number of proliferated-shoots per shoot and recorded ±1.5 months after inoculation.

Rooting Induction

Proliferated shoots derived from the previous experiment were cut individually and selected for the experiments. The shoots with 5 cm in height and 3 pairs of leaves were cultured in rooting media. The rooting media (RM) applied in this experiment were MS medium supplemented with 2 mg L^{-1} IAA and 1 g L^{-1} AC (RM-1), 2 mg L^{-1} NAA and 1 g L^{-1} AC (RM-2), 2 mg L^{-1} IBA and 1 g L^{-1} AC (RM-3). The experiment was arranged in a CRD with 5 replications. Each treatment consisted of 3 shoots per bottle. Periodical observation was carried out to know root formation process. Variables observed in the study were number of roots and length of roots (cm) and recorded ± 1.5 months after culture.

Table 1 Effect of varieties and initiation media on callus and shoot formation.

Varieties/Initiation media	Callus score per explant	Number of shoots per explant
Voyage 2 Green	+	8.0 b
Reina White	+	9.7 a
IM-1: LS medium containing 0.1 mg L^{-1} 2,4 D and 0.5 mg L^{-1} BAP	+	2.8 dc
IM-2: LS medium supplemented with 0.1 mg L^{-1} IAA and 0.5 mg L^{-1} BAP	+	5.3 c
IM-3: LS medium augmented with 0.1 mg L^{-1} NAA and 0.5 mg L^{-1} BAP	+	3.0 dc
IM-4 B5 medium fortified by 0.1 mg L^{-1} 2,4 D and 0.5 mg L^{-1} BAP	+	18.7 a
IM-5: B5 medium 0.1 mg L^{-1} IAA and 0.5 mg L^{-1} BAP	+	16.0 b
IM-6: B5 medium added by 0.1 mg L^{-1} NAA and 0.5 mg L^{-1} BAP	++	14.5 b
IM-7: MS medium supplemented with 0.1 mg L^{-1} 2,4 D and 0.5 mg L^{-1} BAP	+	14.2 b
IM-8: MS medium with 0.1 mg L^{-1} IAA + 0.5 mg L^{-1} BAP	+	4.3 dc
IM-9: MS medium containing 0.1 mg L^{-1} NAA and 0.5 mg L^{-1} BAP	+	1.2 e

Note: -, no callus formed; +, 1-25%; ++, 26-50%; total explant produced callus. Means followed the same letter at the same coloum are not significantly difference based on Duncan Multiple Range Test, *p=0.05*.

Acclimatization

Well-developed plantlets were removed from the agar medium, washed the roots of plantlets under running tap water to remove agar from them, immersed in 1% solution of 50% Benomyl and 20% Kanamicyn Sulphate for 3 min and then air-dryed for moment. The plantlets were then planted in plastic pots (±25 plantlets per pot) containing mixture media of cocopeat and burned rice husk (1:1, v/v) and covered the pots with transparent plastic for ±1.5 months. Total plantlets acclimatized were 150 plantlets. Variables observed in the study were percentage of survivability (%) and number of survival plantlets.

Data analysis

All data collected from the experiments were analyzed using analysis of variance (ANOVA) with SAS program Release Windows 9.12. Significant differences between means were assessed by Duncan Multiple Range Test (DMRT) at $P= 0.05$ (Mattjik and Sumertajaya, 2006).

Results

Callus Induction and Shoot Formation

Initial callus formation was occured 7-21 days after culture, and then initial shoots was visible 21-28 days after in culture (Figure 2A, 2B, 2C). Total number of shoots per explant varied between 1-20 shoots. The highest response of callus and shoot formation was recorded on leaf explants of *E. grandiflorum* 'Reina White' cultured on B5 medium supplemented with 0.1 mg L^{-1} 2,4 D and 0.5 mg L^{-1} BAP.

The single treatment of varieties and types of media gave significant effect on callus and shoot formation, however the interaction of both treatment did not give significant interaction effect in all parameters observed. In variety treatment, the highest callus formation and number of shoots per explant were determined on *E. grandiflorum* 'Reina White' with 9.7 shoots. While the suitable medium for the same purpose was established on B5 medium supplemented with 0.1 mg L^{-1} 2,4 D and 0.5 mg L^{-1} BAP with 18.7 shoots (IM-4 medium; Figure 2D). The lowest response on callus and shoot formation was recorded on MS containing 0.1 mg L^{-1} 2,4 D and 0.5 mg L^{-1} BAP with 1.2 shoots (IM-7 medium). From the study it was revealed that *E. grandiflorum* 'Reina White' and IM-4 medium induced higher callus induction and higher shoots formation (Table 1).

Multiplication Rate of Shoots Derived from Young Leaf Explants

Multiplication rate of individual shoot at 3 periodical subcultures on two different media showed that MS containing 0.02 mg L^{-1} NAA and 0.2 mg L^{-1} BAP induced higher multiplication rate than that of MS PGR free medium. The multiplication rate of individual shoot on MS containing 0.02 mg L^{-1} NAA and 0.2 mg L^{-1} BAP was 6.47 in first subcultures, 8.92 in second subculture and 8.66 in third subculture (Figure 1 and Figure 2E) (Table 2). Although the medium 2 stimulated high multiplication of shoots, the performance of shoots was lower compared to medium 1 with smaller size of leaves, lower height of shoots, and rosette morphologically.

Application of GA_3 to Improve the Shoot Growth

Based on periodical observation, it was known that acceleration growth of shoots, indicating by initial elongation of shoot tip, was observed ± 7 days after inoculation The elongation of shoot tip followed by raising pairs of new leaves was occurred 10-15 days after culture with the good performance of shoots observed on MS medium supplemented by 10 mg L^{-1} GA_3 at *E. grandiflorum* 'Voyage 2 Green'. Furthermore different concentrations of GA_3 and media gave significant effects on number of shoots per plantlet and height of shoots. However the both treatments did not indicate significant interaction effect in all parameters observed. *E. grandiflorum* 'Voyage 2 Green' had higher number of shoots than *E. grandiflorum* 'Reina White' with 1.8 shoots per plantlet and5.7 cm average of shoot height. MS medium supplemented with 10 mg $L^{-1}GA_3$ influenced the highest number of shoot per plantlet (3.0) and height of shoots (9.3 cm) (Table 3).

Table 2 Effect of different varieties and culture media in the second subcultures on multiplication rate of shoots

Varieties/Media	Multiplication Rate
Voyage 2 Green	4.92 b
Reina White	5.71 a
MS	1.47 b
MS + 0.02 mg L^{-1}NAA + 0.2 mg L^{-1}BAP	9.16 a

Means followed the same letter at the same colum are not significantly difference based on Duncan Multiple Range Test, *p=0.05*

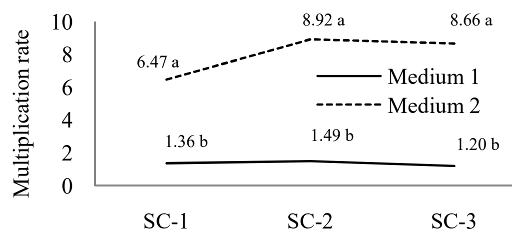

Figure 1. Multiplication rate of shoots on two selected-media at 3 periodical subcultures (SC-1, SC-2 and SC-3). Medium 1= MS, Medium 2= MS supplemented with 0.2 mg L^{-1}BAP and 0.02 mg L^{-1}NAA.

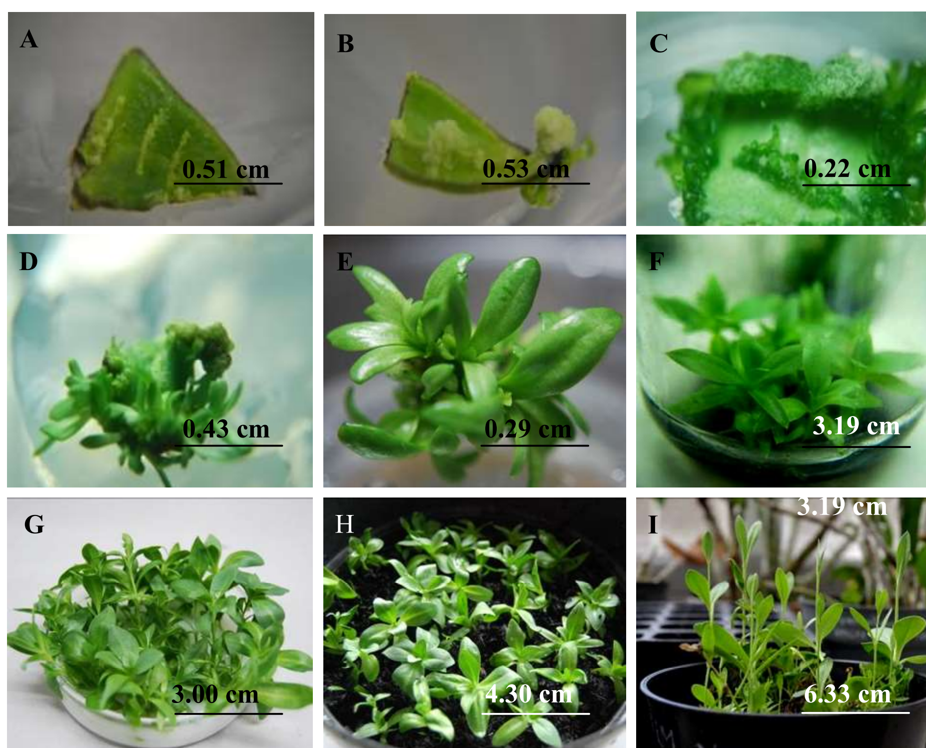

Figure 2 The new *in vitro* propagation protocol of Lisianthus. (A) Initiated-callus derived from young leaf ± 7 days after culture; (B). callus produced 15 days after culture; (C) initial shoots derived from leaf callus 21 days after culture; (D) regenerated shoots on B5 medium containing 0.5 mg L^{-1}BAP and 0.mg L^{-1}2,4-D ± 35 days after culture; (E) well-growth of multiple shoots on MS medium plant growth regulator free; (F) well-rooted shoots on MS medium supplemented with 2 mg L^{-1}IAA and 1 g L^{-1}AC ± 1.5 months after culture; (G) plantlets treated by 1% pesticide solution before acclimatization; (H) acclimatized plantlets ± 1.5 months after transferring to *ex vitro* condition; and (I) well-growth plants ± 3.5 months after acclimatization.

Rooting Induction

In the root induction experiment, initial root formation occurred ± 14 days after start of cultivation. The roots formed initially continued to grow and produced 0-3 roots per plantlet with 1-4 cm in length easily observed 4 weeks after culture. In this study, there was no significant difference on number of roots per plantlet between varieties tested (Table 4; Figure 2F). However, *E. grandiflorum* 'Reina White' produced higher number of roots per plantlet and wider root length compared than *E. grandiflorum* 'Voyage 2 Green'. While, MS medium

Table 3 Growth of plantlets derived from two varieties and under different GA_3 concentrations.

Varieties/GA concentrations	Shoot (No. Plantlet^{-1})	Shoot height (cm)
Voyage 2 Green	1.8 a	5.7 a
Reina White	1.6 a	4.5 b
0.0 mg L^{-1} GA	0.0	0.0
2.0 mg L^{-1} GA	1.6 c	1.7 c
5.0 mg L^{-1} GA	1.8 c	6.2b
7.0 mg L^{-1} GA	2.3 b	8.7 a
10 mg L^{-1} GA	3.0 a	9.3 a

Mean followed the same letter at the same coloum are not significantly difference based on Duncan Multiple Range Test, *p=0.05*.

Table 4 Rooting induction of shoots from two varieties under different rooting media.

Varieties/Media	Root (No. Plantlet^{-1})	Root height (cm)
Voyage 2 Green	1.60 a	1.74 a
Reina White	1.77 a	1.86 a
MS+2mg L^{-1}IAA +1 g L^{-1}AC	1.90 a	1.85 a
MS+2mg L^{-1}NAA+1 g L^{-1}AC	1.60 b	1.73 a
MS+2mg L^{-1}IBA +1 g L^{-1}AC	1.56 b	1.82 a

Means followed the same letter at the same coloum are not significantly difference based on Duncan Multiple Range Test, *p=0.05*.

containing 2 mg L^{-1} IAA and 1 mg L^{-1} AC was the appropriate medium in inducing root formation. The medium induced 1.9 roots per plantlet and 1.85 cm root length.

Acclimatization

Acclimatization of plantlets derived from rooting induction experiment was successfully established in the study. The high survavibility up to 80% with ± 20 survival of planlets per pot were recorded in 1.5 months after acclimatization. The survival plantlets with vigor performances were observed about 3.5 months after acclimatization (Figure 2H).

Discussion

A new *in vitro* propagation protocol for *E. grandiflorum* was successfully established in this study. The new protocol was initiated by high callus formation derived from young leaf explants of *E. grandiflorum* 'Voyage 2 Green' and 'Reina White' on MS medium supplemented with 0.1 mg L^{-1}NAA and 0.5 mg L^{-1} BAP (regenerating up to 50% callus formation). Razaee et al. (2012) reported that callus formation was obtained on LS medium containing 3 mg L^{-1} IAA, 3 mg L^{-1} NAA, 0.1 mg L^{-1} kinetin, and B5 medium containing 0.225 mg L^{-1} BA and 1.86 mg L^{-1} NAA, while Zhou et al. (2014) found that the highest frequency of callus formation induced from anthers up to 100 % with 60 % shoot regeneration was established on MS medium containing 8.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA. In this study, the result was higher than other basalt media and BAP-auxins.

Successfully advent if shoots formation on *in vitro E. grandiflorum* were established with different results. Popa et al (2006) applied basalt MS medium in combination with the applications of auxine and cytokinin (0.5 mg L^{-1} BA and 0.1 mg L^{-1} NAA) to produce 31.1 shoots/explant. The same medium containing 0.5 mg L^{-1} GA_3 and 1.5 mg L^{-1} BA obtained 7.6 adventitious shoots (Mousavi et al., 2012a); 0.5 mg L^{-1} BA and 0.02 mg L^{-1} NA with 8 shoots/explant (Winarto et al., 2015); 0.5 mg L^{-1} BA resulted 8.86 shoots/explant (Esizad et al., 2012) and 8.75 shoots per explants (Kaviani et al., 2014). While in this study the highest shoots formation up to 18.7 shoots per explant were proved on B5 medium supplemented with 0.1 mg L^{-1} 2,4 D and 0.5 mg L^{-1} BAP and this result higher compared to other basalt media and BAP-auxins.

Multiplication rate study in *in vitro* culture of lisianthus under periodical subcultures was rarely reported, however it was also revealed in this study. The high multiplication rate of shoots increased gradually till the second subculture with 8.92 for medium 2 and 1.49 for medium 1, respectively, then decreased thereafter. While Winarto et al. (2015) recorded that the multiplication of shoots increased progressively up to the third subculture with 1.74, and lowered afterwards.

High number of shoots successfully regenerated in the initiation and multiplication stage did sometime not lead to producing shoots with healthy and vigor performance. The shoots were proved by culturing shoots on MS medium containing 10 mg L^{-1} GA_3, while Popa et al. (2006) cultured shoots on

MS supplemented with 5 mg L^{-1} GA_3 and 1 mg L^{-1} BAP. MS medium with 0.5 mg L^{-1} BAP and 0.02 mg L^{-1} NAA was applied for *E. grandiflorum* 'White Lavender' shoots (Winarto et al., 2015).

The shoots of *E. grandiflorum* 'Reina White' produced in the study were easily rooted on MS medium augmented with 2 mg L^{-1} IAA and 1 g L^{-1} AC. Meanwhile Paek and Hahn (2000) rooted shoots on MS medium containing 1 mg L^{-1} IAA, 2.40 roots per shoot stimulated on MS medium supplemented with 2 mg L^{-1} Kin and 0.5 mg L^{-1} NAA (Esizad et al., 2012), 2.55 roots on MS medium fortified with 1 mg L^{-1} Kin and 0.5 mg L^{-1} NAA (Kaviani 2014), and 62.0 roots and 4.97 cm root length on B5 medium with 1.5 mg L^{-1} NAA respectively (Mousavi et al., 2012a; Akbari et al. 2014), 3.9 roots on MS medium augmented with by 0.1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA (Winarto et al., 2015). The transgenic elongated shoots were 93 % rooted on MS medium supplemented 3.0 μM IAA and 100 mg L^{-1} kanamycin (Thiruvengadam and Chung, 2015).

Completing of the *in vitro* propagation protocolis significantly noted when transferring well-rooted shoots is occurred with high survivability. The new *in vitro* propagation protocol also successfully ensured high survivability of plantlets during acclimatization up to 80% on a mixture media of burned rice-husk and cocopeat (1:1, v/v). High survivability of plantlets during acclimatization (up to 100%) in a mixture of peat and perlite (1:1, v/v) was also reported by Esizad et al. (2012) and Kaviani (2014), 90% in burned-rice husk and organic manure (1:1, v/v) (Winarto et al., 2015). These results are presumably related to well-drainage of media containing burned-rice husk and cocopeat.

Conclusion

An *in vitro* propagation protocol for*E. grandiflorum* was successfully established. *E. grandiflorum*'Reina White'. B5 medium supplemented 0.1 NAA and 0.5 mg L^{-1} BAP stimulated callus formation up to 50%. The callus was easily regenerated on B5containg 0.1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP with 18.67 shoot per explant at 8 weeks under light incubation. Well growth of shoots with 9.33 cm shoot height at 4 weeks after culture was established on MS medium augmented with 10 mg L^{-1} GA_3, easily rooted on MS supplemented with 2 mg L^{-1} IAA and 1 g L^{-1} AC produced 1.9 roots per shoot and 1.8 cm root length at 4 weeks after culture. Plantlets were successfully transferred to ex vitro condition with 80 % survivability on a mixture of burned-rice husk and cocopeat (1:1, v/v).

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