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In vitro PROLIFERATION AND *ex vitro* ROOTING OF MICROSHOOTS OF LISIANTHUS USING AUXIN AND CYTOKININ ON THE SOLID, LIQUID AND DOUBLE-PHASE CULTURE SYSTEMS

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ABSTRACT

A protocol was developed for high frequency and low cost of *in vitro* shoot proliferation and *ex vitro* rooting of *Eustoma grandiflorum* (*Gentianaceae*) on solid medium. Shoot tips as explants were cultured on Murashige and Skoog (MS) medium enriched with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0.00, 0.01, 0.10 and 1.00 mg l^{-1}) and 6-benzylaminopurine (BAP) (0.00, 0.50, 2.00 and 5.00 mg l^{-1}). Three culture media systems (solid, liquid and double-phase) were applied. None of the explants cultured on liquid and double-phase media resulted in live plant production. Maximum axillary shoot number (54.45) was recorded in the plantlets treated with 0.10 mg l^{-1} 2,4-D in combination with 5.00 mg l^{-1} BAP. Treatment of 0.01 mg l^{-1} 2,4-D along with 0.50 mg l^{-1} BAP produced maximum node number and internode length. Some shoots produced on medium containing plant growth regulators (PGRs) were rooted in soil. The largest number (5.50/plantlet) and longest length of root (7.75 cm/plantlet) were obtained in *ex vitro* condition on the base of shoots produced in culture medium enriched with 0.10 mg l^{-1} 2,4-D along with 0.50 mg l^{-1} BAP. The combination of 1.00 mg l^{-1} 2,4-D and 0.50 mg l^{-1} BAP was found to be the most suitable PGRs for obtaining the highest callus weight. The most fresh weight was calculated from plantlets grown on the medium containing 0.10 mg l^{-1} 2,4-D along with 5.00 mg l^{-1} BAP. Maximum dry weight was obtained in free-PGRs medium. About 90% of the rooted plantlets were established successfully in cultivation beds. Acclimatized plants were morphologically similar to the mother plants.

Key words: lisianthus, micropropagation, ornamental plants, plant growth regulators

INTRODUCTION

In vitro propagation techniques could allow for the production of physiologically uniform clonal plants and potentially rapid multiplication. Micropropagation has been extensively applied for the rapid production of many plant species and cultivars. In many micropropagation studies, a high number of treatments, PGRs, and dosages are examined in an effort to find the best way to obtain a proper propagation protocol. Lisianthus (*Eustoma grandiflorum*; *Gentianaceae*), a relatively new floral crop to the international market, quickly ranked in the top ten cut flowers worldwide due to its rose-like flowers, excellent post-harvest life, and being available in various colors [Armitage and Laushman 1993, Kunitake et al. 1995]. In recent years, plant breeders have developed a variety of cultivars with respect to many traits such as uniform flowering throughout the year, flower color, and flower size and form, etc. [Harbaugh 2006]. *E. grandiflorum* is commonly propagated by seed or cutting. A large number of seedlings can be produced by seed propagation, but

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the quality is not uniform due to variations in flowering time, plant height and the number of flowers. In some cultivars, seedlings show a wide range of variation because of their heterozygous characteristics [Furukawa et al. 1990]. The methods for micropropagation of prairie gentian [*E. grandiflorum* (Griseb.) Schinners] have been developed and many plants were regenerated from stem, leaf and meristem explants [Furukawa et al. 1990]. The success of the micropropagation method depends on several factors like genotype, physical and chemical state of the culture medium, PGRs and type of explants, which should be observed during the process [Pati et al. 2005, Nhut et al. 2010]. The culture medium in a double-phase and liquid system can enhance the growth and development rate, mainly due to a better nutrient availability [Scherwinski-Pereira et al. 2012, de Oliveira et al. 2013]. The use of double-phase has improved the efficiency of *in vitro* proliferation of some species [Costa et al. 2007, Pullman and Skryabina 2007, Scherwinski-Pereira et al. 2012, de Oliveira et al. 2013]. There is not much research on micropropagation of *E. grandiflorum*. A few studies on *in vitro* propagation of *E. grandiflorum* have been reported by Paek and Hahn [2000], Ordogh et al. [2006] and Ghaffari Esizad et al. [2012]. Most of these workers have been using BA, KIN, NAA and IBA as PGRs. Therefore, the objective of the present study was to evaluate the effect of different concentrations of 2,4-D and BAP on regeneration of axillary shoot *in vitro* and rooting of micro-shoots *ex vitro* in *E. grandiflorum*. We also investigated liquid and double-phase media in addition to the solid medium.

MATERIALS AND METHODS

Source materials and disinfection. The mother plants of *Eustoma grandiflorum* were prepared from a greenhouse in Isfahan, Iran during November–December 2013. Shoot tips with height of roughly 10 mm were dissected from mother plants and used as explants for tissue culture establishment in all experiments. The explants were washed under running tap water and 1–2 drops of hand washing liquid for 45 min. Then explants were dipped in water (40°C) for 50 min. followed by immersion in 20% H_2O_2 and two drops of Tween-20 for 10 min. then rinsed with sterilized distilled water thrice. Explants were disinfected by immersion in 20% sodium hypochlorite (NaClO) (v/v) with a few drops of Tween-20 for 23 min, followed by three rinses in sterile distilled water under a laminar flow hood. Finally, shoot tips were treated with 300 mg l^{-1} silver nano-particles for 35 min and washed thoroughly three times in sterilized distilled water.

Culture media and growth conditions. Surface sterilized shoot tips were placed in 250 ml bottles, with 50 ml of the full-strength MS [Murashige and Skoog 1962] culture medium containing 30 g l^{-1} sucrose. The culture media were enriched with concentrations of 0.00, 0.01, 0.10 and 1.00 mg l^{-1} of 2,4-D, and 0.00, 0.50, 2.00 and 5.00 mg l^{-1} of BAP, individually or/and in combination. With the exception of liquid culture media, the solidification of culture media was done using 7 g l^{-1} agar-agar (Merck, Germany). In addition to the solid and liquid media, double-phase culture medium was also used in this experiment. In the double-phase system, 15 ml of liquid medium was added to the surface of solid medium. The pH of the media was adjusted to 5.7 ±0.1 before adding agar. The culture glass bottles containing the media were plugged firmly and autoclaved at 121°C and 104 kPa for 20 min. Experiments were carried out in three replications and each replication consisted of three explants. The cultures were maintained at temperature of 22 ±2°C, 16-h photoperiod (irradiance of 50 µmol m^{-2} s^{-1}), provided by cool daylight fluorescent lamps.

Effect of PGRs on shoot proliferation. Traits including axillary shoot number, node number, internode length, chlorophyll content, callus weight and fresh and dry weight of plantlets were calculated after 45 days. Internode length was measured with a ruler. Shoot and node number were counted with the naked eye. In order to measure the dry and fresh weight of plantlets, they quickly were weighed after harvest on a digital scale (fresh weight). Then, the samples were dried in an oven at the temperature of 105°C for 24 h and reweighed by a digital scale (dry weight). Callus obtained from the media was removed and weighed on a digital scale after 45 days. Leaf chlorophyll (Chl) content was measured (5 leaves from each treatment randomly selected from different plantlets) by hand-held Chlorophyll Content Meter (Hansatech CL 01, England) on expanded leaves.

Rooting and acclimatization. After 45 days of establishment of explants on media and for hardening

and acclimatization, the glass bottles with plantlets (fully expanded leaflets with a height of 6–7 cm) were kept open for a week after removing the plugs from the culture room. Then the plantlets were removed from the culture media and washed with distilled water followed by transfer to *ex vitro* conditions. During transfer of plantlets from *in vitro* to *ex vitro* conditions, all the shoots formed on each plantlet were separated from each other and planted into the plastic cups (10-cm in diameter) containing a mixture of peat and perlite (1 : 1). Plantlets were kept in a greenhouse at 24 ±2°C, 70% relative humidity and photosynthetic photon flux density of 450–500 µmol $m^{-2}s^{-1}$ under 16 h light/8 h dark photoperiod with periodic irrigation. After 30 days of cultivation under *ex vitro* conditions, root number and length (cm) were evaluated. Plants transferred to *ex vitro* conditions (control and treated plants) were morphologically compared with each other and with mother plants to view any changes.

Data analysis. The experimental design was R.C.B.D. Each experiment was done in three replicates and each replicate includes three specimens. Data processing of the results was carried out by an EXCEL. Analysis of variance (ANOVA) was done using SAS and MINITAB statistical software and means were compared using the Least Significance Difference Test (LSD) at 5% probability level.

RESULTS AND DISCUSSION

Influence of 2,4-D and BAP on *in vitro* multiplication of *E. grandiflorum*. Maximum axillary shoot number (54.45) was recorded in the plantlets treated with 0.10 mg l^{-1} 2,4-D plus 5.00 mg l^{-1} BAP (Tab. 1, Fig. 1A). These axillary shoots were not very extensive. While the number of axillary shoots in the control plantlets were 2.80. The large number of axillary shoot was produced on explants treated with 0.10 mg l^{-1} 2,4-D in combination with 2.00 mg l^{-1} BAP (37.03), 2.00 mg l^{-1} BAP without 2,4-D (32.30) and 0.01 mg l^{-1} 2,4-D in combination with 2.00 mg l^{-1} BAP (27.53). Minimum axillary shoot number (0.63) was induced in media containing 1.00 mg l^{-1} 2,4-D. Low axillary shoot number (2.33, 2.50 and 2.53) was induced in media without BAP (Tab. 1). None shoot was produced in media containing 1.00 mg l^{-1} 2,4-D in combination with 0.50, 2.00 and 5.00 mg l^{-1} BAP (Tab. 1). Callus was produced in these media. Concerning the axillary shoot number per plantlets, BAP at 2.00 mg l^{-1} induced the maximum number (24.21). Differences of axillary shoot number in explants grown under combination of 2,4-D and BAP were highly significant ($p \leq 0.01$) (Tab. 2). Similar results were observed by some other researchers [Paek and Hahn 2000, Ghaffari Esizad et al. 2012]. Study of Ghaffari Esizad et al. [2012] on micropropagation of *E. grandiflorum* showed the positive effect of cytokinin KIN on multiplication rate. So that, shoot tip medium supplemented with 1.00 mg l^{-1} KIN resulted in the largest number of shoots per explant (2.62). BAP played more important role than 2,4-D for induction of shoot production in current study. Study on some other ornamental plants revealed that cytokinins are necessary for shoot multiplication [Jain and Ochatt 2010, Kaviani et al. 2011]. Several studies showed that some species require a low concentration of auxin along with cytokinin to enhance shoot multiplication [van Staden et al. 2008, Hashemabadi and Kaviani 2010, Ghaffari Esizad et al. 2012, Kaviani et al. 2014]. Current study is in accordance with this finding.

Node number varied with 2,4-D and BAP concentrations (Tab. 1). Minimum node number was recorded in the plantlets treated with highest concentration of 2,4-D (1.00 mg l^{-1}) with all concentrations of BAP (0.50, 2.00 and 5.00 mg l^{-1}) (Tab. 1). Largest number of node (6.10/plantlet) was achieved on MS medium supplemented with 0.01 mg l^{-1} 2,4-D along with 0.50 mg l^{-1} BAP (Tab. 1). Probably, the explants have enough auxin to induce shoot formation. Concerning the node number per plantlets, BAP at 0.50 and 2.00 mg l^{-1} induced the maximum number (4.46). Differences of node number in explants grown under combination of 2,4-D and BAP were significant ($p \leq 0.01$) (Tab. 2). PGRs (2,4-D and BAP) had a significant effect on increasing internode length. Maximum internode length (8.10 mm/plantlet) was observed on MS medium supplemented with 0.01 mg l^{-1} 2,4-D along with 0.50 mg l^{-1} BAP (Tab. 1, Fig. 1B). Minimum internode length was recorded in the plantlets treated with highest concentration of 2,4-D (1.00 mg l^{-1}) with all concentrations of BAP (0.50, 2.00 and 5.00 mg l^{-1}) (Tab. 1). Among all concentrations of BAP, 0.50 mg l^{-1} induced the longest internode (4.70 mm/plantlets). Also, among all concentra-

Table 1. Mean comparison of the effect of different concentrations of 2,4-D and BAP, individually and in combination with each other, on shoot number, node number, internode length, root number (*ex vitro*), root length (*ex vitro*), fresh weight, dry weight, callus weight and chlorophyll content of *Eustoma grandiflorum*

PGRs (mg l^{-1})	Shoot number	Node number	Internode length (mm)	Root number (*ex vitro*)	Root length (*ex vitro*) (cm)	Plantlet fresh weight (g)	Plantlet dry weight (g)	Callus weight (g)	Chlorophyll content
2,4-D 0.00	14.00 ±0.25b	3.77 ±0.07ab	3.11 ±0.07b	0.86 ±0.02c	1.40 ±0.02c	0.50 ±0.02c	0.08 ±0.01a	0.89 ±0.04bc	32.01 ±1.27a
2,4-D 0.01	12.42 ±0.22c	3.97 ±0.09a	3.35 ±0.08b	1.67 ±0.04b	2.25 ±0.03b	0.70 ±0.03b	0.05 ±0.00b	0.71 ±0.03c	26.39 ±1.10b
2,4-D 0.10	27.05 ±0.15a	3.55 ±0.06b	4.25 ±0.22a	1.99 ±0.05a	2.88 ±0.04a	0.99 ±0.05a	0.08 ±0.01a	0.95 ±0.05b	26.08 ±2.00b
2,4-D 1.00	0.63 ±0.02d	0.77 ±0.01c	0.67 ±0.02c	0.00 ±0.00d	0.00 ±0.00d	0.11 ±0.00d	0.02 ±0.00c	1.31 ±0.06a	16.53 ±0.67c
BAP 0.00	2.54 ±0.14d	3.64 ±0.05a	3.79 ±0.08b	2.37 ±0.06a	3.54 ±0.06a	0.36 ±0.01d	0.06 ±0.01ab	0.24 ±0.00d	50.70 ±2.47a
BAP 0.50	9.82 ±0.10c	3.84 ±0.05a	4.70 ±0.20a	2.15 ±0.05b	2.99 ±0.04b	0.49 ±0.02c	0.05 ±0.01bc	1.55 ±0.07a	22.10 ±1.74b
BAP 2.00	24.21 ±0.17a	2.51 ±0.04b	1.40 ±0.27c	0.00 ±0.00c	0.00 ±0.00c	0.68 ±0.03b	0.04 ±0.00c	1.29 ±0.06b	13.80 ±0.87c
BAP 5.00	17.53 ±0.27b	2.07 ±0.03c	1.50 ±0.29c	0.00 ±0.00c	0.00 ±0.00c	0.76 ±0.04a	0.07 ±0.02a	0.79 ±0.03c	14.20 ±0.27c
2,4-D 0.00 + BAP 0.00	2.80 ±0.08ef	3.86 ±0.05ab	3.20 ±0.09a-d	3.45 ±0.05b	5.60 ±0.11b	0.62 ±0.02b-f	0.10 ±0.02b	0.00 ±0.00d	45.56 ±3.39ab
2,4-D 0.00 + BAP 0.50	12.75 ±0.36d	4.46 ±0.07ab	4.90 ±0.23a-c	0.00 ±0.00e	0.00 ±0.00f	0.01 ±0.00bc	0.08 ±0.01b	1.00 ±0.06b-d	35.40 ±1.23c
2,4-D 0.00 + BAP 2.00	32.30 ±0.09b	4.46 ±0.07ab	2.00 ±0.05b-d	0.00 ±0.00e	0.00 ±0.00f	1.10 ±0.19b	0.08 ±0.01b	1.96 ±0.08ab	28.36 ±1.12cd
2,4-D 0.00 + BAP 5.00	8.16 ±0.11d-f	2.30 ±0.04bc	2.36 ±0.19b-d	0.00 ±0.00e	0.00 ±0.00f	0.28 ±0.03d-f	0.06 ±0.02b	0.63 ±0.03cd	18.72 ±0.86de
2,4-D 0.01 + BAP 0.00	2.50 ±0.5ef	3.30 ±0.05ab	2.20 ±0.07b-d	3.58 ±0.08b	4.78 ±0.09c	0.35 ±0.03c-f	0.06 ±0.00b	0.00 ±0.00d	52.55 ±3.87ab
2,4-D 0.01 + BAP 0.50	11.46 ±0.10de	6.10 ±0.08a	8.10 ±0.58a	3.10 ±0.06c	4.23 ±0.06d	1.19 ±0.26b	0.08 ±0.01b	0.78 ±0.03cd	24.70 ±1.17d
2,4-D 0.01 + BAP 2.00	27.53 ±0.13c	3.50 ±0.06ab	1.83 ±0.21b-d	0.00 ±0.00e	0.00 ±0.00f	0.93 ±0.06b-d	0.06 ±0.02b	1.76 ±0.07a-c	18.73 ±0.86de
2,4-D 0.01 + BAP 5.00	8.20 ±0.06d-f	3.00 ±0.03ab	1.30 ±0.43cd	0.00 ±0.00e	0.00 ±0.00f	0.34 ±0.01c-f	0.02 ±0.00b	0.30 ±0.02d	9.60 ±0.65ef
2,4-D 0.10 + BAP 0.00	2.33 ±0.07ef	4.30 ±0.07ab	7.06 ±0.32a	2.47 ±0.06d	3.78 ±0.06e	0.24 ±0.00d-f	0.08 ±0.03b	0.00 ±0.00d	57.63 ±4.02a
2,4-D 0.10 + BAP 0.50	15.10 ±0.27d	4.80 ±0.08ab	5.80 ±0.41a	5.50 ±0.09a	7.75 ±0.14a	0.78 ±0.03b-e	0.05 ±0.02b	1.75 ±0.07a-c	28.60 ±2.71cd
2,4-D 0.10 + BAP 2.00	37.03 ±0.15b	2.10 ±0.03bc	1.80 ±0.18b-d	0.00 ±0.00e	0.00 ±0.00f	0.71 ±0.03b-f	0.04 ±0.00b	0.95 ±0.05b-d	8.40 ±0.62f
2,4-D 0.10 + BAP 5.00	54.45 ±0.18a	3.00 ±0.04ab	2.36 ±0.05b-d	0.00 ±0.00e	0.00 ±0.00f	2.24 ±0.41a	0.15 ±0.05a	1.13 ±0.06b-d	9.70 ±0.81ef
2,4-D 1.00 + BAP 0.00	2.53 ±0.14ef	3.10 ±0.04ab	2.70 ±0.03b-d	0.00 ±0.00e	0.00 ±0.00f	0.26 ±0.02d-f	0.03 ±0.00b	0.96 ±0.04b-d	47.16 ±3.41b
2,4-D 1.00 + BAP 0.50	0.00 ±0.00f	0.00 ±0.00c	0.00 ±0.00d	0.00 ±0.00e	0.00 ±0.00f	0.00 ±0.00f	0.00 ±0.00b	2.67 ±0.09a	0.00 ±0.00f
2,4-D 1.00 + BAP 2.00	0.00 ±0.00f	0.00 ±0.00c	0.00 ±0.00d	0.00 ±0.00e	0.00 ±0.00f	0.00 ±0.00f	0.00 ±0.00b	0.50 ±0.01d	0.00 ±0.00f
2,4-D 1.00 + BAP 5.00	0.00 ±0.00f	0.00 ±0.00c	0.00 ±0.00d	0.00 ±0.00e	0.00 ±0.00f	0.18 ±0.07ef	0.06 ±0.01b	1.12 ±0.06b-d	18.96 ±0.75de

In each column, means with the similar letters are not significantly different at 5% level of probability using LSD test

Table 2. Analysis of variance (ANOVA) for the effect of different concentrations of 2,4-D and BAP, individually and in combination with each other, on shoot number, node number, internode length, root number (*ex vitro*), root length (*ex vitro*), fresh weight, dry weight, callus weight and chlorophyll content of *Eustoma grandiflorum*

Source of variations	DF	Shoot number	Node number	Internode length (mm)	Root number (*ex vitro*)	Root length (*ex vitro*) (cm)	Fresh weight (g)	Dry weight (g)	Callus weight (g)	Chlorophyll content
2,4-D	3	140.20**	27.10**	28.12**	1.53**	18.66**	0.804**	0.0090**	0.761**	494.00**
BAP	3	105.80**	8.81**	32.78**	2.45**	43.30**	0.383**	0.0020*	4.015**	3635.00**
2,4-D × BAP	9	45.10**	4.06**	9.32**	0.619**	10.90**	1.041**	0.0035**	1.339**	283.00**
Error	32	4.11	0.251	0.1406	0.0492	0.077	0.0056	0.00027	0.0311	25.20
CV (%)		14.99	16.57	13.15	19.62	16.92	13.02	27.85	18.21	19.70

*, **: significant at $\alpha = 5\%$ and $\alpha = 1\%$, respectively

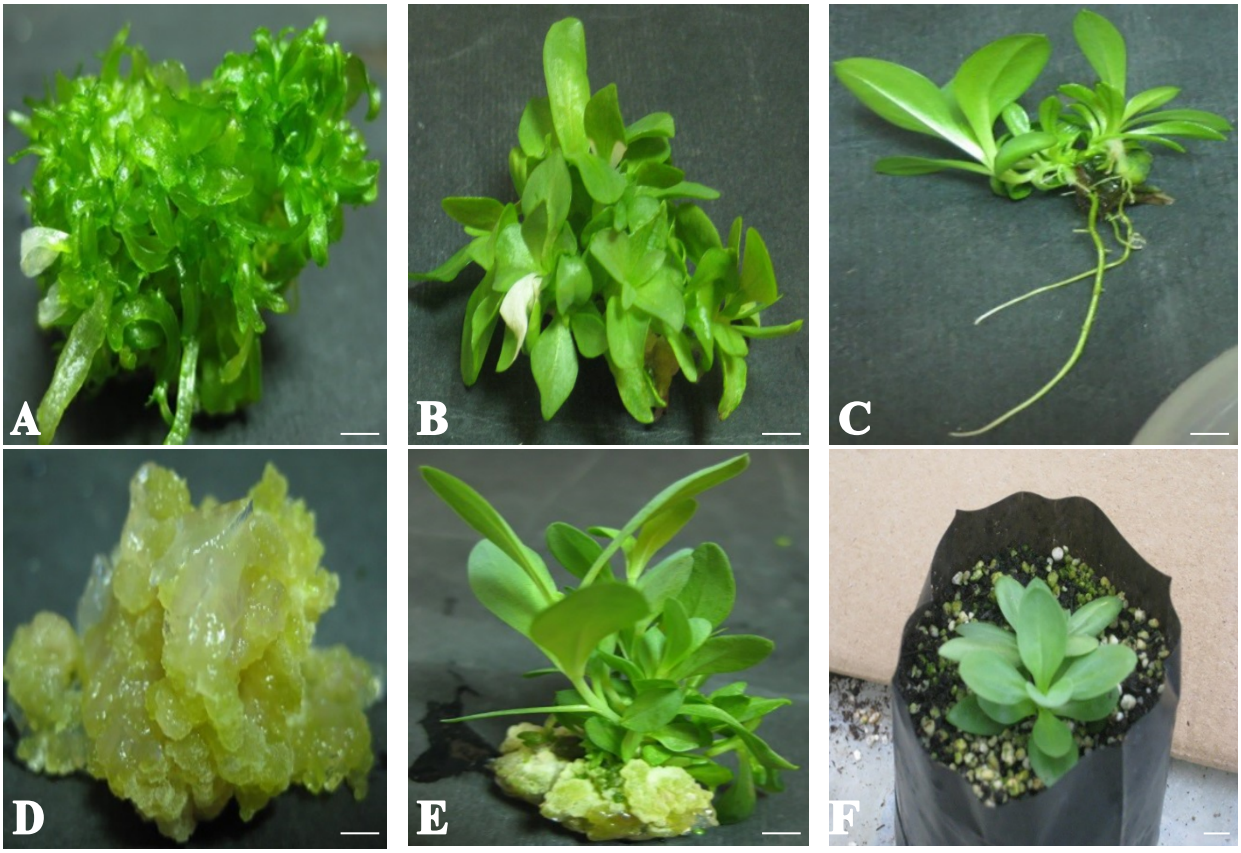

Fig. 1. (A) Aspect of *E. grandiflorum* shoot tip treated with 0.10 mg l^{-1} 2,4-D + 5.00 mg l^{-1} BAP after 45 days included the maximum shoot number; (B) Aspect of *E. grandiflorum* shoot tip treated with 0.01 mg l^{-1} 2,4-D + 0.50 mg l^{-1} BAP included the highest internode length; (C) Aspect of *Eustoma grandiflorum* shoot tips in medium without 2,4-D and BAP-control, rooted *ex vitro*; (D) Aspect of *E. grandiflorum* shoot tip treated with 1.00 mg l^{-1} 2,4-D + 0.50 mg l^{-1} BAP containing maximum callus weight; (E) Aspect of *E. grandiflorum* shoot tip treated with 0.10 mg l^{-1} 2,4-D + 0.50 mg l^{-1} BAP containing callus produced at the base of shoots. All plantlets and callus were produced in solid medium; (F) Micropropagated *E. grandiflorum* plantlets acclimatized in greenhouse after 30 days (scale bar = 5 mm)

tions of 2,4-D, 0.10 mg l^{-1} induced longest internode per plantlets (4.25 mm) (Tab. 1). Differences of internode length in explants grown under combination of 2,4-D and BAP were significant ($p \leq 0.01$) (Tab. 2). Current study revealed that the highest concentration of 2,4-D (1.00 mg l^{-1}) inhibited shoot, node and internode production when used at all concentrations of BAP. Thus, the content of 2,4-D and/or other auxins in apical buds is enough. Low concentration of 2,4-D and BAP is suitable for axillary shoot and node induction and growth in *E. grandiflorum* explants. Similar findings were reported by other researchers working on micropropagation of *E. grandiflorum* [Ordogh et al. 2006, Ghaffari Esizad et al. 2012]. Cytokinins are usually applied to stimulate shoot multiplication and node production [van Staden et al. 2008, Chawla 2009, Gomes et al. 2010]. The present study showed important role of suitable concentrations of an auxin and cytokinin on formation of shoots, nodes and internodes of *E. grandiflorum*. This study is agreement with Kaviani et al. [2014] worked on *E. grandiflorum* with BA and NAA. These researchers showed that the maximum shoot number (5.80 per plant) was produced in medium containing 0.10 mg l^{-1} BA along with

0.20 mg l^{-1} NAA. Also, the most shoot length (2.07 cm/plant) was obtained on medium supplemented with 0.1 mg l^{-1} BA (without NAA).

Influence of 2,4-D and BAP on *ex vitro* rooting of *E. grandiflorum*. Rooting of shoots under *ex vitro* conditions depends on the vigor and growth of shoots and leaves produced *in vitro*. Approximately, 35% of shoots produced *in vitro* were rooted *ex vitro*. Shoots with larger leaves produced more roots. Small shoots with compact leaves did not produce roots (data not shown). The largest number of roots (5.50/plantlet) was obtained on the base of shoots produced on medium enriched with 0.10 mg l^{-1} 2,4-D along with 0.50 mg l^{-1} BAP *in vitro* (Tab. 1). Poor rooting was noticed on shoots cultured on high callus induction media. Shoots without callus or with small callus produced more roots (Fig. 1C). Large callus inhibited root induction *ex vitro*. The plantlets with more roots showed faster growth of the root in relation to the other plantlets with 5.00–8.00 cm length at 30 days. The highest values of the length of the root under *ex vitro* conditions (7.75 cm length) were obtained on the base of shoots treated with 0.10 mg l^{-1} 2,4-D along with 0.50 mg l^{-1} BAP under *in vitro* conditions. LSD showed significant differences among reciprocal effect of 2,4-D and BAP on the root length ($p \leq 0.01$) (Tab. 2).

Roots were produced on media without any PGRs, solely. Explants grown on other media did not produce any roots. Current result is not in agreement with many studies on micropropagation of ornamental plants and some studies on *E. grandiflorum*. Probably, the content of indigenous PGRs is enough for root induction and growth. Sometime, lower and higher concentrations of PGRs than optimum concentration inhibit differentiation of organs. Rooting in soil saves much money, because root induction is the most expensive step during micropropagation process. Shoots produced on culture media supplemented with 0.01 and 0.10 mg l^{-1} 2,4-D both without BAP and along with 0.50 mg l^{-1} BAP were rooted *ex vitro*. Some studies on *E. grandiflorum* showed the positive effect of auxins on rooting [Ghaffari Esizad et al. 2012, Kaviani et al. 2014]. This result showed in many other ornamental plants [Jain and Ochatt 2010, Kaviani et al. 2011]. Some other investigations revealed the positive effect of cytokinins on root induction [Gomes et al. 2010, Ghaffari Esizad et al. 2012, Kaviani et al. 2014]. Similarly to our findings, root formation was inhibited in culture medium of *Lilium longiflorum* enriched with BA [Han et al. 2004]. Study of Kaviani et al. [2014] on micropropagation of *E. grandiflorum* showed that the largest number of root (14.53/plant) was produced in media containing 0.20 mg l^{-1} NAA (without BA).

Influence of 2,4-D and BAP on fresh and dry weight, chlorophyll content and callus weight of *E. grandiflorum*. The effects of different concentrations of 2,4-D and BAP were significant on the fresh and dry weight of plantlets (Tab. 2). The highest average fresh (2.24 g) and dry (0.15 g) weight of plantlets was found with 0.10 mg l^{-1} 2,4-D along with 5.00 mg l^{-1} BAP (Tab. 1). Average fresh (0.00 g) and dry (0.00 g) weight of plantlets was minimum in 1.00 mg l^{-1} 2,4-D along with 0.50 and 2.00 mg l^{-1} BAP (Tab. 1). Callus was produced in these two media (Fig. 1D). Interaction effect of 2,4-D and BAP had a significant effect on increasing chlorophyll content of leaflets (Tab. 2). The different concentrations of 2,4-D increased chlorophyll content of leaflets more than different concentrations of BAP (Tab. 1). Also, 2,4-D at concentrations of 0.01 and 1.00 mg l^{-1} induced the higher content of chlorophyll than other treatments except for 0.10 mg l^{-1} (Tab. 1). Average chlorophyll content of leaflets was minimum in 1.00 mg l^{-1} 2,4-D along with 0.50 and 2.00 mg l^{-1} BAP (Tab. 1). Callus was produced in these two media (Fig. 1D). No callus formation occurred from explants cultured on media containing 0.01 and 0.10 2,4-D without BAP and control (Tab. 1). Callus induction and formation (2.67 g/explant) was maximum in the medium supplemented with 1.00 mg l^{-1} 2,4-D along with 0.50 mg l^{-1} BAP. Also, callus weight calculated in media containing 2.00 mg l^{-1} BAP and 0.10 mg l^{-1} 2,4-D + 0.50 mg l^{-1} BAP was high and average, respectively (Tab. 1, Fig. 1E). LSD showed significant differences among reciprocal effect of 2,4-D and BAP on the callus weight ($p \leq 0.01$) (Tab. 2).

Some concentrations of 2,4-D, BAP and interaction effect of these PGRs promoted callus formation and growth. Paek and Hahn [2000] demonstrated that the increased NAA concentrations adversely affected root formation and led to increased callus formation. Pierik [1987] indicated that with high concentrations of auxins such as 2,4-D and NAA, root formation fails to occur and callus formation takes place. Used con-

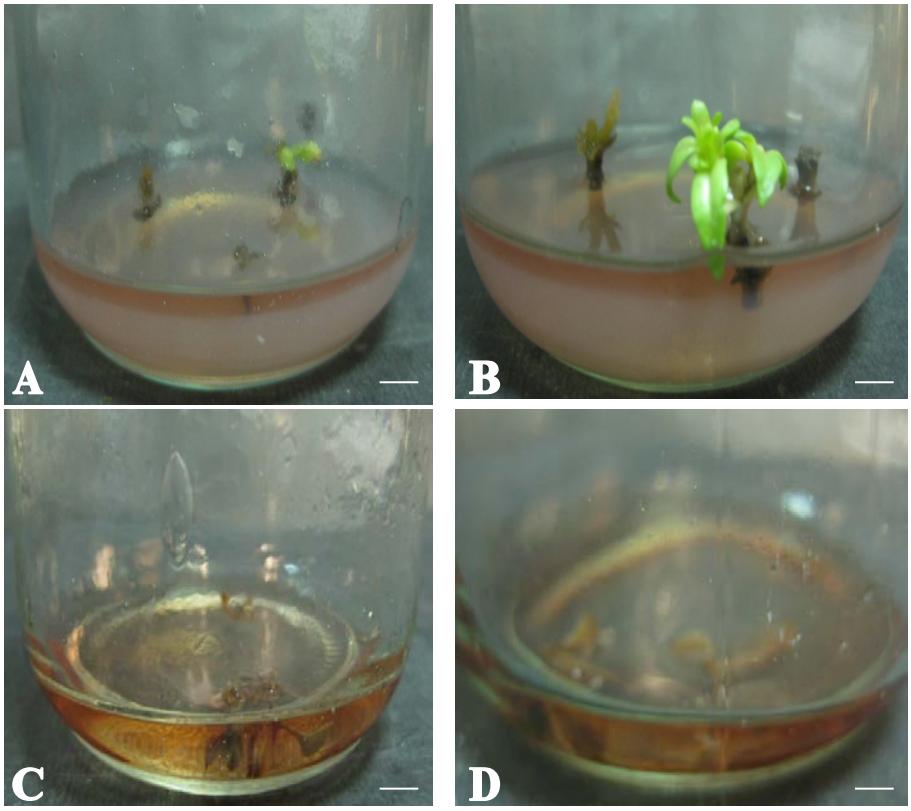

Fig. 2. (A) Aspect of *Eustoma grandiflorum* shoot tips in double-phase medium without 2,4-D and BAP-control; (B) Aspect of *E. grandiflorum* shoot tips treated with 0.50 mg l^{-1} BAP without 2,4-D after 45 days; (C) Aspect of *E. grandiflorum* shoot tips in liquid medium without 2,4-D and BAP-control; (D) Aspect of *E. grandiflorum* shoot tips treated with 0.01 mg l^{-1} 2,4-D + 2.00 mg l^{-1} BAP. Eventually, all the explants and plantlets died (scale bar = 5 mm)

centrations of NAA in Ghaffari Esizad et al. [2012] study had no significant effect on root induction of *E. grandiflorum* and caused callus formation on the base of shoots. Study by Khorrami Raad et al. [2012] on the effect of culture medium on callus weight of *Anthurium andreanum* revealed that BA and NAA had important effect on callus weight. The highest callus weight (0.74 g) was observed on MS medium containing 3.00 mg l^{-1} BA along with 0.50 mg l^{-1} NAA. Hormonal regulation of auxin and cytokinin balance is a key factor in the control of cell division and callus formation in tissue culture. The importance of auxins (especially 2,4-D) and cytokinins (BA, BAP, KIN and zeatin) for callus induction in many ornamental plants was demonstrated by some researchers [Jain and Ochatt 2010]. Consistently to our study, some researchers obtained callus from various explants, when placed on MS medium with BAP and 2,4-D [Viégas et al. 2007, Jahan et al. 2009, Reddy et al. 2011]. Study of Kaviani et al. [2011] on micropropagation of *Matthiola incana* (an ornamental plant) showed that MS medium containing NAA and KIN was most effective for callus induction on leaf micro-cuttings. Cytokinins and auxins are usually known to promote the formation of callus in many excited and *in vitro* cultured organs [Jain and Ochatt 2010]. Proper type and concentration of these hormones are different for each species. Similarly to our findings, many researchers showed that cytokinins and auxins induced callus formation in ornamental plants [Pati et al. 2005, Hashemabadi and Kaviani 2010, Jain and Ochat 2010]. Callus plays an important role in organogenesis, embryogen-

Table 3. Roots or callus development on the base of shoots after transfer from *in vitro* to *ex vitro*

PGRs (mg l^{-1})	First replicate	Second replicate	Third replicate
2,4-D 0.00 + BAP 0.00	Rooting	−	Rooting
2,4-D 0.00 + BAP 0.50	+	−	+
2,4-D 0.00 + BAP 2.00	+	+	+
2,4-D 0.00 + BAP 5.00	+	+	+
2,4-D 0.01 + BAP 0.00	Rooting	Rooting	−
2,4-D 0.01 + BAP 0.50	−	Rooting	−
2,4-D 0.01 + BAP 2.00	+	+	+
2,4-D 0.01 + BAP 5.00	+	−	−
2,4-D 0.10 + BAP 0	−	−	Rooting
2,4-D 0.10 + BAP 0.50	Rooting	Rooting	Rooting
2,4-D 0.10 + BAP 2.00	+	+	+
2,4-D 0.10 + BAP 5.00	+	+	+
2,4-D 1.00 + BAP 0	+	+	−
2,4-D 1.00 + BAP 0.50	+	+	+
2,4-D 1.00 + BAP 2.00	+	+	+
2,4-D 1.00 + BAP 5.00	+	+	+

+: with callus, −: without callus

esis (especially somatic embryogenesis), production of secondary metabolites, protoplast fusion and genetic manipulations.

Effect of 2,4-D and BAP on micropropagation of *E. grandiflorum* in double-phase and liquid media. None of explants cultured on free-hormones medium and media containing different concentrations of 2,4-D and BAP were survived. Some of explants cultured on double-phase system were grown slightly, but they eventually died after a few days (Fig. 2). This finding is not in agreement with some other findings on some ornamental plants like orchids [de Oliveira et al. 2013]. These researchers revealed that axillary shoot multiplication in vanilla was greatest when double-phase medium with 1.00 mg l^{-1} BA was used, representing more than a 2.5-fold increase over the rates obtained with semi-solid medium, after 90 days of cultivation. Shoots taken from double-phase medium were rooted at a frequency of 100% [de Oliveira et al. 2013]. Double-phase and liquid culture media has improved the efficiency of micropropagation of some species such as conifers and pineapple [Pullman and Skryabina 2007, Scherwinski-Pereira et al. 2012]. The reason for the loss of specimens in double-phase and liquid culture media is unknown to us. Therefore, we cannot discuss more about that.

Acclimatization of *E. grandiflorum* plantlets. Rooted plantlets were successfully transferred to the soil (Fig. 1F). The results of acclimatization showed that 90% of plantlets survived to grow under greenhouse conditions and were morphologically similar to the mother plants. A mixture of light soil containing peat and perlite (1 : 1) with good drainage is suitable for acclimatization of *E. grandiflorum* (Fig. 1F). Similar results were reported by some other researchers working on *E. grandiflorum* [Ghaffari Esizad et al. 2012, Kaviani et al. 2014]. Rooted plantlets were successfully transferred to the soil.

CONCLUSIONS

1. It is concluded that *Eustoma grandiflorum* can be well-multiplied and grown on MS medium supplemented with suitable concentrations of BAP and

2,4-D (0.10 mg l^{-1} 2,4-D in combination with 5.00 mg l^{-1} BAP and 0.10 mg l^{-1} 2,4-D in combination with 2.00 mg l^{-1} BAP).

2. Plantlets produced *in vitro* can be rooted *ex vitro*. *Ex vitro* rooting process is faster and cheaper than that of *in vitro*.

3. This study introduces 0.10 mg l^{-1} 2,4-D in combination with 5.00 mg l^{-1} BAP to produce maximum axillary shoot number. Plantlets grown on this medium were easily acclimatized.

4. In current research, double-phase and liquid culture media were not suitable for micropropagation of *E. grandiflorum* at all. The reason is unknown to us. We recommend further research in this subject.

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