***IN VITRO* FLOWERING AND MICROPROPAGATION OF LISIANTHUS (*EUSTOMA GRANDIFLORUM*) IN RESPONSE TO PLANT GROWTH REGULATORS (NAA AND BA)**

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**Abstract**

Here, a protocol was developed for flowering and high frequency *in vitro* micropropagation of Lisianthus (*Eustoma grandiflorum*), an ornamental plant. Micropropagation is an effective tools for propagation of ornamental plants in large scale. The aim of the present study was to evaluate the effect of different concentrations of NAA and BA on micropropagation and flowering of Lisianthus *in vitro*. Used culture medium was MS enriched with 0, 0.1, 0.2 and 2 mg l-1 of NAA and BA. In establishment process of explants the most shoot length (2.07 cm/plant) was obtained on medium supplemented with 0.1 mg l-1 BA (without NAA). Maximum shoot number (5.80/plant) was produced in medium containing 0.1 mg l-1 BA along with 0.2 mg l-1 NAA. Bud explants in culture media containing 0.2 mg l-1 NAA (without BA) and 0.1 mg l-1 NAA along with 2 mg l-1 BA produced maximum node number (3.20/plant). The largest number of root (14.53/plant) and root length (3.87 cm/plant) were produced in media containing 0.2 mg l-1 NAA (without BA), also 0.2 mg l-1 BA plus 0.2 mg l-1 NAA and 0.2 mg l-1 BA without NAA, respectively. Explants produced flower on medium containing 0.1 mg l-1 BA along with 0.1 mg l-1 NAA without transition of callus formation. Flower was produced from callus in medium containing 0.1 mg l-1 BA along with 2 mg l-1 NAA. Regenerated plants showed 98% survival in greenhouse during acclimatization. Acclimatized plants were morphologically similar to the mother plants.

**Key words:** Axillary buds, callus, ornamental plants, plant tissue culture

**INTRODUCTION**

Lisianthus (*Eustoma grandiflorum*) (Gentianaceae), quickly ranked in the top ten cut flowers worldwide due to its rose-like flowers and being available in various colors (Kunitake et al. 1995). In recent decades, breeders have developed a variety of cultivars with respect to many traits such as uniform flowering throughout the year, lack of rosetting, heat tolerance, flower color, and flower size and form, including double flowers, etc. (Harbaugh 2006). *Eustoma grandiflorum* is commonly propagated by seed or cutting. A large number of seedlings can be produced by seed propagation but the quality is not uniform due to variations in flowering time, plant height and the number of flowers. Lisianthus has the qualities of an “ideal cut flower” and should continue to increase in popularity throughout the next century.

The number of papers dealing with the *in vitro* propagation of *E. grandiflorum* is relatively low. Some studies on micropropagation of *E. grandiflorum* have been reported by Paek and Hahn (2000), Ordogh et al. (2006), Ming-xia et al. (2008), Xue-hua et al. (2009) and Ghaffari Esizadet al. (2012). Most of these researchers have been used of shoot tips as explants and BA, KIN, NAA and IBA as plant growth regulators. The success of the micropropagation method depends on several factors like genotype, media, plant growth regulators and type of explants, which should be observed during the process (Pati et al. 2005, Nhut et al. 2010).

Plant flowering is controlled by several factors, both genetic and environmental such as medium pH, carbohydrate, agar, light intensity and size of explants (Kulpa and Nowak 2011). The most important of these are kind and concentration of plant growth regulators (Taylor and van Staden 2006). Gibberellic acid (GA3) and cytokinins are considered plant growth regulators responsible for induction of flower *in vitro* (Kulpa and Nowak 2011). Some studies have been indicated flowering of ornamental plants *in vitro* such as in roses (Wang et al. 2002), orchids (Tee et al. 2008), *Spathiphyllum* (Dewir et al. 2007) and *Petunia* (Kulpa and Nowak 2011). Flowering plantlets *in vitro* have good commercial potential as ornamentals as well (Kulpa and Nowak 2011). The objective of the present study was to determine the influence of the concentrations of BA and NAA in the medium of *E. grandiflorum* propagated *in vitro* and also the choice of the optimal media for shoot and root production as well as the induction of flowering *in vitro*.

**MATERIALS AND METHODS**

***Plant materials and surface sterilization***

Lisianthus (*Eustoma grandiflorum*) mother plants were prepared from a commercial greenhouse, Mahallat city, Iran. Lateral buds were cut from the mother plants as explants and washed thoroughly under running tap water and a few drops of hand washing for 20 min. After three times rinses with distilled water, explants were sterilized for 40 sec in 70% ethanol followed by three times rinses with sterile distilled water (15 min each). Then, explants were disinfected with 300 mg l-1 nano-silver for 15 min then rinsed three times in sterile distilled water (10 min each).

***Culture media and culture conditions***

Five explants were cultivated in culture flasks on half strength macro- and micro salts of MS (Murashige and Skoog 1962) basal medium supplemented with 0, 0.1, 0.2 and 2 mg l-1 of both BA and NAA (16 treatments). The media were adjusted to pH 5.6-5.8 and solidified with 7 g l-1 Agar-agar. The media were pH adjusted before autoclaving at 121°C, 1 atm. for 30 min. The cultures were incubated in growth chamber whose environmental conditions were adjusted to 26 ± 2°C and 75-80% relative humidity, under a photosynthetic photon density flux 50 µmol/m2/s with a photoperiod of 16 h per day.

***Measurements and acclimatization***

Characters including shoot length, shoot number, node number, root number and flowering were calculated after 36 days. Matured plantlets were washed with sterile distilled water and transferred into the plastic bags (10-cm in diameter) containing a mixture of peat and perlite (1:1). Plantlets were kept in a greenhouse at 24 ± 2°C and 70% RH with periodic irrigation.

***Statistical analysis***

The experimental design was R.C.B.D. Each experiment was carried out in three replicates and each replicate includes five specimens (totally; 15 specimens for each treatment). Data processing of the results was carried out by an EXCEL. Analysis of variance (ANOVA) was done using MSTATC statistical software and means were compared using Duncan's test.

**RESULTS**

***Shoot multiplication***

Studied characteristics on shoots were shoot length, shoot number and node number. The results are summarized in Table 1. Our data revealed that there are differences in the effect of the different concentrations of BA, NAA and interaction between these two growth regulators on these characters except for the effect of BA on node number (Table 1, Fig. 1). When different BA concentrations were tested, the best results were obtained with 0.1 mg l-1. When this concentration of BA was applied, the shoot length (2.07 cm/plantlet) and shoot number (5.80/plantlet) were significantly higher than the other concentrations tested (p≤0.01). The medium enriched with 0.2 mg l-1 BA along with 2 mg l-1 NAA and 2 mg l-1 NAA without BA resulted in the lowest shoot length (0.52 cm/plantlet) and shoot number (1.26/plantlet), respectively (Table 1). The results showed that BA was more effective than NAA on enhancing the shoot length and shoot number (Table 1). The media containing 0.1 mg l-1 BA without NAA (with 4.68 shoots/plantlet), also 0.1 mg l-1 BA along with 0.1 mg l-1 NAA (with 4.13 shoots/plantlet) were proper treatments for increasing shoot number (Fig. 1). When the explants were inoculated in the media containing 0.2 mg l-1 NAA without BA, also 2 mg l-1 BA along with 0.1 mg l-1 NAA the best results were observed for node number (3.20) (Table 1, Fig. 1). The media containing 0.1 mg l-1 NAA without BA, also 0.1 mg l-1 BA along with 0.1 mg l-1 NAA (both with 3.13 nodes/plantlet) were suitable treatments for increasing node number. The least node number (1.60) was calculated in medium supplemented with 0.1 mg l-1 BA along with 2 mg l-1 NAA (Table 1). Most callus was observed in the base of shoots grown on this medium. Analysis of variance showed that the interaction effect of BA and NAA on the node number was significant (p≤0.01).

***Flower induction***

The studies show that the addition of plant growth regulators, especially cytokinin to the media has a significant influence on *E. grandiflorum* flowering. Our study revealed the flower induction on the both of callus and shoots. Flower induction occurred on the shoots grown on the media containing 0.1 mg l-1 BA without and with 0.1 mg l-1 NAA (Fig. 2). Flower also appeared on the callus produced on the medium supplemented with 0.1 mg l-1 BA along with 2 mg l-1 NAA (Fig. 2). Full floral structure was not observed in callus. Flower development in shoot tips grown on the mentioned above media was done completely (Fig. 2).

***Rooting and plant acclimatization***

When roots induced on media containing different concentrations of BA and NAA, it was found that the best frequencies of root formation (14.53/plantlet) were obtained on a medium supplemented with 0.2 mg l-1 of both of BA and NAA (Table 1, Fig. 1). However, shoots cultured on the media enriched with 0.2 mg l-1 NAA without BA (with 14.13 roots/plantlet) and 0.2 mg l-1 BA along with 2 mg l-1 NAA gave the number of root higher than other treatments (Table 1). Minimum root number (0.33/plantlet) was observed on media containing 0.1 mg l-1 BA along with both of 0.1 and 0.2 mg l-1 NAA (Table 1). Shoots grown on the medium with 0.2 mg l-1 BA produced the longest root (3.87 cm/plantlets) (Fig. 1). NAA was unable to improve the length of root. Comparing the root length per explants, the medium without BA and NAA (control) and medium containing 0.2 mg l-1 BA along with 0.1 mg l-1 NAA were no effective for root length and induced minimum of that (0.16 cm/plantlet) (Table 1). All treatments higher than 0.2 mg l-1 BA along with 0.1 mg l-1 NAA have negative effects on root length. The data clearly show that root number and length are strongly affected by BA and NAA concentrations (p≤0.01). Rooted plantlets were successfully transferred to the soil (Fig. 3). The results of acclimatization showed that the 98% of plantlets were 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 Lisianthus (*E. grandiflorum*) (Fig. 3).

**DISCUSSION**

The results indicate that the multiplication rate increased in the presence of optimal concentrations of BA and NAA. Cytokinins are usually used on the micropropagation media to stimulate shoot multiplication (van Staden et al. 2008, Chawla 2009, Gomes et al. 2010). The ideal concentrations differ from species to species and need to be established accurately to obtain the effective rates of multiplication (Gomes et al. 2010). Our results showed that NAA at particular concentrations along with BA was able to increase the multiplication rate. Some concentrations of BA, NAA and interaction effect of BA and NAA promoted callus formation and growth. Current study showed important role of BA on micropropagation of Lisianthus. Studies of Ghaffari Esizad et al. (2012) on micropropagation of Lisianthus (*E. grandiflorum*) showed the positive effect of cytokinin KIN on multiplication rate. Xue-hua et al. (2009) findings on micropropagation of Lisianthus (*E. grandiflorum*) showed that MS basal medium supplemented with 0.1-0.5 mg l-1 BA + 0.05 mg l-1 NAA was suitable for adventitious shoot differentiation. Our finding confirms this result. Ordogh et al. (2006) revealed that the highest number of shoots in Echo cultivars of *E. grandiflorum* was obtained on MS medium with 0.1 mg l-1 BA. Reduction of the shoot number occurred on the medium without BA. Studies of Ming-xia et al. (2008) on *E. grandiflorum* showed that the best proliferation medium was MS + 0.8 mg l-1 BA + 0.04 mg l-1 NAA. Evaluation of Paek and Hahn (2000) on *E. grandiflorum* demonstrated that BA and KIN at high concentrations (13-22 and 14-23 µM) resulted in good shoot formation, but high percentages of hyperhydric shoots. Studies of Fukai et al. (1996) showed that the medium containing 0.1 mg l-1 BA + 0.01 mg l-1 NAA produced the highest number of healthy shoots per explants in *E. grandiflorum*. Explants of shoot tips of Lisianthus developed into the multiple shoots on a medium supplemented with 3 mg l-1 BA + 0.2 mg l-1 NAA (Semeniuk and Griesbach 1987). Studies on other ornamental plants showed the role of cytokinins on proliferation (Jain and Ochatt 2010, Kaviani et al. 2011, Ahmadi Hesar et al. 2011). In current study, the highest rates of shoot production were obtained when shoot tips were cultured on the medium supplemented with 0.1 mg l-1 BA. In contrary with our finding, Gomes et al. (2010) showed that NAA was unable to improve the multiplication rate. Some species may require a low concentration of auxin in combination with cytokinins to increase shoot proliferation (van Staden et al. 2008, Hashemabadi and Kaviani 2010). Fuller and Fuller (1995) showed that the most shoot percentage (88.3%) of *Brassica* spp. obtained in medium containing 2 mg l-1 IBA + 4 mg l-1 KIN.

Our findings demonstrated that the addition of NAA in culture media was effective for increasing the root number but not for root length. Some studies showed the positive effect of NAA on rooting (Gautam et al. 1983, Xilin 1992, Hammaudeh et al. 1998, Lee-Epinosa et al. 2008, Jain and Ochatt 2010, Kaviani et al. 2011). Rooting is a crucial step to the success of micropropagation. Some studies showed the positive effect of cytokinins on rooting (Gomes et al. 2010). Our study demonstrated the positive effect of low concentration of BA (0.1 mg l-1) along with NAA on root number and length. Contrary to our findings, root formation was inhibited in the medium culture of *Lilium longiflorum* Georgia containing BA (Han et al. 2004). Also, Fuller and Fuller (1995) demonstrated that the most percentage of explants regeneration with root percent (65.0%) in *Brassica* spp. obtained in culture medium supplemented with 2 mg l-1 IBA without KIN. Studies of Gomes et al. (2010) on *Arbutus unedo* L. showed that shoots produced on higher cytokinin-containing medium are more amenable to root induction than shoots obtained with the lowest concentrations of BA. In a study on *in vitro* micropropagation of orchid, NAA stimulated root growth (Kalimuthu et al. 2007). Hartmann et al. (1997) have recommended brief exposure to auxins for root induction and not for prolonged growth. The highest root number per shoot (2.40) was seen in medium supplemented with 2 mg l-1 KIN + 0.5 mg l-1 NAA. Shoot tips grown in medium containing 2 mg l-1 NAA without KIN showed the most callus formation. Studies of Ghaffari Esizad et al. (2012) on micropropagation of Lisianthus (*E. grandiflorum*) showed that the largest number of root per shoot (2.40) was obtained in medium supplemented with 2 mg l-1 KIN + 0.5 mg l-1 NAA. Shoot tips grown in medium containing 2 mg l-1 NAA without KIN showed the most callus formation.

*In vitro* flowering was observed in some ornamental plants (Wang et al. 2002, Zhang and Leung 2002, Dewir et al. 2007, Tee et al. 2008). The study of Kulpa and Nowak (2011) on *in vitro* flowering of *Petunia* × *atkinsiana* D. Don using different concentrations of auxins, cytokinins and GA3 showed that the nodes cultured on MS medium enriched with 0.5 mg l-1 KIN had the largest number of flower and the most flowering percentage. Some other observations confirm more impact of cytokinings on the flowering *in vitro* than the other growth regulators (Dielen et al. 2001, Lin et al. 2007). Our study showed the positive effect of 0.1 mg l-1 BA on the flower induction of *E. grandiflorum*. Some researchers revealed that GA3 and cytokinins are considered growth regulators responsible for inducing flowering *in vitro* (Dielen et al. 2001, Lin et al. 2007, Huang et al. 2009, Masmoudi-Allouche et al. 2010, Kulpa and Nowak 2011). Flowering *in vitro* has been successful in many species using other conditions and products (Chaari-Rkhis et al. 2006).

**CONCLUSION**

2 mg l-1 of NAA was not suitable for increasing the shoot length and number at all. Also, none of NAA concentrations are proper for enhancing the root length, while NAA at 0.2 mg l-1, singularly or in combination with 0.2 mg l-1 BA are good for root induction. Current study showed that the different concentrations of BA are not suitable for increasing the shoot and root length, except for 0.1 mg l-1. A combination of 0.1 and 0.2 mg l-1 BA along with 0.1 and particularly 0.2 mg l-1 NAA are well for quantitative and qualitative promotion of all traits. Flowering was induced in plantlets grown on the media containing 0.1 mg l-1 BA without or with 0.1 mg l-1 NAA.

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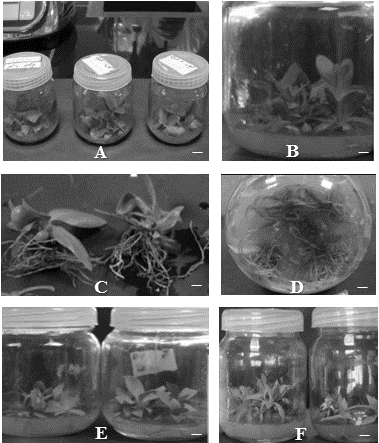
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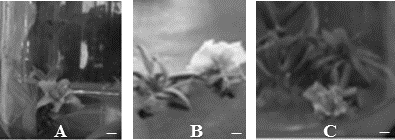
**Table 1. Mean comparison of the effect of different concentrations of BA and NAA on shoot length, shoot number, and shoot and root number of *Eustoma* *grandiflorum*.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Phytohormones (mg l-1) | Shoot length (cm) | Shoot number | Node number | Root length (cm) | Root number |
| BA 0 + NAA 0 | 1.96ab | 3.02c | 2.53bcd | 0.16h | 60.40cdef |
| BA 0 + NAA 0.1 | 1.20cd | 1.73de | 2.80ab | 1.40def | 7.66bc |
| BA 0 + NAA 0.2 | 1.18cd | 3.13c | 3.00a | 1.15de | 13.14a |
| BA 0 + NAA 2 | 0.54e | 1.26e | 1.80fg | 1.44cd | 5.46bcd |
| BA 0.1 + NAA 0 | 2.07a | 4.68b | 2.93abc | 1.52cd | 1.76efg |
| BA 0.1 + NAA 0.1 | 1.83ab | 4.13b | 3.13ab | 1.86c | 0.33g |
| BA 0.1 + NAA 0.2 | 1.49bc | 5.80a | 2.80bcd | 1.92c | 0.33g |
| BA 0.1 + NAA 2 | 1.24cd | 1.66de | 1.60g | 2.96b | 1.13fg |
| BA 0.2 + NAA 0 | 1.14cd | 2.63cd | 2.90abc | 3.87a | 3.53defg |
| BA 0.2 + NAA 0.1 | 0.98de | 2.46cd | 2.86abcd | 0.16h | 4.80cde |
| BA 0.2 + NAA 0.2 | 1.28cd | 2.40cd | 2.93abc | 0.17h | 14.53a |
| BA 0.2 + NAA 2 | 0.52e | 1.80de | 2.00efg | 0.49gh | 13.40a |
| BA 2 + NAA 0 | 1.61abc | 2.61cd | 2.35cdef | 0.57fgh | 7.66bc |
| BA 2 + NAA 0.1 | 1.58bc | 2.06cd | 3.20a | 0.60fgh | 1.63efg |
| BA 2 + NAA 0.2 | 0.90de | 2.13cd | 2.26def | 0.74efg | 5.40bcd |
| BA 2 + NAA 2 | 0.66e | 1.33e | 2.06efg | 0.95efg | 8.60b |

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



**Fig. 1.** Micropropagation process of *Eustoma* *grandiflorum* through lateral buds. (A) Lateral buds used as explants (Bar = 5 mm). (B) Lateral buds cultured on MS medium containing 0.2 mg l-1 NAA (largest number of node) (Bar = 10 mm). (C and D) Lateral buds cultured on MS medium containing 0.2 mg l-1 BA along with 0.2 mg l-1 NAA (largest number of root) (Bar = 5 mm). (E) Lateral buds cultured on MS medium containing 0.2 mg l-1 BA (maximum root length) (Bar = 5 mm). (F) Lateral buds cultured on MS medium containing 0.1 mg l-1 BA (maximum shoot number and length and containing flower) (Bar = 5 mm).



**Fig. 2.** *In vitro* flowering. (A and B) Flower induction on lateral buds formed in medium supplemented with 0.1 mg l-1 BA without or with 0.1 mg l-1 NAA (Bar = 5 mm). (C) Flower induction on callus formed in medium supplemented with 0.1 mg l-1 BA along with 2 mg l-1 NAA (Bar = 5 mm).



**Fig. 3.** The process of plantlets acclimatization. Acclimatization was done in a greenhouse using peat and perlite (1:1).