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Original Contribution

CALLUS INDUCTION AND PLANT REGENERATION IN LISIANTHUS (*EUSTOMA GRANDIFLORIUM*)

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ABSTRACT
This study was conducted to evaluate the effect adding hormones on callus induction and *in vitro* regeneration from callus of Lisianthus (*Eustoma grandiflorum*) by using a completely randomized design and factorial experiment. Sterile explants were placed on basal medium B5 along with different concentration of 2, 4-D and NAA under dark and light conditions. In regard to the explants location collogenesis was happened just from leaf explants. Increasing the amount of NAA, in referring the hormonal balance, lead to more collogenesis than 2, 4-D ($P<0.05$). For indirect regeneration callus was subcultured in B5 medium supplemented with different concentration of Kin or BAP which applied individually or in combination with NNA, IAA and GA_3. Media contains GA_3 and Kin induced maximum proliferation from callus.

Key words: Lisianthus, callus, proliferation, *in vitro* culture

INTRODUCTION

Lisianthus (*Eustoma grandiflorum*) belongs to the *Gentinaceae* family which is one of the newest ornamental plants in international market because of rose-like flower and vase life among 10 top cut flowers in the world (1). Lisianthus grows to 50–75 cm in height with 20–40 flowers, flowering mainly in summer. The longevity of cut flowers and pot flowers is 2 weeks and more than 5 weeks, respectively (2). Cutting is the traditional method of Lisianthus propagation which is considered a slow as well as seed that produces seedling which are not true to the type (3). *In vitro* culture technique can be effectively used to over comes these barriers. Also micro propagation is an important asexual method that can be used for the production of virus-free plants.

The present study was designed to identify the ideal conditions for micro propagation of this plant. This study was conducted to identify the best type of explants from *in vitro* grown seedling and growth regulator concentration and combination for callus induction, shoot regeneration from callus by using *in vitro* techniques.

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METHODS AND MATERIALES

Plant material:

Explants of (*Eustoma grandiflorum*) were collected from 2 months old seedling in the green house at Isfahan University of Iran. These explants were surface sterilized with 70% (v/v) ethanol for 1 min, followed by 10% (v/v) solution of commercial bleach (Clorox, 5.25% (w/v) of sodium hypochlorite) added with one drop of poly ethylene sorbitan monoleate for 15 min, then rinsed 3 times with sterile distilled water. The explants were aseptically cut into small pieces. Young leaves with the size 0.5× 0.5 cm, stem segments with the size 1 cm and tip roots were placed on different mediums supplemented with different combinations of hormones.

Medium:

Medias consisting MS (Murashig – Skoog) and B5 (Gamborg), 2% sucrose were provided. Different hormones added to media. A volume of 20 ml of nutrient medium was dispensed into 3 glass jar that was capped with a polystyrene screw cap. The pH of media was

adjusted to 5.7 with 0.1N NaoH or 0.1N Hcl prior to autoclaving at 1.05 kg/cm², 121°c for 20 min.

Callus induction:
For callus induction, sterile explants of leaves, stem segments, tip roots were aseptically placed on B5 medium supplemented with different combination of 2,4- D (0 - 1.5 mg/L) and NAA (0 - 1.5 mg/L), containing 25 g/L (w/v) of sucrose and 7 g/L (w/v) agar. The explants were incubated at 25±1 °c under dark and light (1200 lux) condition. After 8 weeks of culture, the percentage of explants producing callus was recorded. The observation was made on the morphology of callus formed in different phytohormones tested.

In vitro shoot induction:
Well-proliferated calli derived from the leaf segments 5 weeks after culture were used for regeneration studies. Approximately 2 grams of fresh callus were placed on the B5 medium containing 1.5 mg / L BAP + 0.5 mg/L NAA or IAA or GA3 and 1.5 mg/L K + 0.5 mg/L IAA or NAA or GA_3. Data on the mean number of transferable shoots per explants and percentage of shoot formation and shoot length were observed after 12 weeks of culture. Each experiment consisted of 3 replicates. The cultures were incubated at 25±1 °c under an illumination of 1200 lux during a 16/8 h photoperiod obtained from Gro – Lux fluorescent lamps.

Experimental design and statistical analysis
The experiments were done in factorial based on completely randomized design (CRD). Data were analyzed by using MSTATC. In order to determine significances, Duncan Multiple Test will be done (5%, 1% level).

RESULTS AND DISCUSSION
Callus induction of *(Eustoma grandiflorum)*
In order to establish the most suitable condition for indirect regeneration we tried some concentrations and combination of hormones such as NAA (0 - 1.5 mg/L) and 2, 4-D (0 - 1.5 mg/L), under dark and light (1200 lux) conditions. Callus was optimally induced from leaf segments derived from 2 mounts old seedling within 28 – 29 days in darkness and 26 – 27 days in light of incubation in all treatments containing NAA. Callusing was initiated at the middle of explants and eventually extended all over explants. The callus which induced from leaf explants on B5 basal medium containing NAA in dark condition was yellow and friable whereas those in light condition were green and friable **(Figure 1 A, B)**. It was found that light condition which promote higher rate of chlorophylls synthesize could be the possible reason (4) As results were shown **(Table 1)**, 1.5 mg/L NAA was found to produce significantly higher callus as compare to other treatments. The result indicated that light appeared to have an effect on growth of callus. It was observed that the fresh weight of callus by the end of incubation period in the light was higher than fresh weight of callus incubated in the dark condition. Similar result on the effect of light on callus growth was observed in the culture of *cistanche deserticola* where the presence of light was found to increase the production of callus (5). The increase of callus growth in the light condition might be related to the rate of nutrient uptake which was found higher than those in dark condition (6). However, the results tend to contrast with observation in callus culture of *azarichta indica* where the maximum fresh weight of callus incubated in dark were 61% and 64% higher compared to those incubated in light condition (7). Dark condition was also found to be preferable for the growth of *Lycopersicum esculanthum*, *Dacus carota* and *Arabidopsis thaliana* callus. The reduced growth caused by light can be attributed primarily to photochemical alterations of the culture medium rather than to phytosensory functions of the plant tissues. The result showed that light-induced growth reduction was primarily due to photo degradation of components in medium other than auxin (8).

It was found that from 2 to week 6, there was no callus formed for root and shoot explants. Similar results were also attained by Lee *et al*., 2003 in studying production of callus in root cultures of *Cucurbita maxima* (9).

Callusing wasn't occurred in all treatments containing different concentrations 2, 4-D (0-1.5mg/L). Although, auxin is able to promote the growth of callus, however according to Wernicke and Mikovitis, high concentrations of 2, 4-D was able to inhibit callusing of basal segments and it had effect as the herbicide (10).

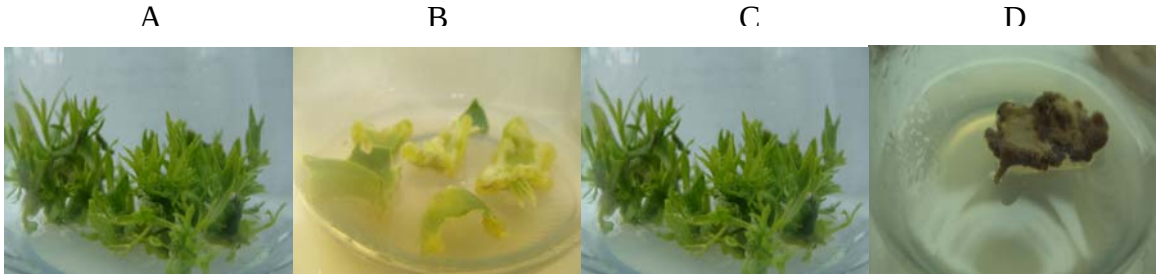

Figure 1. (A): Callus induction on leaf explants on the B5 medium containing 1.5 mg/L NAA in light condition. (B): Callus induction on leaf explants on the B5 medium containing 1.5 mg/L NAA in dark condition. (C): Proliferation from callus. (D): Callus were necrosesed after during 20 days

Table 1. *Percentage callus induction, fresh eight, morphology of callus, days of callus form from Eustoma grandiflorum in medium supplemented with different levels of 2.4-D and NAA after 4 weeks of culture in darkness and light*

Darkness &light	Hormonal combination(mg/l)	Percentage of callus induction%	Days of callus induction	Fresh weight of callus (gr)	Morphology of callus
Darkness	0 NAA	-	-	-	-
Darkness	0.5 NAA	94	27±1	0.04417 ^{f}	yellow/friable
Darkness	1 NAA	96	26±2	0.1352 ^{e}	yellow/friable
Darkness	1.5 NAA	100	26±2	0.2088 ^{d}	yellow/friable
light	0 NAA	-	-	-	-
light	0.5 NAA	96	29±1	0.3524 ^{c}	Green/friable
light	1 NAA	98	28±2	0.5390 ^{b}	Green/friable
light	1.5 NAA	100	28±1	0.7106 ^{a}	Green/friable
Darkness	0.5 2,4-D	-	-	-	-
Darkness	1 2,4-D	-	-	-	-
Darkness	1.5 2,4-D	-	-	-	-
light	0.5 2,4-D	-	-	-	-
light	1 2,4-D	-	-	-	-
light	1.5 2,4-D	-	-	-	-

Shoot regeneration from callus cultures

Calli derived from leaf explants, were used for shoot regeneration. Regeneration of green shoots buds occurred in these calli after 6 weeks of culture and maximum number of shoot bud emergence occurred in the 10 weeks. These shoot buds elongated and attained on average height of 2.5 cm in B5 medium containing 1.5 mg/L K +0.5 mg/L GA_3. Skoog and Miller 1957 demonstrated contrast between auxines and cytokinines. Kinetine is more effective than adenin, because more shooting were observed in 10^{-6} gr/L kinetine in comparison to $3*10^{-8}$ gr/L auxin, so there is a weight relation about 1: 35 between 2 complexes, however about adenin this ratio must be about 1:1500 (11). Hence, exogenous auxin was needed for multiple shoot formation of *Eustoma grandiflorum*, the role of BAP and K seems essential for regeneration.

All the calluses cultured in B5 medium with NAA+BAP or NAA+K, did not induce shoot from explants and callus were necrosesed during 20 days **(Figure 1. D)**. Tissue necroses might be made due to high concentrations of auxin and low concentration of cytokinin (12-13). Similar results were obtained in Lisianthus by using NAA and BA (14). **(Table 2)**

Table 2. *Percentage of shoot induction (shoot length, No. of shoot, days of shoot regeneration) from Eustoma grandiflotum in medium supplemented with different levels of GA3, NAA, BAP, Kin, IAA after 7 weeks of culture*

Hormone combination In B5 basal medium (mg/l)	Shoot regeneration%	Days of shoot regeneration	Shoot length(cm)	No. of shoot
0.5 NAA +1.5K	-	-	-	-
0.5 IAA +1.5K	10	41±1	0.13 [c]	1.2 [c]
0.5 GA3 +1.5K	80	38±1	1.8 [b]	4.3 [b]
0.5 NAA +1.5BAP	-	-	-	-
0.5 IAA +1.5BAP	15	40±2	0.17 [c]	1.3 [c]
0.5 GA3 +1.5BAP	98	38±2	2.50 [a]	7.6 [a]

CONCLUSION

The conducted study reveals that collogenesis was seen just from leaf explants in light condition. Stem and tip roots were not effective for callus induction positively. In our experiment NAA was more effective than 2, 4-D with respect to callus induction. Overall findings of the present study are significant in obtaining the maximum Callogenesis with NAA.

In the other experience for shoot proliferation data demonstrated that the highest shoot formation was obtained in medium supplemented with BAP and GA_3, whereas combination of NAA with BAP or K have negative effect on shoot induction. Conclusion, we have developed a method for an efficient regeneration from callus of *Eustoma grandiflotum* by using BAP and GA_3. The protocol could be benefit for large scale production.

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