Promotion of callus initiation, shoot regeneration and proliferation in *Lisianthus*

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Abstract

The effects of plant growth regulators were examined in order to optimize the callus induction, regeneration, and proliferation of lisianthus (*Eustoma grandiflorum*). *In vitro* leaves provided the explants for callus induction. Explants were cultured on Murashige and Skoog (MS) medium with different concentrations of indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D). Maximum callogenesis was obtained on MS medium supplemented with 100 µM NAA. Calluses were cultured on MS medium containing 6-benzyladenin (BA) (4.4, 13.3 or 22.2 µM) with or without 0.5 µM IAA and NAA for regeneration. The highest number of shoots (12.3 shoots/explant) developed on MS media with 22.2 µM BA plus 0.5 µM NAA. Individual shoots 1 cm in length were excised and multiplied. The maximal shoot proliferation with an average of 10.2 and 11.2 shoots/explant after 4 weeks of culture was achieved when the shoot tips were cultured on MS medium supplemented with 2.2 µM BA with or without 0.5 µM NAA. These results indicate that an efficient callus induction and micropropagation protocol of lisianthus had been established.

Key words: Lisianthus; callus initiation; shoot regeneration; proliferation;BA; NAA

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid; IAA: indole-3-acetic acid; MS: Murashige and Skoog; NAA: 1-naphthaleneacetic acid

Key words: proline; salinity pretreatment; tomatoes; salinity; mycorrhiza

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Introduction

Eustoma grandiflorum (Raf) Shinn., commonly known as lisianthus, belongs to the Gentianaceae. Although lisianthus is native to the United States, all commercially important hybrids have been bred in Japan (Roh and Lawson, 1984). It is a relatively new cut flower crop when compared with established floricultural crops such as cut roses, carnations, or chrysanthemums. Lisianthus quickly ranked in the top ten cut flowers worldwide due to its rose-like flowers, excellent post-harvest life, and blue flowers. It is also widely used as a flowering potted and bedding plant. In addition to blue, a wide range of flower colors are available, as well as floral patterns (Harbaugh,

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2007). The annual increase in production is remarkable and this popularity can be attributed to progress in breeding with new flower colors, diverse forms, excellent vase life, and availability of flowers all year round (Ohkawa and Sasaki, 1999; Harbaugh, 2007).

Lisianthus is propagated by seeds, although tissue culture and stem tip cutting methods can be utilized (Roh and Lawson, 1984). Tissue culture can be used very effectively to produce novel genetic variations in lisianthus (Griesbach and Semeniuk, 1987). Somaclonal variants (dwarf, miniature, or branched compared to the parent that was a tall single stem cultivar) were identified in a tissue-cultured population of lisianthus (Harbaugh, 2007). Somaclonal variation may occur during adventitious shoot regeneration from callus (Davey and Anthony, 2010).

Callus induction and plant regeneration have been obtained in lisianthus using different explants such as leaves (Mousavi *et al*., 2012a), protoplast (Kunitak et al., 1995), root (Furukawa *et al*., 1990), internodes, petals, and anthers (Rezaee *et al.,* 2012). Additionally, shoot proliferation from shoot tips has been reported (Damiano *et al*., 1989; Ghaffari Esizad *et al.,* 2012). Despite the availability of the sufficient literature, there is a need to study the tissue culture of lisianthus efficiency. In this paper, callus induction, plant regeneration, and shoot multiplication from *in vitro* cultured explants of lisianthus by using different combinations of growth regulators is described.

Material and Methods

In vitro plants of lisianthus were used for explant preparation (Mousavi *et al.,* 2012b). MS basal medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar (Agar-Agar, Sigma) was employed in all experiments. Growth regulators were added to the medium and pH was adjusted to 5.7 before autoclaving. Cultures were incubated at 26 ± 2 °C under a 16/8 h photoperiod with 25 $mM.m^{-2}.s^{-1}$ light intensity provided by fluorescent tubes.

For callus induction, the basal MS medium was supplemented with varying concentrations (0, 0.1, 1, 10, and 100 µM) of indole-3-acetic acid (IAA), 1-aphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Leaves (1 cm long) were excised from *in vitro* grown plants and cultured with their abaxial surface on the medium. After 4 weeks of incubation, callus induction (%), fresh weight (FW), texture, and color of calluses were recorded. Fresh weight was obtained by weighting callus immediately after the removal of adhering agar.

Green and normal calluses were cut into pieces (approximately 1 cm in diameter) and transferred to fresh media supplemented with BA (6-benzyladenin) (4.4, 13.3 or 22.2 µM) with or without 0.5 µM IAA and NAA for shoot induction. After 4 weeks culture, shoot regeneration (%), and number of shoots per explant were measured.

Shoots 1 cm in length, obtained from regeneration, were cultured on MS medium containing BA (0.4, 2.2 or 4.4 µM) in combination with IAA or NAA (0 or 0.5 µM) for shoot proliferation. All cultures were maintained under similar conditions as described above. Data for shoot number per explant were recorded after 4 weeks of culture.

Callus induction experiments were done as complete randomized design (CRD) and plant regeneration and proliferation were performed in a factorial based on CRD. Two explants were cultured per Petri dish with 25 ml of medium with at least 3 replicates per treatment. The exercise was repeated two times. Data from each experimental stage were analyzed separately by analysis of variance (ANOVA) using SPSS Ver. 16 software and means were compared using Duncan's test at 5% level of significance. Arcsine transformation was applied for the data expressed in percentages.

Results

The effect of four different concentrations of auxins, NAA, IAA, and 2.4-D was assessed on the callus induction. Approximately 1 to 2 weeks after culture, leaf explants increased in size and, in some treatments, began to produce callus from cut surfaces. The whole surface of the explants was covered with a mass of callus within 3-4 weeks (Fig. I. A). Data in Table 1 shows that the callus formation was greatly influenced by the auxins used, and no callus was produced in 0.1 µM concentration and control (MS medium without any growth regulators). Abundant callus

formation could be found from cut pieces of the leaf in media containing NAA, the optimum level being 100 µM with 100% callus induction frequency and 1.92 g fresh weight. This suggests that the higher concentration of NAA had influenced the callus growth. Callus fresh weight was slightly higher in 2, 4-D than in IAA, and it increased considerably when 2,4-D was increased to 100 µM.

Callus was properly grown after 3-4 weeks of culture with different morphologies. Friable yellow to brown-green callus was observed in 10 and 100 µM both NAA and 2, 4-D, whereas compact and brown callus was formed in 1 to 100 µM IAA. The medium supplied with 1 µM in all types of auxins gave rise to compact callus.

Adventitious and axillary shoot formation is a reliable technique for clonal propagation. One-month-old callus derived from leaf tissues was further subcultured on MS media supplemented with varying levels of BA and auxins for organogenesis. Table 2 shows the responses of explants to cytokinin-auxin combinations. Shoot primordia could be observed after two weeks and developed completely after another 10 days (Fig. 1B). Some calli had shown root regeneration. Shoot development could not be induced from the callus on MS medium supplemented with 4.4 µM of BA. When MS medium containing 4.4 µM of BA and auxin was used, organogenesis was very low, but when the concentration of BA was further increased to 22.2 µM, well-developed shoots were obtained. The highest numbers of shoots (12.3 and 10.2 shoots/explant) were obtained in the presence of 22.2 µM BA with or without 0.5 µM NAA.

The effect of BA in combination with NAA or IAA was assessed on shoot multiplication (Fig. I. C). Proliferation of shoots was observed in all shoot multiplication media (Table 3). Treatment 4.4 µM BA + 0.5 µM IAA was excluded because of contamination. Shoots proliferated very well on MS medium supplemented with 2.2 µM BA ± 0.5 µM NAA. Most shoot tips (55.2-100%) were less than 1 cm in length. Shoots less than 1 cm produced on medium with 2.2 µM BA + 0.5 µM NAA combination were significantly higher (11.0 shoots/explant) than those cultured on medium containing any of the other growth regulator combinations. However, the treatments had no significant effect on the number of shoots longer than 1 cm.

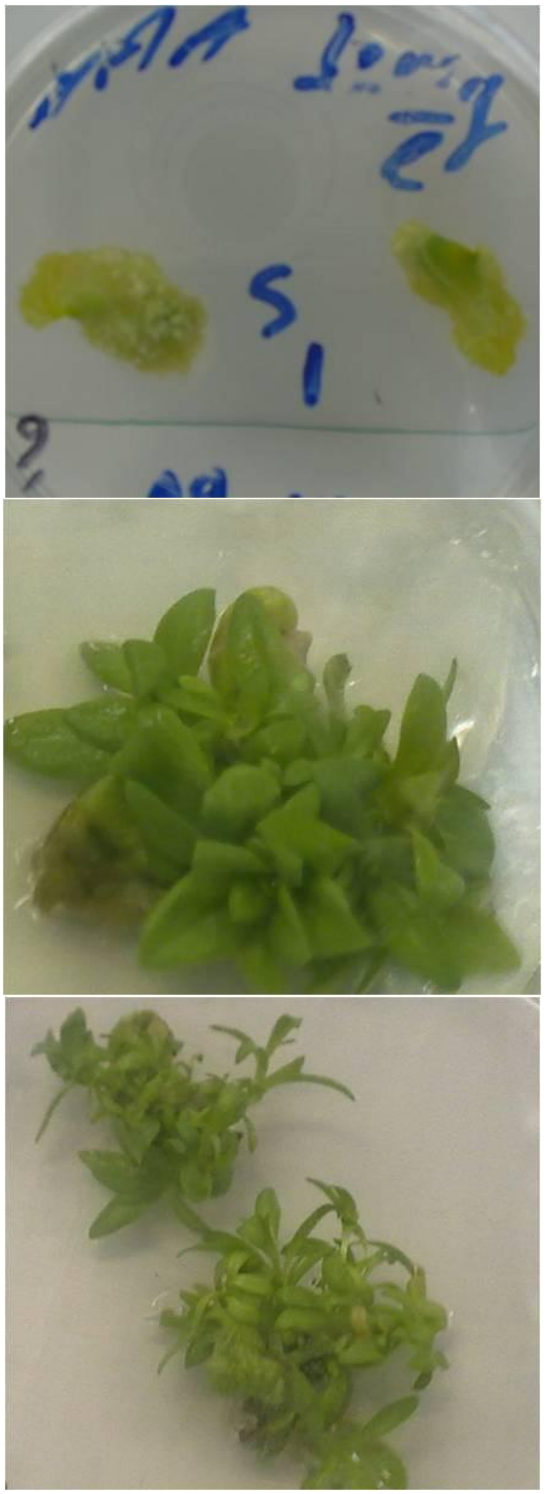

Fig. I. Callus formation (A), adventitious shoot regeneration (B) and shoot proliferation (C) of *lisianthus* in 4 weeks

Table 1
Effect of plant growth regulators on the callus induction in lisianthus

Plant growth regulators (μM)		Callus induction (%)	Fresh weight (g)	Texture	Color
Control	0.0	0.0 c	0.00 c	-	-
NAA	0.1	0.0 c	0.00 c	-	-
	1	100 a	0.51 b	Compact	Green-Yellow
	10	100 a	0.81 b	Friable	Brown
	100	100 a	1.92 a	Friable	Brown-Green
IAA	0.1	0.0 c	0.00 c	-	-
	1	100 a	0.02 c	Compact	Brown
	10	100 a	0.01 c	Compact	Brown
	100	100 a	0.01 c	Compact	Brown
2,4-D	0.1	0.0 c	0.00 c	-	-
	1	80.0 b	0.03 c	Compact	Green
	10	100 a	0.19 c	Friable	Yellow
	100	100 a	0.72 b	Friable	Yellow

Means with similar characters in each column for each factor show no significant differences (Duncan's multiple range test, α= 0.05).

Table 2
Effect of BA, NAA, and IBA on shoot regeneration in callus of lisianthus

Plant growth regulators (μM)			Shoot regeneration (%)	No. of shoots/explant
BA	NAA	IAA		
4.4	0.0	0.0	0.0 d	0.0 c
	0.5	0.0	33.0 c	0.3 c
	0.0	0.5	66.0 b	2.3 c
13.3	0.0	0.0	66.0 b	2.6 c
	0.5	0.0	3.03 c	1.0 c
	0.0	0.5	100 a	7.6 b
22.2	0.0	0.0	100 a	10.0 ab
	0.5	0.0	100 a	12.3 a
	0.0	0.5	100 a	6.3 b

Means with similar characters in each column for each factor show no significant differences (Duncan's multiple range test, α= 0.05).

Discussion

Most callus induction media contain an auxin, usually NAA or 2.4-D. Root formation in high concentrations of NAA, fails to occur and callus formation takes place (Paek and Hahn, 2000; Neumann et al., 2009). NAA proved most efficient for the induction of callus after 4 weeks. This result is in agreement with Ghaffari Esizad et al., (2012) and Mousavi *et al*., (2012a), who reported NAA showed the most callus formation in lisianthus. Several studies had been reported regarding the effects of plant growth regulators on callus growth of lisianthus. Jin *et al*., (2009) reported MS basal medium supplemented with 0.1-0.5 mg.L^{-1} BA + 0.05 mg.L^{-1} NAA was suitable for callus inducement. According to Su (2011), the suitable culture medium for callus differentiation of *E. grandiforum* was MS + 0.1-0.5 mg.L^{-1} BA + 0.1-0.5 mg.L^{-1} NAA. Rezaee *et al.*, (2012) also found that basal LS (Linsmaier and Skoog, 1965) medium containing 3 mg.L^{-1} IAA, 3 mg.L^{-1} NAA, 0.1 mg.L^{-1} kinetin, and B5 (Gamborg *et al*., 1968) medium containing 0.225 mg.L^{-1} BA and 1.86 mg.L^{-1} NAA were the best media for induction of callus from leaf explants.

The presence of BA with a low concentration of auxin enhanced regeneration. These results are similar to findings of other researchers. Gong (2008) showed that for the induction effect of the adventitious buds from lisianthus leaves, the combination of 6-BA and IBA was better than that of 6-BA and NAA. The best bud induction medium was MS + 0.6 mg.1^{-1} BA +

Table 3
Effect of BA, NAA and IBA on shoot proliferation of lisianthus

Plant growth regulators (µM)			No. of shoots/explant		
BA	NAA	IAA	No. of shoots < 1 cm	No. of shoots > 1 cm	Total
0.4	0.0	0.0	6.0 b	0.5 a	6.5 b
	0.5	0.0	4.3 b	0.0 a	4.3 b
	0.0	0.5	4.7 b	1.7 a	6.5 b
2.2	0.0	0.0	8.5 b	1.7 a	10.2 a
	0.5	0.0	11.0 a	0.2 a	11.2 a
	0.0	0.5	3.7 b	3.0 a	6.7 b
4.4	0.0	0.0	8.3 b	0.0 a	8.3 b
	0.5	0.0	7.0 b	0.0 a	7.0 b
	0.0	0.5	-	-	-*

Means with similar characters in each column for each factor show no significant differences (Duncan's multiple range test, α= 0.05).
*: shoot tips were contaminated

0.04 mg.L^{-1} IBA, and the mean number of the normal induced buds was 8.3 per month. Additionally, Jin *et al*., (2009) observed 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. Although these combinations were used for regeneration, Kunitake *et al*., (1995) reported that the highest regeneration frequency of adventitious shoots (70%) from protoplast-derived calluses was obtained on 1/2 MS medium containing 2 mg.L^{-1} BA. Da *et al*., (2008) also mentioned that MS basal medium supplemented with 1 mg.L^{-1} BA was suitable for leaf adventitious shoot formation.

Currently, the most frequently used micropropagation method for commercial mass production of plants utilizes axillary shoot proliferation from isolated apical or axillary buds under the influence of cytokinin (Davey and Anthony, 2010). Application of a cytokinin to the axillary buds can overcome the apical dominance effect and stimulate the lateral buds to grow in the presence of the terminal bud. In cultures, the rate of shoot multiplication by enhanced axillary branching can be substantially enhanced by growing shoots in a medium containing a suitable cytokinin at an appropriate concentration, with or without auxin (Bhojwani and Razdan, 1996). A remarkable enhancement of multiple shoot formation (10.2 and 11.2 shoots/explant) by axillary branching was observed in MS medium containing 2.2 µM BA ± 0.5 µM NAA. This result was an improvement over the findings of the study reported by Ghaffari Esizad *et al*., (2012). They found that shoot tip medium supplemented with 1 mg.L^{-1} kinetin (Kin) without NAA resulted in the best shoot number/explant (2.62).

Conclusion

A highly efficient protocol for the callus induction, plantlet regeneration, and proliferation of lisianthus was established. The results of this study show that higher regeneration rate of lisianthus could be attributed to high BA concentration. Maximum callogenesis, shoot regeneration and proliferation were noted on MS medium supplemented with 100 µM NAA, 22.2 µM BA ± 0.5 µM NAA and 2.2 µM BA ± 0.5 µM NAA, respectively. This protocol could be most useful for future studies on genetic transformation of lisianthus.

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