Micropropagation of Lisianthus (*Eustoma grandiflorum* L.) from different explants to flowering onset

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Abstract

Lisianthus (*Eustoma grandiflorum* L.) is a very popular, ornamental plant. Due to low germination of its seeds, usual techniques for its propagation are not efficient. The present study was aimed to establish callus from explants of different plant parts of *Eustoma grandiflorum* L. Raf Shinners in B5, LS and MS media with modifications. The results showed that among different plant parts, leaf explants were pioneer to produce callus. Basal LS medium containing 3 mg/L IAA, 3 mg/L NAA, 0.1 mg/L kinetin, and B5 medium containing 0.225 mg/L BA and 1.86 mg/L NAA were the best media for induction of callus. The calluses in LS media started for organogenesis (rhizogenesis) earlier than those in B5, and MS medium containing 3 mg/L IAA, 3 mg/L NAA, and 2 mg/L Glycin. All calluses from aforesaid media were able to regenerate new plants. Flowering onset occurred after 84 days growing under 16/8 h photoperiod, 25±2 ºC.

Keywords: B5; Lisianthus; LS; Micropropagation; MS

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Introduction

Lisianthus is an ornamental, herbaceous annual plant from Gentianaceae. The plant grows to 15 - 60 cm tall, with bluish green, slightly succulent leaves, mature rapidly, and produce beautiful funnel shaped flowers growing on long straight stems. In 2005, the total wholesale value of Lisianthus in the United States for operations with sales worth $100,000 or more was $4.89 million, with California accounting for 89.4% of those sales (Wegulo and Vilchez, 2007). The plant is well known for its long vase life, size, and different colors of its flowers (Hecht et al., 1994). In addition, the plant is tolerant to pathogen, soil acidity condition, and high temperature stresses. However, because of small size of the Lisianthus seeds (19,000 seed /gm or 545,000 seeds /oz) it is hard to handle in field plantings. In our preliminary studies we found low rate of germination of Lisinthus seeds. Due to high demand for this ornamental plant, its propagation through tissue culture seems helpful. *In vitro* micropropagation of the shoot tips, internodes stem sections, and leaf segments of Lisianthus via tissue culture method have been previously conducted in modified MS medium (Murashige and Skoog, 1962) (Semeniuk and Griesbach, 1987). In addition, more research has been devoted to the investigation and improvement of Lisianthus by genetic engineering, such as regulation of floral transition

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(Zaccai et al., 2001; Zaccai and Edri, 2002), or production of Lisianthus flowers with altered pigment metabolic pathways (Aranovich et al., 2007). In present study, we examined induction and maintenance of calluses from different Lisianthus organs in three types of modified media with various compositions (B5, LS and MS media), regeneration of plants from calluses and their sustainable growth until anthesis and seed production.

Materials and Methods

Culture conditions

Segments of young leaves, internodes, roots, petals and anthers (2–4 mm in diameter) were obtained from one year old rooted cuttings of *Eustoma grandiflorum* L. Raf Shinners. The samples were surface sterilized by subsequent washing with detergent, sodium hypochlorite (containing 5% active chlorine, 20 min), ethanol (75%, 30 s) with rinsing in sterile distilled water intervals. Surface sterilization was accomplished under laminar air flow. To remove probable contaminations from root samples, they were washed with potassium permanganate (2%, 3 min) before washing with detergent. Solidified basal media of B5 (Gamborg et al., 1968) supplemented with 0.225 mg/L BA and 1.86 mg/L NAA, LS (Linsmaier and Skoog, 1965) supplemented with 3 mg/L IAA, 3 mg/L NAA, and 0.1 mg/L kinetin, and MS basal media supplemented with 3 mg/L IAA, 3 mg/L NAA, and 2 mg/L Glycin, were used. The composition of aforesaid media was decided according to previous studies which have been continuously conducted in our lab (Ghanati et al., 2002; Ghanati and Rahmati Ishka, 2009). Culture vessels were transferred to darkness, 25± 2 °C, allowing the emergence and growth of the calluses.

Growth condition of regenerated plants

Growth of regenerated plants was accomplished in sterile growth chambers at 25±2 ˚C with 16/8 h photoperiod and photosynthetic photon flux of 115 µMs-1m2 at the flask level. When plants were regenerated from the calluses, they were transferred to a hydroponic culture system on 1/2 Hoagland nutrient solutions (Hoagland and Arnon, 1950), pH 6.0 and were allowed to grow until anthesis. Temperature and light conditions were as mentioned above. Nutrient solutions were continuously aerated and renewed weekly.

Histochemical analysis

Viability of the cells was examined using Evans blue (0.1%) solution (Morita et al., 2006). Differentiation of tissues and somatic embryogenesis was checked by staining with Phloroglucirol – HCl staining (Ghanati et al., 2002). Observations were achieved under light or fluorescence microscopes equipped with digital camera (Olympus BH-2, Tokyo, Japan).

Statistical analysis

The factorial design based on completely random design (CRD) was used to examine the effects of culture media and type of explants. All experiments and observations were repeated at least 4 times, each with 10 samples.

Results

Except for root, explants from other organs were able to establish callus (Table 1). The highest percentage of callus was emerged from leaf and the lowest one from internodal stem explants. Among three applied media, LS was the best medium for induction of callus (Table 1).

Calluses originated from leaf in LS medium showed the highest growth rate, while those of internods in the same medium had the lowest growth rate (Table 2).

Table 1.
Percent of callus induction from different explants of Lisianthus on different media. Data are presented as average of 40 samples ± SD.

	Callus induction (%)				
Medium	Leaf	Internode	Anther	Petal	Root
LS	95±2	45±5	40±3	70±6	N
B5	80±6	60±5	55±5	75±7	N
MS	85±5	60±3	55±4	75±5	N

N: not emerged

As shown in Table (3), except for internodal stem segments whose calluses were very compact, the calluses of other explants were friable and their color varied from white to dark yellow (Table 3).

Discussion

Microscopic examination of the calluses did not show somatic embryos. Instad, direct organogenesis occurred in calluses of different organs. In dark conditions, rhizogenesis was the most rapid phenomenon occurred in LS medium, compared to B5 and MS media (Fig. I, a-e). Rhizogenesis in B5 and MS media occurred at longer periods of darkness. Gong (2008) reported that the combination of 6-BA and IBA was better than that of 6-BA and NAA for the induction of the adventitious buds from leaves of Polestar yellow variety of Eustoma grandiflorum. Our results is to some extent coincident with those of DA Ke-dong et al. (2003) who reported that MS basal medium supplemented with BA 1mg/L was suitable for leaf adventitious shoot formation,

Table 2
Growth of the calluses from different explants of Lisianthus on different media, after 20 days; data are presented as average of 40 samples ± SD.

Fresh Weight (g)				
Medium	Leaf	Internode	Anther	Petal
LS	2.0±0.2	0.95±0.05	1.0±0.03	1.4±0.06
B5	1.4±0.3	1.07±0.05	1.2±0.04	1.5±0.05
MS	1.2±0.1	1.07±0.03	1.07±0.04	1.4±0.05

Table 3
Morphologic features of the calluses from different explants of Lisianthus on different media; data are presented as average of 40 samples ± SD.

Color and Compactness			
	LS	B5	MS
Leaf	Cream Friable	Dark yellow Friable	Light Yellow Friableе
Internode	Dark yellow Friable	Light Yellow Friable	Cream Very Compact
Anther	Cream Friable	Cream Friable	White Friable
Petal	Light Yellow Friable	Cream Friable	Light Yellow Friable

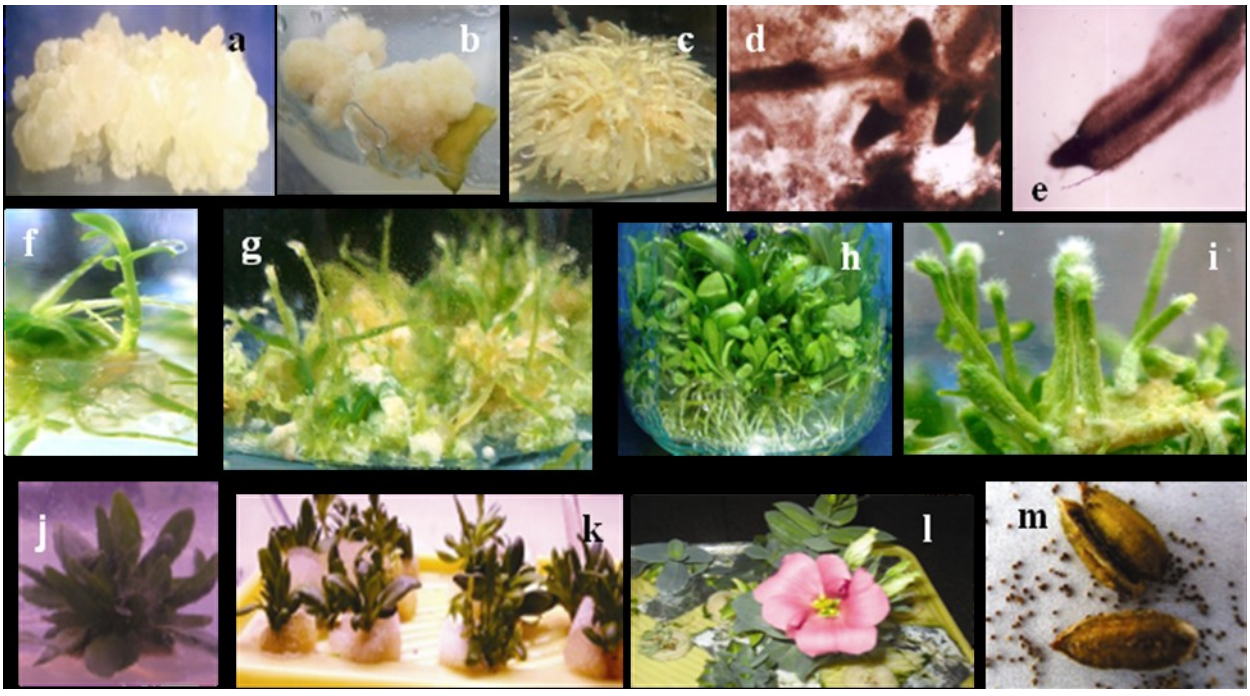

Fig. I. Different stages of induction of callus and regeneration of plantlets of Lisianthus. a-b, The calli induced in B5 and MS media, respectively; c, Rapid rhizogenesis from callus in LS medium; d-e, Frequent regenerated roots in LS medium, stained with phloroglucinol-HCl; f-h, Further regeneration of shoots in light conditions; i, Multiple adventitious shoot production in MS medium; j-l, Growth and flowering of regenerated plants in hydroponic culture; m, Seeds in capsules.

and MS + IAA 0. 1mg/L medium was suitable for plantlet rooting of Lisianthus. However, Paek and Hahn (2000) induced shoots from cultured shoot-tip using much lower concentration of IAA (0.25 mg/L) which can be attributed to existence of auxin in shoot tip explants.

Transfer to light condition with a PPFD of 115 µMs-1m2 at the flask level at 25±2 ˚C, reinforced the growth of shoots which have been already regenerated in LS medium in dark conditions (Fig. I, f-h) and resulted to emergence of multiple shoots directly from leaves in MS medium (Fig. I, i).

A problem in seedlings of Lisianthus is that after rising, the plant has tendency to remain in the rosette stage because of inadequate conditions in the plant exigency (temperature, light, etc) (Ohkawa et al., 1991). It is accepted that in regenerated plants, development of the stem does not happen and the flowering time is delayed (Popa et al., 2006). In our experiment however, when the new plants were allowed to grow in hydroponic culture systems under a photoperiod of 16/8 h, anthesis happened after 8 weeks (i.e., total of 84 days after explants culture) (Fig. I, j-l). This was another interesting result obtained in the present research, since according to Harbough (1995), Lisianthus plants flowered in a minimum of 98 days when seedlings were grown under long day photoperiod at higher temperatures and a maximum of 162 days with short day at cold conditions. New seeds were used for establishing new offsprings and further biotechnological experiments (Fig. I, m).

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