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A REVIEW

Lisianthus micro propagation

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Abstract : Lisianthus [*Eustoma grandiflorum* (Raf) Shinn.] is becoming one of the most highly ranked cut flowers in the international market. Propagation by seeds is complicated and difficult exercise due to slow germination and growth and seedling populations are disadvantageously variable with respect to flowering time, stem length and flower qualities. Vegetative propagation of selected cultivars might provide a useful alternative to seed propagation as the limited availability of cuttings from each stock resulting in apical dieback is a major setback. Therefore, micro propagation serves as potent tool to develop a method of rapid propagation of selected/ elite genotypes of lisianthus. The clonal multiplication of lisianthus especially through tissue culture might provide a useful alternative to seed propagation, thus resulting in to production of better quality planting stock. *In vitro* multiplication of elite plant genotypes offers immense opportunities to multiply large number of disease free, healthy and vigorous planting material in shortest possible time. In this review, the scattered information on clonal multiplication of *Eustoma* through micro propagation are being tried to put together. This could eventually be helpful in drawing the attention of the researchers and scientists to work on it, besides would be benefitted by utilizing the knowledge review in this paper so as to popularize it as a cut flower to make its place among top 10 cut flowers.

Key Words : *Eustoma*, Tissue culture, GA_3, BA, NAA, IBA, In vitro rooting, Hardening

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Eustoma grandiflorum (Raf.) Shinn. syn. *Lisianthus russelianus* Hook., belongs to family Gentianaceae and is native to southern parts of the United States (Popa *et al.,* 2004). Lisianthus is also known as 'Texas Blue Bell' and Prairie Gentian. Lisianthus is becoming one of the most highly ranked cut flowers in the international market due to its rose like flowers, excellent post harvest life and blue / purple colour. It gained importance on account of a variety of cultivars developed with respect to many traits like uniform flowering throughout the year, lack of rossetting, heat tolerance, flower colour, flower size and form including double flower etc. (Toa, 2006). Tsukada *et al.* (1982) classified the corolla shape of lisianthus into four groups: funnel-shape, cup-shape, shallow bowl shape and bell shape where as Harbough (2006) described flower shapes as flat/open petals, bell shape and tubular shape.

Lisianthus is a seed produced pot plant florist crop, with flowers that appear quite similar to those of tulip. The colour range include pink, white and purple (Asen *et al.*, 1986). The stem is monopodial at the base and branches apically. Stem length of most commercially available cultvars varies from 500-750mm. Individual flowers last for 2 weeks and a whole plant can remain in bloom for up to 5 weeks (Ruffoni and Savona, 2006; Gnesback *et al.*, 1988). Lisianthus is normally grown as an annual crop. The young plants have a high light requirement and optimum temperature of 20^0 C at sowing and 26^0 C / 18^0 C (day/night) during the growing season. The crop requires a fairy light, free draining soil, with good organic matter content and a pH range of 6-7 (Tsukada *et al.*, 1995). Lisianthus can be used either as cut flowers or as flowering pot plants and flowers are available in various colours like blue, purple, plum, white, pink and bicolours (Kunitake *et al.*, 1995).About three cut flower stems are produced per plant in the first harvesting cycle and retaining the plant for the second crop is considered uneconomic (Halevy and Kofranek, 1984).

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Cut flower lisianthus may be divided into single and double flowering forms. Single flower forms have four to five petals, while double flower form can range from six to seven to as high as twenty five. A further division in lisianthus is flowering response time. *Eustoma grandiflorum* (Raf.) Shinn. cultivars are quantitative long day plants characterized by promoted flowering by long days (Ohkowa, 2003; Yamada *et al.,* 2008a; Yamada *et al.*, 2009; Zacci and Edri, 2002). Plants grown under higher light integral flowered earlier than those under a low light integral of the same day length. However, growth and flowering of *Eustoma* plants are either promoted or delayed by night break treatment using different type of light sources (Yamada *et al.*, 2008b).

Lisianthus takes about 6 months from seed to flowering (Griesbach *et al.*, 1988; Ordogh *et al.,* 2006). This period can be reported in to two stages, first stage begins at germination and last for about 3 months during which plant grow in rosette forming only four pairs of true leaves whereas, second stage starting after 3 months involves stem elongation and flower stalk elongation (Ruffoni and Savona, 2006). Propagation by seeds is complicated and difficult exercise due to slow germination and growth and seedling populations are disadvantageously variable with respect to flowering time, stem length and flower qualities(Ordogh *et al.,* 2006). Hybrids would be expected to more uniform than seedling populations; however, true hybrids have not been produced, since highly selfed inbred lines are very weak and difficult to keep alive. Vegetative propagation of selected cultivars might provide a usefull alternative to seed propagation (Griesbach *et al.,*1988) as the limited availability of cuttings from each stock resulting in apical dieback is a major setback (Roh and Lawson, 1984).

Therefore, micro propagation serves as potent tool to develop a method of rapid propagation of selected/ elite genotypes of lisianthus. *In vitro* multiplication of elite plant genotypes offers immense opportunities to multiply large number of disease free, healthy and vigorous planting material in a shortest possible time.Tissue culture propagation of selected clones has great importance as uniform inbred seed population does not exist for lisianthus. Such seedlings could be mass propagated through tissue culture. Thus tissue culture offers the hope of obtaining new genes that can be used in further improvement of this crop (Griesbach *et al.,* 1988). The relevant literature available on micro propagation of lisianthus is reviewed under the following heads:

- Explants
- Surface sterilization
- Cultures establishment and shoot multiplication
- *In vitro* rooting
- Acclimatization

Explants :

Semeniuk and Griesbach (1987) ascertained the condigness of various explants *viz*., shoot tips (10 mm long), internodal stem sections (20 mm long) and leaf segments (10 mm x10 mm) of lisianthus (*Eustoma grandiflorum*) cultivar 'Dwarf Purple' cultured *in vitro* on modified MS medium and reported developmental of multiple shoots from shoot tips and stem sections. Whereas, leaf segments did not produce shoots and later they turned chlorotic (Griesbach *et al*., 1988). Le (1994) cultured shoot explants of *Eustoma grandiflorum* cultivar 'Sakata Yodel Blue' on MS medium containing different combinations of cytokinins and auxins *in vitro* and reported successful culture establishment. Fukai *et al.* (1996) obtained *in vitro* adventitious shoot regeneration from leaf segments of *Eustoma grandiflorum* on MS medium containing various concentrations of BA and NAA.Murayama *et al*. (1996) isolated protoplasts enzymatically from young leaves of *Eustoma grandiflorum* seedlings and cultured them aseptically on MS medium to establish *in vitro* cultures in 50 days. Similarly, Dong *et al*. (2003) reported high frequency adventitious shoot formation and plantlet regeneration from leaf cultures of *Eustoma grandiflorum*. Popa *et al.* (2004) tried various explants comprising shoot tips (10 mm), stem sections of (20 mm) and leaf sections (10 mm x 10 mm) for *in vitro* propagation of *Lisianthus russellianus*. They further investigated the morphogenetic capacity of these explants as well as phytohormone balance for plant regeneration of *Lisianthus russellianus*.

Surface sterilisation:

Surface sterilization is the pedestal of aseptic culture initiation. After the selection and identification of suitable explant, the major problem is the incidence of heavy contamination, especially when the explants are collected from the field grown plants. A number of chemicals and techniques have been employed to accomplish this step. Semeniuk and Griesbach (1987) surface sterilized various explants *viz*., shoot tips, stem sections and leaf segments in 20 per cent bleach for 25 minutes and then rinsed them twice in sterilized distilled water to establish aseptic cultures *in vitro*. However, Daminano *et al*. (1989) used 1 per cent NaOCl along with one drop of tween twenty for 15 minutes to surface sterilize the axillary buds of *Eustoma grandiflorum* taken from the field grown plants. Then, these surface sterilized explants were rinsed twice in sterile water for the establishment of *in vitro* cultures. Murayama and his co-workers (Murayama *et al.*, 1996) accomplished the surface sterilization of explants with 70 per cent ethanol for 5 seconds and 1 per cent sodium hypochloride for 5 minutes, respectively and then rinsed explants thrice in sterilized distilled water to establish aseptic *in vitro* cultures of *Eustoma grandiflorum.* Similarly, Ordogh and his associates (Ordogh *et al.*, 2006) excised the shoot tips, axillary buds and flower buds from the field grown plants of four echo cultivars ('E. White', 'E. Rose', 'E. Blue', 'E. Blue Picotee') of *Eustoma grandiflorum* and surface sterilized them in 50 per cent ethanol for 5 minutes and afterwards sterilized in 0.2 per cent $HgCl_2$ for

5 minutes and rinsed in sterile distilled water three times for the successful culture establishment *in vitro*.

Culture establishment and multiplication:

Damiano *et al*. (1986) reported the best proliferation of *Lisianthus russelianus (Eustoma grandiflorum)* in a medium containing mineral nutrients supplemented with 0.5 ppm nicotinic acid, 0.5 ppm pyroxidine, 0.1 ppm thiamine, 100 ppm myo-inositol, 2 ppm glycine, 0.3 ppm BA, 30 g/l sucrose and 8 g/l bacto-agar, at pH 5.8 for proliferation. Griesbach *et al.* (1988) cultured various explants *viz*., shoot tips, internodal stem sections and leaf segments of lisianthus (*Eustoma grandiflorum*) cultivar 'Dwarf Purple' *in vitro* on modified MS medium and recorded multiple shoot development explants shoot tips and stem sections with BA @ 3 mg/l and NAA @ 0.2 mg/l. The shoot proliferation was also obtained on shoot tips and leaf segments with BA @ 3 mg/l, but internodal stem sections became necrotic and died on this medium. Skrzypczak *et al*. (1988) obtained the callus cultures on MS medium containing GA_3 1.0 mg/l and kinetin 0.5 mg/l with leaf explant. Shoots were obtained when the callus was transferred to MS medium containing GA_3 (0.2-0.5 mg/l) and kinetin (0.1-0.25 mg/l). They further reported formation of adventitious buds, lateral buds and apical buds when BA was incorporated in the MS medium.

Damiano *et al*. (1989) reported 98-100 per cent germination when seeds of *Lisianthus russelianus* (*Eustoma grandiflorum*) were cultured *in vitro* on Brooks and Hough medium supplemented with GA_3 1 mg /l and active charcoal. The terminal root segments (1 cm long) of *Eustoma grandiflorum* were cultured *in vitro* on MS medium containing 0-1 mg NAA or IBA and 20 g sucrose/l and reported increased production of laterals (19.7 shoots/plant) at the lowest concentration of IBA. Brien and Lindsay (1993) isolated protoplasts from either cotyledons or leaves of 5 cultivars of *Eustoma grandiflorum* and cultured them in agarose beads surrounded by liquid V-KM media containing 5.4 µM NAA and 2.3 µM zeatin. When microcalli were approximately 1 mm in diameter, the agarose beads were transferred to shoot regeneration media containing 0.1 µM IBA and 4.4 µM BA. Shoots were produced from the calli during several subculture periods. Protoplast viability and subsequent regeneration of plants were dependent on calcium levels and growth regulator present in the *in vitro* seed germination media, osmolality of the protoplast purification solution, and osmolality and pH of the culture media. Shoots were rooted in MS media containing 5.7 µM IAA. Plantlets derived from protoplasts of cultivars Fresh White, Hakusen, Miss Lilac, Fresh Purple and Doremi Wine Red were successfully transferred to the glasshouse. Le (1994) reported establishment of shoot explants of *Eustoma grandiflorum* cultivar 'Sakata Yodel Blue' on MS medium containing different combinations of cytokinins and auxins. He recorded best shoot proliferation (4.73 shoots/explant) on MS medium supplemented with 0.53 µM NAA + 4.44 µM BA.

Fukai *et al*. (1996) obtained the adventitious shoot regeneration from leaf segments of *Eustoma grandiflorum* when cultured on MS medium containing various concentrations of BA and NAA. Medium containing BA 0.1 mg/l + NAA 0.01 mg /l produced very healthy and more shoots per explant. Cultivar differences in shoot regeneration were observed. Out of 9 cultivars used, four cultivars namely Fukushihai, Royal Pink, Royal Purple and Royal Light Purple gave good results while 2 cultivars Royal Violet and Holy Creamy White gave poor results. The age of the mother plants (*in vitro* cultured seedlings) from which the explants were taken notably influenced the regeneration and shoot formation. The explants excised from older seedlings resulted in the formation of quality shoots and the explants of younger seedlings failed to form shoots.

Murayama *et al*. (1996) isolated the protoplasts enzymatically from young leaves of *Eustoma grandiflorum* seedlings cultured aseptically on MS medium. The isolated protoplasts were cultured at a density of 1-2x104/ml in a modified MS medium containing 1.0 per cent sucrose, 9.0 per cent mannitol, NAA 1.0 mg /l and BA 0.1 mg /l. Cell division occurred after 4-5 days in culture. A fresh medium was added weekly to promote colony formation. The colonies (0.5-1.0 mm in diameter) were transferred to a modified MS agar medium containing BA 0.1 mg/l + NAA 1.0 mg /l for callus proliferation. Shoots regenerated rapidly when these calli were transfered to MS medium containing BA 0.3 mg /l. The regenerated shoots were multiplied on MS agar medium containing BA 0.1 mg/l + GA31.0 mg /l. Paek and Hahn (2000) investigated the effects of cytokinins, auxins and activated charcoal on organogenesis and anatomical characteristics of *Eustoma grandiflorum* cultured *in vitro*. They found that BA and kinetin at high concentrations (13.32-22.2 and 13.94-23.23 µM) resulted in good shoot formation but high percentages of hyperhydric shoots. Both shoot and root developments were suppressed by activated charcoal. The highest percentage of regeneration and the largest number of glaucous shoots with an average of 15 shoots per explants after 4 weeks of culture were obtained when the shoot-tips were cultured on MS medium supplemented with 4.44 µM BA and 1.47-4.92 µM IAA and IBA.

Dong *et al*. (2002) reported high frequency adventitious shoot formation and plantlet regeneration were established from leaf cultures of *Eustoma grandiflorum*. They also investigated the effects of plant hormones and their combinations on adventitious shoot regeneration and plantlet growth and recorded better shoot formation when MS medium was supplemented with BA (1mg/l). The plantlet growth was found to be better on MS medium containing BA (0.1 mg/l) and NAA (0.05 mg/l). Popa *et al.* (2004) cultured shoot tips (10 mm), stem sections (20 mm) and leaf sections (10x10 mm) of *Lisianthus russellianus in vitro* on MS medium containing

varied concentrations of growth regulators. After 8 weeks, the formation of adventitious shoots from each explant was observed. Shoot tips produced the highest number of shoots on MS medium supplemented with BA 3 mg/l, whereas the presence of NAA in MS medium proved to be beneficial for production of adventitious shoots per internodal explants. However, the leaf explants proliferated better when MS medium was supplemented with GA_3 0.5 mg/l.

Tao (2006) reported MS medium containing BA 0.5-1 mg/l and NAA 0.5-1 mg /l as the most suitable for callus formation and bud multiplication of Lisianthus (*Eustoma grandiflorum*). The multiplication was best when MS medium was supplemented with BA (0.5-1.0 mg/ l), NAA (0.5-1 mg/ l), GA (0.5-1 mg / l) and casein (50-100 mg / l). Ordogh *et al*. (2006) studied the effect of various concentrations of BA on four 'Echo' cultivars (E. White, E. Rose, E. Blue, E. Blue Picotee) of lisianthus (*Eustoma grandiflorum*) on shoot multiplication cultured *in vitro* on MS basal medium with 11 g/l agar and 20 g/l sucrose. They found that highest number of shoots with smallest leaves in cultivar E. White on medium supplemented with 0.10 mg/l BA. The other three cultivars developed the longest leaves when MS basal medium was supplemented with 0.10 mg/l BA. However, they obtained tallest plants with the longest leaves in the proliferation medium containing 0.25 mg/l BA, in cultivar E. Blue Picotee.

***In vitro* rooting:**

Damiano *et al*. (1986) obtained the best rooting of *in vitro* raised shoots on half-strength MS medium supplemented with IAA @ 1 mg/l. Similarly, Semeniuk and Griesbach (1987) obtained the *in vitro* rooting of shoots on half-strength MS medium supplemented with IAA @ 2 mg / l and these plantlets were successfully transferred to soil. Damiano *et al*. (1989) rooting of shoots were obtained on MS media supplemented with 5.7 µM IAA. Similarly, roots were obtained when *in vitro* raised shoots were transferred to a medium containing IAA (0.5-1 mg/l) and 84-100 per cent rooting was achieved *in vitro* when medium is supplemented with IAA @1 mg /l (Skrzypczck *et al.*, 1988).

Le (1994) cultured young shoots on MS medium containing different concentrations of NAA and obtained the highest percentage of rooted shoots (85%) in MS medium containing 2.5 µM NAA. Murayama *et al*. (1996) reported rooting in IBA- treated shoots which grew into normal plants. However, the percentage of bicoloured flowers in protoplast-derived plants was significantly lower than that in plants obtained from seeds, the usual means of propagation.Paek and Hahn. (2000) recorded this the increased IAA and IBA concentrations in rooting medium favored root formation, while increased NAA concentration adversely affected root formation. Both shoot and root development was suppressed by activated charcoal. However, MS medium supplemented with 0.1 mg l IAA was found to be much suitable for plantlet rooting (Dong *et al*., 2002).

Dong *et al.* (2002) reported this MS medium containing BA (0.1 mg/l and NAA (0.05 mg/l) was suitable for plantlet growth and whereas MS medium supplemented with IAA (0.1 mg/l) resulted in the best rooting of plantlet. Tao (2006) reported that half strength MS medium supplemented with 0.3-1 mg NAA/l, 0.5 mg IBA/l and 0.5 mg IAA/l was the most suitable for root formation and rooting per cent was found to be 95 per cent within 25 days. Ordogh *et al*. (2006) obtained highest rooting percentage and number of roots was on MS medium supplemented with 1.0 mg/l NAA, irrespective of the cultivars 'E. Rose' developed the maximum roots on this medium.

Acclimatization:

Harbaugh and Woltz (1991) reported that foliar chlorosis or bleaching, interveinal chlorosis, leaf edge and tip necrosis, a poor root system, and stunted growth of *Eustoma grandiflorum* seedlings were associated with a growing medium (a 2:1:1 mixture of vermiculite, sand and perlite) pH of 5.0 or 5.4 but not when the values ranged from 6.4 to 7.5. The range in growing medium pH resulting in the best growth of seedlings and flowering plants was 6.3-6.7. Responses to growing medium pH were similar, regardless of nutrient solution pH or cultivar (Saga Pink, White and Yodel Mixture were compared). *E. grandiflorum* seedling and shoot fresh weights at pH <5.5 were 23-66 per cent of corresponding values for plants grown at pH 6.4. Leaf tissue Zn concentration was extremely high (1050 mg/kg) at a growing medium pH of 5.0, but other macro- and micronutrients in leaves were not at abnormal levels.

Dong *et al*. (2002) studied the effect of different media composition on growth and floral characters of Lisianthus. Sivakumar *et al*. (2002) reported plants grown on media M3 (2 coir peat:2 sand:1 soil) recorded the maximum number of branches per plant, more number of leaves per stem at flower opening, maximum leaf area and highest fresh root weight. The plants in same media flowered earlier than other media compositions and recorded the earlier harvest. In case of floral attributes, the media M3 recorded the highest stem length and diameter, maximum cut flower weight and maximum flower diameter. The media M2 (1 coir peat: 2 sand:1 soil) recorded the maximum flower length. Thus, it was found that media M3 containing 2 parts of coir peat, 2 parts of sand and 1 part of soil was best for cut flower cultivation of lisianthus.

Salvador and Minami (2004) reported that substrate densities of 0.75 and 0.95 g cm^{-3} recorded the highest values for all parameters studied, *i.e.* percentage of pots with flowers, number of flowers, number of buds, plant height, fresh top weight, dry top weight, fresh root weight, dry root weight and root volume. Lisianthus developed well under the following conditions: 72.60-75.10 per cent porosity, 12.81-19.53 per cent air space, 19.79-26.03 per cent available water and 17.41-23.71

per cent easily available water. In the second experiment, cultivated peat + vermiculite, eucalyptus bark + conventional peat + sand and control showed the best results and produced plants with good commercial quality. Pine bark + earthworm humus was inadequate for lisianthus cultivation.

Salvador and Balas (2006) reported the best substrate density for lisianthus cultivation was 0.75 gcm^{-3}. Substrates suitable for lisianthus growth should present 72-75 per cent porosity, 12-19 per cent air-filled porosity, 19-26 per cent available water and 17-23 per cent easily available water. Different mixtures of substrate materials, which had suitable physical characteristics, were analyzed in a second experiment. Lisianthus grew best in cultivated peat: vermiculite: eucalyptus bark: conventional peat: sand substrates and in controls. The survival rate of the sprouts was 96 per cent in a substrate of vermiculite, perlite and humus at a 4:4:2 ratios (Tao *et al.*, 2006).

Fascella *et al*. (2009) carried study on growth and flowering of lisianthus hybrids under protected cultivation. Plants of the double-flower cultivars Echo White, Echo Yellow, Dream White Blue and Dream White Pink were grown in polypropylene containers filled with a mixture of perlite and coconut coir dust (1:1, v/v) in an open-loop system. The 4 hybrids significantly varied in growth, yield and quality. Echo White and Dream White Pink showed earlier flowering (150 days from transplanting) but had lower yields (61.5 stems m^{-2}) than Echo and 67.0 stems m^{-2}). Dream White Bluer produced cut flowers with the longest stems (68.3 cm), and greatest fresh weight (178.6 g) and number of flower buds (29.3 per stem).

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