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RESEARCH PAPER

HORTICULTURAL STUDIES

Micropropagation of Lisianthus [*Eustoma grandiflorum* (Raf.) Shinn.] Leaf Explants and Single Nodes in Response to Plant Growth Regulators

Hakan AKTAŞ[1] Gizem Gökçe AKKAYA[2] Ebru AKYÜZ ÇAĞDAŞ[2] Gülden HASPOLAT[3] Şeküre Şebnem ELLİALTIOĞLU[4]

[1] Isparta University of Applied Sciences Faculty of Agriculture Department of Horticulture, 32200, Isparta, Türkiye
[2] Has Biotech Company, Antalya, Türkiye
[3] Aegean Agricultural Research Institute, 35660, İzmir, Türkiye
[4] Ankara University Technopolis Doqutech Academy Company, Ankara, Türkiye

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Corresponding Author
E-mail: aktashakan33@gmail.com

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Abstract

Lisianthus has long-lasting, white, blue-purple, velvet or pink flowers. Varieties that can be used in pots, outdoors, or cut flowers are widely used and it is essential to develop new varieties to increase their commercial value. Breeding studies with classical methods can take a long time in lisianthus. Techniques to create variation in these plants and facilitate their reproduction always contribute to shortening the breeding period and one of the best known of these methods is plant tissue culture. This study aimed to investigate the micropropagation possibilities in lisianthus and examine the effects of auxin and cytokinin applications and doses on shoot reproduction. Supplemented with 10 different plant growth regulators in Murashige and Skoog (MS) medium, leaf explants from lisianthus plants that were germinated under *in vitro* conditions (seedlings) were cultivated. 6-benzyl aminopurine (BAP) was utilized in 5 doses (0.5, 1.0, 2.0, 3.0, and 4.0 mg L^{-1}) alone in the first 5 groups. Naphthalene acetic acid (NAA) was also added to the BAP mediums at a concentration of 0.5 mg L^{-1}. Shoot formation, shoot lengths, the number of shoots longer and shorter than 1 cm, and regeneration of new shoots after subcultures were determined. According to the results, MS mediums containing 3.0 and 4.0 mg L^{-1} BAP could be good starting mediums for regenerating new lisianthus shoots from leaf explants. These treatments got the highest values both in shoot formation and the number of shoots longer than 1 cm. It has been determined that 3.0 mg L^{-1} BAP alone can be used for both organogenesis from leaf explants and shoot proliferation in single node culture. *In vitro* shoots of the lisianthus species were easily rooted and both PGR-free MS or ½ MS mediums. Lisianthus shoots propagated under *in vitro* conditions can be rooted under *ex vitro* conditions both in the float hydroculture and in the soil mixture. The acclimatization stage was optimized also successfully.

1. Introduction

Lisianthus (*Eustoma grandiflorum* (Raf.) Shinn.) is an ornamental plant, belongs to the Gentianaceae family, has silky-looking but very durable flowers in bright and pastel colors. It is among the top 10 cut flowers in the ornamental plants trade with long post-harvest life of flowers (Harbaugh, 2007; Hutchinson, 2011; Bertoldo et al., 2015; Ozkan, 2017). Tall types are preferred for cut flower production in the field and greenhouse, and multi-branched dwarf varieties come to the fore when it was planned to be used as potted plants (Harbaugh and Zhanao, 2006; Hanks, 2014; Menge, 2019).

Lisianthus is usually a seed-propagated plant. Root cuttings or tissue culture methods can also be used for propagation. Although the most commonly

used method of propagation is seed propagation, the very small size of the seeds (10,000 seeds in 1 g) causes the need for seed pelleting (coating) for commercial production. Inbreeding depression is an important limiting feature for lisianthus breeding. Self-depression causes poor seed formation or poor plant growth. However, strong inbred lines need to be used in the generation of F_1 hybrids from strongly developing homozygous parents (Harbaugh, 2007).

However, seed propagation is provided by seedling cultivation, obtaining homogeneous seedlings in double-colored and double-flowered types can sometimes be a problem. There may be differences in flowering time, plant height, and number of flowers in plants grown from seeds in varieties with high heterogeneity (Furukawa, 1993). Micropropagation of the plant is also an issue of interest in lisianthus, as it allows obtaining very high numbers of plants in a short time with a homogeneous structure. Propagation by tissue culture is important for large-scale propagation of high-quality selected or breeding plants (Semeniuk and Griesbach, 1987; Miri et al., 2016; Rahaman et al., 2018). Success in effective micropropagation of lisianthus depends on several factors such as genotype, culture medium, plant growth regulators (PGR), and explant type (Ordogh et al., 2006; Uddin et al., 2017). The importance of micro-propagation in the reproduction of elite clones is indisputable. *In vitro* propagation of *Eustoma*; either by direct organogenesis using explants such as shoot tip, lateral buds, internode stem segments, and leaf fragments (Semeniuk and Griesbach, 1987; Mousavi et al., 2012), organogenesis via callus (Rezaee et al., 2012; Akbari et al., 2014) can also be obtained. Duong et al. (2006) developed a protocol for optimal somatic embryo formation from leaf explants and made it available for plant breeding programs (Yumbla-Orbes et al., 2020).

The explant/shoot tip consists of the shoot apical meristem, non-elongated leaves of various stages of development, and a series of leaf primordia approximately 1 cm long. Cytokinin-rich medium suppresses the apical dominance of shoot-tip explants and promotes the formation of highly branched shoot systems (Saeed et al., 1997). Moreover, *in vitro* plants (shoots, seedlings) containing single nodes can be used as explants, because the nodes contain axillary buds and treatment with cytokinins under *in vitro* conditions can induce branch formation (Barna and Wakhlu 1995). In the shoot multiplication stage of propagation by tissue culture, single-node explants are successfully used to establish subcultures to obtain a sufficient number of micro shoots. Martini et al. (2022) reported that the single node explant of the *Salvia tomentosa* plant in the subculture stage gave high shoot numbers. Single node explants are obtained by separating the shoots grown in tissue culture into micro cuttings. Cuttings are a natural method of vegetatively propagating plants in both *in vivo* and *in vitro* conditions. Leaf axillary buds are comparable to stem tips in terms of mass propagation capacity (Bhatia and Sharma, 2015). This method has been used successfully in the micropropagation of many plants such as *Mentha* sp. (Tepe et al., 2002), *Artemisia* sp. (Türközü et al., 2014), *Centauria* sp. (Okay et al., 2014), *Elatostema* sp. (Mizushima, 2017).

Micropropagation is currently the easiest way to produce healthy, disease-free *Eustoma* (Jafari et al., 2017). The purpose of this research is to determine the optimum *in vitro* propagation method using single node culture from leaf explants of *in vitro* seedlings of lisianthus. In light of the literature, the major goals of the research are to identify a technique that can enable quick plant reproduction, identify developing issues, and obtain knowledge that will provide the foundation for additional studies. After the completion of this study, it is aimed to breed local varieties that will be commercially propagated by vegetative propagation method using *in vitro* mutation methods, using biotechnological accelerator techniques.

2. Materials and Methods

The research was carried out at Isparta University of Applied Sciences, Faculty of Agriculture, Department of Horticulture, and in the tissue culture laboratory and greenhouses of Has Biotech Company in Antalya between 2021 and 2022. Plant material from lisianthus plants with certificate number 552640 and supplied by the Ege Plantek Company's Türkiye representative was used in the study. Commercial varieties of the *Eustoma grandiflorum* (Raf.) Shinners species with purple and white flowers were used to test *in vitro* germination and regeneration. Due to the usage of the leaves of *in vitro* seedlings, appropriate PGR tests were conducted.

2.1. General tissue culture techniques applications

All tissue culture applications were carried out under aseptic conditions, and a sterile cabinet (laminar flow cabinet) was used. The scalpel blade and forceps used were pre-sterilized and during the culturing process, a bead sterilizer was used to keep the forceps and scalpels sterile during sewing. MS (Murashige and Skoog, 1962) basic medium composition was used as the nutrient medium. Commercially available premixed powder of MS was used in the preparation of plant nutrient medium (Sigma). These products, whose macro and micronutrients and vitamins have been adjusted at the dose of use, provide a significant convenience in the functioning of the study. Plant growth regulators (PGR) were added to the basic MS medium, which differed according to the treatments. A few drops of 1 N NaOH or HCl solutions were used as needed to adjust the pH.

Finally, 3.0% sucrose and 0.7% agar were added to the nutrient medium. All components were dissolved on a hot plate magnetic stirrer. After the nutrient mediums were autoclaved in 250 or 500 mL autoclave bottles with caps, they were distributed in the previously sterilized Petri dishes as 10 mL each in the sterile cabinet and 40 mL in the jars. Sterilization of the medium in the autoclave was carried out under 1.2 atm of pressure, at 121°C for 20 minutes. After being removed from the autoclave, the bottles containing the sterile nutrient medium were shaken well to ensure that the agar was completely mixed with the medium, and then the medium was distributed in Petri dishes or jars in the cabinet. Following the solidification of the agar at room temperature, the medium was prepared for planting the explants.

2.2. Sterilization of seeds

Lisianthus seeds of two different varieties were brought to the laboratory to germinate on nutrient medium under *in vitro* conditions after being kept at refrigerator temperature for 10 days. The seeds were first placed in small packets prepared from filter paper and these packets were attached with paper clips. Due to the leakage of very small seeds through the wire strainers, it is necessary to immerse them in a disinfectant solution in cheesecloth bags or paper packages. Paper packets containing seeds were dipped in 20% commercial sodium hypochlorite (bleach) for 15 minutes for surface sterilization.

Meanwhile, 3 drops of Tween-20 (0.2% dose) were dropped into the disinfectant solution. After that, it was rinsed with sterile distilled water 3 times for 5 minutes each. After the final rinse water in the glass jar, where sterilization was made, was filtered, the paper packets containing the seeds were taken on sterile blotting paper and the excess water was absorbed into the blotting paper. The paper clips were opened and the seeds stuck on the paper were transferred to the surface of the nutrient medium in February 2021. In this way, the seed planting process has been completed. All these processes were performed in a laminar flow cabinet under aseptic conditions.

2.3. Preparation, planting, and incubation of explants on nutrient medium

After germination of sterile lisianthus plants leaf explants were cultured in different PGR experiments. Under aseptic conditions, the material in the form of rectangular leaf explants was prepared with a scalpel on sterilized filter papers in the cabinet, and the underside of the leaf was placed on the nutrient medium in Petri dishes (Figure 1). After placing 5-6 explants in each Petri dish, they were wrapped with stretch film strips for cutting off their contact with the atmosphere. After planting the explants in MS medium in Petri dishes, the cultures were incubated at 25±2°C and in photoperiodically arranged climate chambers with 16 hours light/8 hours dark.

2.4. Establishment stage

Leaf explants taken from *in vitro* germinated plants (seedlings) of lisianthus were cultured in MS medium with 10 different plant growth regulators. In the first 5 groups, 5 doses (0.5, 1.0, 2.0, 3.0, and 4.0 mg L^{-1}) of BAP (6-benzylaminopurine) were used alone. In the second group, 0.5 mg L^{-1} NAA (Naphthalene acetic acid) was also added to the BAP mediums in the experiment. An average of 4-6 leaf explants were planted in each Petri dish and 10 Petri dishes were used for each treatment. PGR (plant growth regulators) doses and combinations used in the experiment are shown in Table 1. The regenerated shoots from leaf explants about 4 cm in length were cultured by micro-cutting method and single nodes were planted in the mediums.

When the 8th week of culture was completed, observations, counting of the number of shoots, and evaluations were made. In the 12th week, the shoot

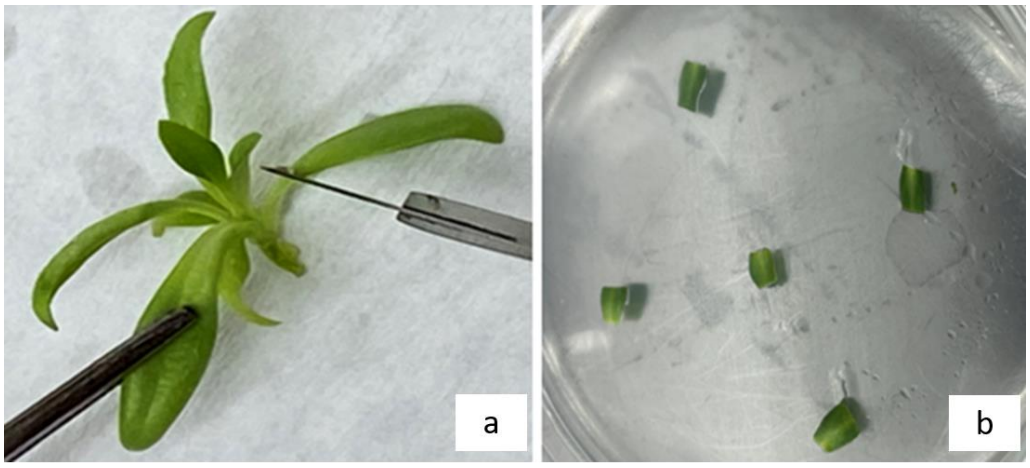

Figure 1. *In vitro* seedlings (a) and leaf explants (b).

Table 1. Plant growth regulators.

Plant growth regulators	Concentration (mg L^{-1})	Plant growth regulators	Concentration (mg L^{-1})
BAP	0.5	BAP+NAA	0.5+0.5
	1.0		1.0+0.5
	2.0		2.0+0.5
	3.0		3.0+0.5
	4.0		4.0+0.5

BAP: 6-benzyl aminopurine, NAA: Naphthalene acetic acid.

Figure 2. Planting the plantlets in vials (a) and placing in covered plastic boxes and keeping them in the climate room (b).

lengths and shoot numbers shorter and longer than 1 cm of the regenerating shoots were estimated and tables were created. After this stage, the explants longer than 1 cm were sub-cultured and incubated for 4 weeks in a 16/8-hour photoperiod in a climate chamber at 25°C. At the end of this period, the number of plants formed after subcultures and the number of plants shorter and longer than 1 cm were also recorded.

2.5. Rooting stage

Rooting experiments were established using shoots longer than 1 cm in subcultures, and 20 shoots representing each growth medium (10 different compositions) were transferred to the PGR-free MS and ½ MS mediums. Thus, it was also investigated whether the propagation medium composition would have an effect on rooting. After 4 weeks, the rooting was observed and the rooted shoots were transferred to the acclimatization stage. On the other hand, after 100 shoots were placed in styrofoam viols, they were taken into floating water culture in plastic containers, where both rooting and acclimatization stages were aimed together. Afterward, 100 shoots were placed directly in a mixture of peat and perlite filled in pots with a diameter of 10 cm, watered, and put into plastic boxes. The lids of the boxes were first kept closed and gradually opened within a week from the second day.

2.6 Adaptation to external conditions

In acclimatization of rooted lisianthus plantlets to external conditions, the plantlets were taken out of glass jars and their roots were washed under tap water and purified from the agar-based nutrient medium. The plantlets (200 pieces in total), which were planted in 5 cm diameter vials in which filled peat: perlite (2:1) mixture, were placed in deep and covered plastic boxes and kept in the climate room. Before the plastic cover was covered, the plants were irrigated and the leaves were sprayed with a mini hand sprayer. Water spraying was continued by opening the cover of plastic boxes (mini-greenhouse) twice a day for 4 days, after which the cover was gradually opened slightly and completely removed at the end of a week. These processes were carried out in the tissue culture climate chamber (25°C) with daily illumination of 16 hours (Figure 2).

The plants were transported to the greenhouse in boxes with the covers completely opened, then transferred to new pots filled with peat (Clasmann) one by one. They were placed on styrofoam under a black net in a shaded area in the greenhouse. Spraying water on the leaves twice a day with a hand sprayer was done for 5 days, after this stage, water was sprayed once a day with an interval of two days, and after the 10^{th} day, the plants were allowed to develop normally.

2.7 Analysis of data

The data of the experiment were statistically evaluated in the Completely Randomized Design, 3 replications and a total of 5 explants were used in each replication (3 replications × 10 combinations of PGR's). Statistical analyses were performed in the SAS-JMP pro13 program (SAS Institute Inc., Cary, North Carolina, USA). Differences between data were tested by analysis of variance, moreover,

means determined to be statistically different were compared with the LSD test.

3. Results and Discussion

3.1. Seed germination studies

Lisianthus seeds belonging to two different genotypes were kept in a closed glass jar in the refrigerator at +4°C for 10 days, and then they were sterilized and planted under sterile conditions in the prepared PGR-free MS medium. The first germination occurred after 17 days. Although the germination of the white-flowered variety, 99.8% germination was observed from 200 seeds sown, and 23.6% heterogeneous germination was observed in the variety with purple-white variegated flowers. This situation was also mentioned by Furukawa (1993) and Haspolat et al. (2020) that there is substantial genetic heterogeneity in seed germination and that physiological requirements might vary significantly between genotypes. As a result of this finding, the study was continued on the white-flowered variety, in which germinated and sterile seedlings were obtained.

3.2. Shoot formation of leaf explants

Leaf explants produced shoot differentiation in all treatments. It was demonstrated that leaf explants can be successfully used in lisianthus tissue culture for organogenesis. Previous studies have shown that different explants can be used in *in vitro* propagation of lisianthus. For example, Paek and Hahn (2000), Ordogh et al. (2006), Nhut et al. (2006), Esizad et al. (2012), and Yumbla-Orbes et al. (2020) were able to provide shoot organogenesis when they used different explants such as internodes, roots, and petals in lisianthus as starting material. However, Rezaee et al. (2012), proved in their study that leaf explants had higher replication capacity than all other explants. Deroles et al. (1993), Semeria et al. (1996), and Handa and Deroles (2001) reported that leaf explants were successful for *in vitro* shoot regeneration and could be the most suitable expander for genetic transformation studies in lisianthus.

3.2.1. Number of shoots

The shoot numbers were measured after the 8th week of culture. In terms of the average maximum number of shoots, all the treatments were not statistically significant. The maximum number of shoots was observed at the mediums with 3.0 and 4.0 mg L^{-1} BAP (14.9 and 14.5). In the 12th week, shoot numbers of shorter and longer than 1 cm were estimated (Figure 3). The average maximum number of shoots measured over 1 cm in length was observed in the medium containing 3.0 mg L^{-1} BAP (9.4 shoots/explant) followed by the 4.0 mg L^{-1} BAP medium. However, since the total number of shoots formed was different, it is clear that these values alone may not be sufficient for medium selection. Because of this, different BAP doses (1.0, 2.0, 3.0, and 4.0 mg L^{-1}) appear to be leading in this regard when the ratio value between shoots longer than 1 cm/shoots shorter than 1 cm was analyzed in terms of providing an idea. In other words, the formation of long shoots was observed more than the number of short and rosette-like shoots in the mediums with BAP concentrations. Within the scope of all these evaluations, it is possible to say that 3.0 mg L^{-1} BAP and 4.0 mg L^{-1} BAP mediums are preferable in terms of both total shoot formation and shoot-forming properties suitable for sub-culturing longer than 1 cm (Table 2).

3.2.2. Shoot lengths

In addition to the number of shoots obtained, it is important that they have reached a size that can be sub-cultured. For this reason, to determine the composition of the nutrient medium to be used, the lengths of the shoots formed in the 12th week were also measured. Different medium treatments were determined to be statistically significant in terms of shoot length values. The average shoot length was measured as the longest in 0.5 mg L^{-1} BAP medium and followed by other BAP treatments which were located in the same statistical group. The maximum

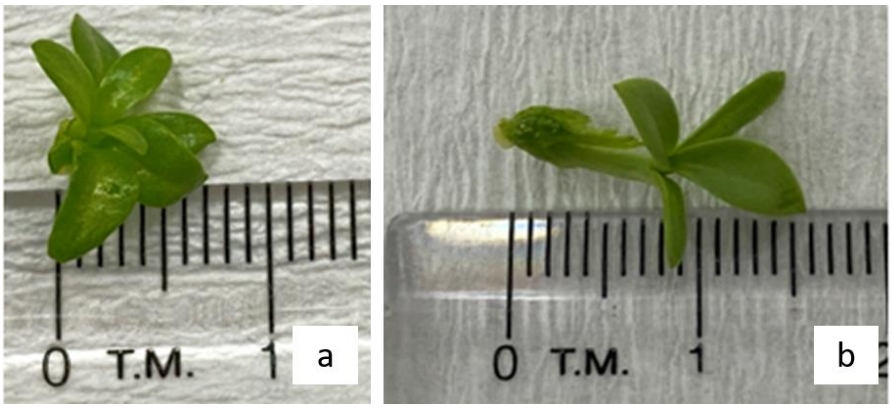

Figure 3. Twelve weeks after planting the leaf explants, the shoots shorter than 1 cm (a), and longer 1 cm (b).

Table 2. The average shoot number, number of shoots less than 1 cm in length, and number of shoots higher than 1 cm.

Plant growth regulators	Concentration (mg L^{-1})	The average shoot number	Number of shoots <1cm (A)	Number of shoots ≥1cm (B)	B/A ratio
BAP	0.5	9.5* ± 3.11	4.0 ± 1.71	5.5 ± 0.23	1.4
	1.0	8.7 ± 3.11	4.0 ± 1.71	4.7 ± 1.56	1.2
	2.0	7.2 ± 0.17	3.0 ± 0.33	4.2 ± 0.17	1.4
	3.0	14.9 ± 5.09	5.5 ± 3.92	9.4 ± 1.54	1.7
	4.0	14.5 ± 4.79	4.0 ± 3.19	7.5 ± 1.91	1.9
BAP+NAA	0.5+0.5	9.7 ± 1.01	4.5 ± 1.03	5.2 ± 1.01	1.2
	1.0+0.5	7.7 ± 0.47	7.2 ± 5.70	6.5 ± 5.69	0.9
	2.0+0.5	11.7 ± 0.39	6.4 ± 0.40	5.3 ± 0.00	0.8
	3.0+0.5	8.9 ± 4.46	6.3 ± 4.25	2.6 ± 0.75	0.4
	4.0+0.5	12.6 ± 5.03	5.5 ± 2.68	7.1 ± 2.54	1.3

*All mediums are non-significant.
BAP: 6-benzyl aminopurine, NAA: Naphthalene acetic acid.

Table 3. The average, maximum, and minimum shoot lengths.

Plant growth regulators	Concentration (mg L^{-1})	The average shoot length (cm)	Minimum shoot length (cm)	Maximum shoot lengths (cm)
BAP	0.5	0.63 a* ± 0.06	0.3	1.4
	1.0	0.58 ab ± 0.16	0.2	1.0
	2.0	0.56 ab ± 0.21	0.2	1.3
	3.0	0.51 ab ± 0.20	0.2	1.4
	4.0	0.59 ab ± 0.18	0.3	0.9
BAP+NAA	0.5+0.5	0.43 bc ± 0.15	0.2	0.8
	1.0+0.5	0.55 ab ± 0.09	0.2	0.8
	2.0+0.5	0.41 bc ±0.09	0.1	0.7
	3.0+0.5	0.30 c ± 0.10	0.2	0.8
	4.0+0.5	0.46 abc ± 0.12	0.2	0.9

* The average shoot lengths, Medium P<0.05, LSD: 0.2; CV: % 20.49.
BAP: 6-benzyl aminopurine, NAA: Naphthalene acetic acid.

shoot length was determined as 1.4 cm both in 0.5 mg L^{-1} BAP and 3.0 mg L^{-1} BAP mediums. The shortest average shoot length was measured in 3.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA medium and took place in a different statistical group (Table 3).

When the BAP cytokine was used in the study alone, it was able to successfully perform vascular meristematic tissue induction and subsequent shoot regeneration. The addition of a single dose of NAA (0.5 mg L^{-1}), used as an auxin source, was not observed to be a significant promoter of organogenesis, and even it was observed that it had some inhibitory effect. It was determined that there was some vitrification in the medium containing NAA. Therefore, the use of BAP alone was determined to be preferable for shoot regeneration and propagation from leaf explants. It can be observed from the literature that different types and doses of growth regulators are used for *in vitro* somatic embryogenesis or organogenesis in lisianthus (Kaviani, 2014; Pop et al., 2016; Yumbla-Orbes et al., 2020).

3.2.3. Sub-culture

Single node explants prepared from shoots longer than 1 cm formed the material for subculture. Images showing the development of single node explants in 5 different doses of BAP and the second 5 mediums combined with NAA are given in Figure 4. The results of average shoot number, number of shoots less than 1 cm in length, and number of shoots higher than were evaluated four weeks after the transfer of single node explants to 10 MS mediums with the same content as the one used at the beginning are given in Table 4.

The most effective medium in terms of the number of plants formed after subcultures were 3.0 mg L^{-1} BAP and 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA and were in the same statistical group in terms of the highest number of plants after subcultures (respectively 152 and 151 regenerated plants/10 plants).

For the number of plants formed under 1 cm in height, the highest value was observed in 1.0 mg L^{-1} BAP+0.5 mg L^{-1} NAA medium, while the lowest number of plants obtained under 1 cm in the treatment of 1.0 mg L^{-1} BAP medium. The number of plants below 1 cm was 80 with the use of 1.0 mg L^{-1} BAP+0.5 mg L^{-1} NAA, whereas the height of 71 plants was recorded above 1 cm. In terms of the number of plants over 1 cm in height, 3.0 mg L^{-1} BAP medium had the highest value, and 1.0 mg L^{-1} BAP medium followed this medium in a different statistical group (Table 4).

An average of 11.5 shoots/explants were obtained from the explants prepared as single node explants and sub-cultured. This number is above the previous studies. Uddin et al. (2017) determined that up to 3.23 new shoots developed from single shoots sub-cultured at 0.1-1.5 mg L^{-1} BAP and GA_3 combinations. It was thought that keeping the doses used lower as well as the genotype difference might have caused this effect. Yumbla-Orbes et al. (2020) reported that 10 µM 2,4-D (2,4-D dimethylamine salt) doses were optimal for somatic embryogenesis

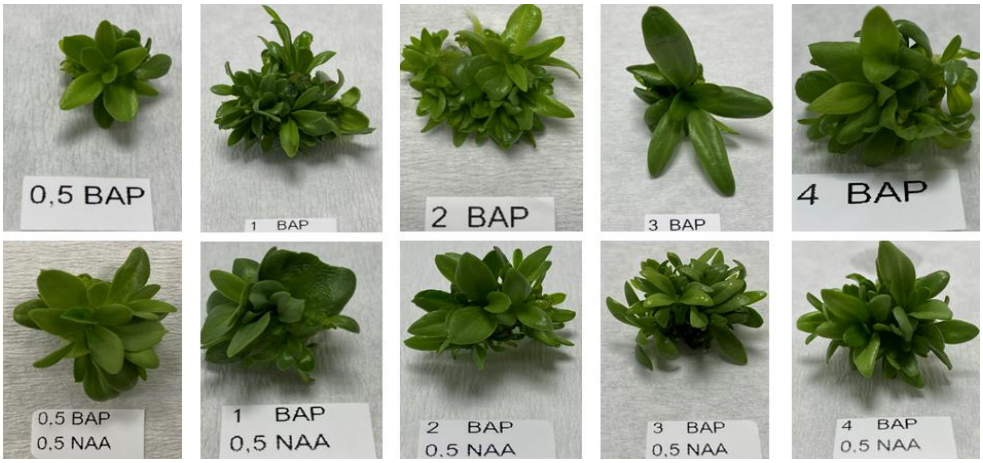

Figure 4. The appearance of 5 doses of BAP (6-benzyl aminopurine), and their second 5 series with 0.1 mg L^{-1} NAA (Naphthalene acetic acid.added) in lisianthus single node explants, 8 weeks after culturing.

Table 4. The average shoot number, number of shoots longer than 1 cm in length, and number of shoots shorter than 1 cm.

Plant growth regulators	Concentration (mg L^{-1})	Number of subcultured plants	Number of plants formed after subcultures	Number of plants <1cm	Number of plants ≥1cm
BAP	0.5	10	84 g* ± 2.52	39 e ±1.15	45 f ± 1.73
	1.0	10	110 d ± 1.53	23 f ± 1.15	87 b ± 1.00
	2.0	10	83 g ± 0.58	39 e ± 1.15	43 f ± 1.53
	3.0	10	152 a ± 1.53	56 c ± 5.51	95 a ± 4.16
	4.0	10	119 c ± 2.31	45 d ± 1.53	74 c ± 3.61
BAP+NAA	0.5+0.5	10	105 e ± 0.58	45 d ± 1.53	61 d ± 2.08
	1.0+0.5	10	151 a ± 2.00	80 a ± 0.58	71 c ± 2.08
	2.0+0.5	10	117 c ± 1.00	65 b ± 1.53	52 e ± 2.52
	3.0+0.5	10	94 f ± 0.58	68 b ± 1.53	26 g ± 1.00
	4.0+0.5	10	134 b ± 1.53	60 c ± 1.53	74 c ± 1.00

*Number of total plants formed after subcultures, Medium P<0.01. LSD: 2.6; CV: 1%; Number of plants <1cm, Medium P<0.01. LSD: 3.7; CV: 4%; Number of plants ≥1cm, Medium P<0.01. LSD: 4.1; CV: 4%.
BAP: 6-benzyl aminopurine, NAA: Naphthalene acetic acid.

induction from leaf explants and (2 µM BA; 2 or 4 µM mT (meta-Topolin)) doses for maturation of the resulting Embryos. Mousavi et al. (2012), applied BAP and GA_3 to the B5 Gamborg medium instead of the MS medium, and determined that the most appropriate dose was 1.0 mg L^1 BAP. Ghanati et al. (2012), compared LS, B5, and MS mediums, and reported that the most shoot regeneration was obtained in B5 medium containing 1.0 mg L^1 BAP and GA_3.

Kaviani (2014), used kinetin as a cytokinin in treatments with combinations with NAA, and stated that the best shoot regeneration was obtained from MS medium containing 0.5 mg L^1 kinetin. In a different study, they obtained callus and shoot organogenesis in MS medium containing a combination of 0.1 mg L^1 BA and 0.2 mg L^1 NAA (Kaviani et al., 2014). In our study, single node explants prepared from *in vitro* regenerated shoots were used in the sub-culturing stage and new axillary shoot development per explant could be achieved up to 15.2 shoots in the most suitable medium composition. Pop et al. (2016), successfully obtained axillary shoots from single node cultures. The low doses of PGR used in their study, resulted in a limited number of axillary shoots obtained. While in our study, like the regeneration numbers, the axillary shoot-forming capacity was also higher due to the relatively high doses used.

It is possible to multiply examples of shoot regeneration studies in lisianthus, but two points come to the fore here: The first is the basic nutrient medium composition in all studies; the lisianthus plant gives shoot regeneration even in different types and doses with PGRs, and the studies suggest a successful PGR combination of nutrient medium. This indicates that lisianthus has a high tissue culture regeneration potential and can form plants without being too selective in terms of nutrient medium and PGRs. Secondly, the reason why different results were obtained from different studies and the optimum composition came out as different suggestions is that it can be considered as genotype x application interaction. As a matter of fact, in our study, genotype reactions were different while the seeds germinated. In general, cytokinin (BAP) doses between 0.1-4.0 mg L^1 were applied in treatments and the best results of the study were obtained in environments with 3.0 mg L^{-1} BAP doses. Although there was an increase in the number of shoots shorter than 1 cm as the cytokinin doses increased with the combination auxins, it was observed that they also grew when the culture period was extended. The results obtained in our study also contributed to the literature in this respect.

The addition of GA_3 to a nutrient medium promotes the elongation of small shoots and it is

frequently used in *in vitro* propagation studies (Ozcelik, 2000). Mousavi et al. (2012) and Uddin et al. (2017) preferred the addition of Gibberellic acid for regeneration of lisianthus. It may be useful to conduct new trials by adding GA_3 to 3.0 mg L^{-1} BAP and 4.0 mg L^{-1} BAP treatments, which are approved by us. Thus, both the number of shoots will be increased and the number of developed shoots that can be separated more easily during the subculture of the shoots.

3.2.4 Rooting stage

The rooting experiment was started since a sufficient number of shoots were obtained in the subculture. For this purpose, 20 shoots longer than 1 cm were taken from each medium and transferred to MS and ½ MS mediums without adding any PGRs. After 4 weeks, it was determined that all the transplanted shoots were rooted. The rooting rate was 100% on both mediums. In fact, it was observed that rooting already occurred in some subculture mediums (especially in mediums containing NAA). There was no difference in rooting rates as a result of transferring lisianthus shoots to PGR-free ½ MS or MS mediums at the *in vitro* rooting stage. The rooting status of lisianthus shoots in MS and ½ MS medium is shown in Figure 5. As can be clearly seen from the image, there is no difference in rooting between the two mediums. Especially as the waiting time increased, the roots surrounded the bottom of the jar. Rooted shoots that had completed 4 weeks were ready for hardening and transferring to external conditions.

It was observed that lisianthus has no problems with rooting and even rooting occurs in environments that are kept for a long time during the shoot propagation stage. However, rooting experiments were established, and results were obtained. As a result of this research, rooting occurred in both used mediums. Rooting medium using full-strength MS salts gives positive results in most plant species (Németh, 1986). However, some conditions where macro and micro elements are diluted in ½, $^1/_3$, or ¼ ratio can sometimes give better results for rooting (Skirvin et al., 1980; Lineberger, 1983). Uddin et al. (2017) also noted that PGR-free ½ MS medium is a suitable choice for rooting in lisianthus.

Lisianthus has been observed as a plant species that responds quickly to *in vitro* applications and does not require intensive doses of PGRs. This is also evident in the rooting phase and plain medium with low strength resulted in healthier plantlets. Both MS and semi-strength PGR-free MS mediums were observed to be successful since 100% rooting occurred in both mediums without any difference in rooting rates.

3.2.5. Aclimatization stage

Rooted lisianthus plantlets were planted in the peat: perlite (2:1) mixture and covered for 10 days. The plantlets, which were gradually acclimated to atmospheric conditions, continued to live in a healthy way at the rate of 100% and were transferred to the greenhouse. Just like the seedlings transferred to the outdoor conditions by transferring to the pots, the seedlings, which were adapted to the external conditions for 5 days by transferring directly to the viols in the greenhouse, continued their vitality without any loss. In addition to being a species that responds positively to tissue culture and can be easily reproduced, lisianthus has proven to be a plant with a strong adaptability with its high survival rate during the acclimatization phase to external conditions.

Peat: perlite (2:1) mixture was determined suitable for the transfer stage to external conditions. No infection was encountered and all plants developed healthily. Kabakci (1996), reported that the lisianthus seedlings obtained under *in vitro* conditions were first acclimatized to external conditions in the pumice medium and then transferred to the soil medium. At this stage, an impression was formed that different applications can be made on *ex vitro* rooting experiments.

The *in vitro* lisianthus shoots can be rooted at the same time during the transfer of external conditions in subsequent studies. Rooting was also

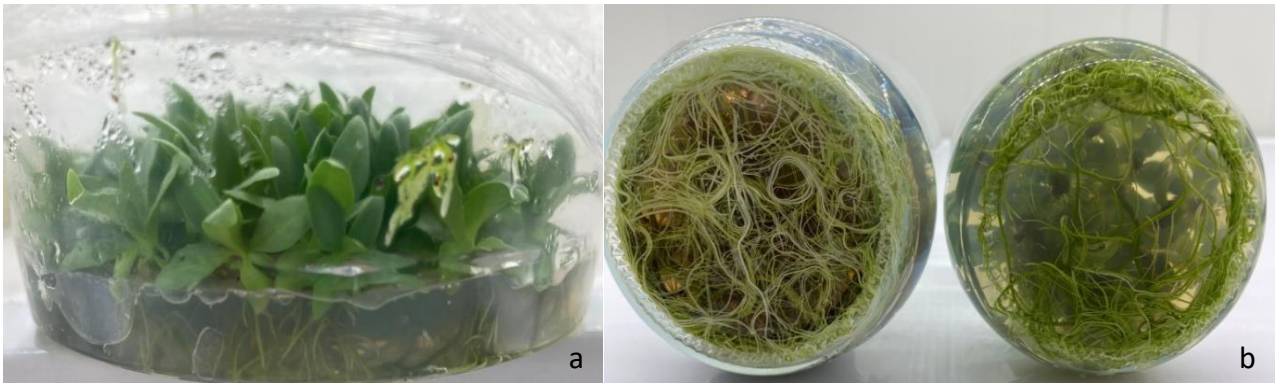

Figure 5. Formed roots of lisianthus shoots, 6 weeks after transferring to rooting mediums (a) and after 2 months to MS (left jar) and ½ MS (right jar) medium (b).

successfully achieved in ex vitro rooting experiments. Moreover, 87% of rooting occurred in the float hydroponic system. Among the shoots transferred directly to the soil mixture, 91% were rooted. It may be useful to address this issue in future studies. Thus, it seems possible to provide a more economical production cycle by reducing the time spent in *in vitro*. For the first time, Clapa et al. (2011), induced roots successfully and acclimatized the rooted shoots to external conditions. Ibrahim (2022), reported that *ex vitro* rooting was not obtained in *Pyrus* sp., while, using the float hydroculture technique, *in vitro* plantlets were successfully acclimatized in 4 weeks. Bejaoui (2022), achieved 100% rooting and acclimatization when transferring *in vitro Kalanchoe* shoots to the floating system. According to the study, *ex vitro* rooting enables tissue culture methods to be more affordable and allows for faster transportation of plantlets to greenhouses. Figure 6 shows lisianthus plantlets rooted in the float hydroponic system and Figure 7 directly transferred to the soil and also acclimatized. The seedlings that developed in our study were transferred to larger pots and grown in the greenhouse in Antalya (Figure 7).

4. Conclusions

Eustoma grandiflorum is a species prone to proliferation by tissue culture. Leaf explants are available for tissue culture propagation. When the appropriate medium is selected, direct shoot regeneration occurs after callus development on the cutting surfaces. Leaf explants achieved shoot differentiation in all mediums. It has been demonstrated that the leaf explants can be used successfully in the tissue culture of lisianthus when it comes to applications for organogenesis.

When the cytokinin and auxin structure is well adjusted, both shoot differentiation can be obtained as a sufficient number of starting materials, they can also benefit from each other, and shoots longer than 1.0 cm can be obtained. For the superficial sterilization of seeds, 20% commercial sodium hypochlorite can be used and after waiting for 15 minutes, it should be rinsed 3 times with sterile distilled water. There was no contamination problem because sterile seedlings were used as the explant source. The PGR-free MS medium used as the control medium, and no organ development took place. There was only swelling in the tissues and very slight swelling on the cut surfaces. There are PGR needs for *in vitro* regeneration. It can be concluded that MS medium containing 3 and 4.0 mg L^{-1} BAP could be a good starting medium for regenerating new lisianthus shoots from leaf explants. These treatments had the highest values both in shoot formation and the number of shoots longer than 1 cm. Although the number of shoots increased in high induction of NAA together with BAP, it was difficult to separate the regenerated shoots from each other during the sub-culturing phase, and they tend to develop in the form of bushes. In leaf explants, shoots begin to appear in the 6^{th} week, and 10-15 shoots per explant can be obtained at the end of the 8^{th} week in the above-mentioned mediums. Counts were made on 10 shoots in the subculture stage and it was aimed to reduce the standard error statistically.

Figure 6. Lisianthus shoots in *ex vitro* rooting and acclimatization periods (a), shoots placed for rooting in float hydroponic system in styrofoam viols (b), roots developed in water (c), healthy rooted lisianthus plantlets in the hydroponic culture, (d) lisianthus plantlets after 10 days of transfer to the soil mixture (in the greenhouse).

Figure 7. Lisianthus shoots in *ex vitro* rooting and acclimatization periods (a), transferring shoots to the soil mixture directly (b), *ex vitro* rooted lisianthus shoots (c) lisianthus plants after hardening in larger pots and grown in the greenhouse.

The highest number of shoots (152 shoots/10 explants) and shoots longer than 1 cm (95 shoots/10 explants) were obtained from 3.0 mg L^{-1} BAP medium. On the other hand, shoot number was also high in 1.0 BAP+0.5 NAA (151 shoots/10 explants) medium, and the number of shoots shorter than 1 cm got the maximum number (80 shoots/10 explants). It was determined that the 3.0 mg L^{-1} BAP alone can be used for both organogenesis from leaf explants and shoot proliferation in single node culture. *In vitro* shoots of the lisianthus species are easily rooted and both PGR-free MS and ½ MS mediums can be used for rooting at a 100% rate.

Peat and perlite mixture was determined suitable for acclimatization to external conditions. In the climate room, adaptation to external conditions was achieved easily under controlled conditions, and acclimatization was achieved quickly and successfully in plantlets planted in viols in less controllable conditions in the greenhouse. The survival rate was 100% in all treatments. The plants were grown healthy in the greenhouse.

The float hydroponic system and transfer to the soil mixture directly were both successful methods to obtain the rooted and the acclimatized shoots at the same time. Due to the continuation and improvement of studies on them, both techniques have the potential to be effective *ex vitro* rooting and acclimatization strategies for lisianthus or other readily rooted ornamental plants. It was revealed that the results obtained from the research can be used in breeding studies such as *in vitro* mutation and genetic transformation. This application can serve to accelerate the long cycles of ornamental plants.

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