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DIRECT AND INDIRECT ORGANOGENESIS FROM LEAF EXPLANTS OF LISIANTHUS [*Eustoma grandiflorum* (Raf.) Shinn.]

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ABSTRACT

A highly efficient protocol for the regeneration of plantlets of lisianthus (cut flowers plant) was established via direct and indirect organogenesis induced from leaves explants. In direct pathway, a reliable strategy for mass propagation of shoots was devised after testing several concentrations of BA with Murashige and Skoog (MS) media, it was confirmed that the use of MS medium supplemented with 2 or 3 mg.L^{-1} produced the highest average number of shoots (13.8 and 14.2 respectively). The shoots were easily rooted on MS medium containing 2 mg.L^{-1} of indole-3-acetic acid (IAA) and high percentage of survival rate (80%) was achieved. In the second pathway (indirect organogenesis), leaves explants were cultured on MS medium supplemented with NAA at 0,3, and 4 mg.L^{-1} for callus induction. Results appeared the maximum formation of callus 11.2 mm (in diameter) was recorded on a medium containing 4mg.L^{-1}. Shoots proliferation was achieved using MS medium enriched with GA3 at 0.5 mg.L^{-1} +BA at 1.5 mg.L^{-1} as it was vigorous and produced the highest average of shoot numbers (23) and shoot length (3.6 mm) on callus tissue but hyper hydric shoots were observed. The root formation was occurred on MS medium devoid of growth regulators. Based on the results of our experiments, the direct pathway protocol should be useful for the rapid propagation of lisianthus plant, as good quality and quantity of shoots proliferation were obtained as well as it produced healthy plants that could still live in the field to produce one of the most beautiful cut flowers in the world.

Keywords: Propagation, Lisianthus plant, Callus tissue, organogenesis.

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INTRODUCTION

Lisianthus (*Eustoma grandiflorum* (Raf.) Shinn), is a member of the Gentianaceae family [20 and 32]. It has other common names, which are Texas bluebell, prairie gentian and nandini [29]. It is becoming one of the most highly prized cut flowers [22]. Lisianthus known for beautiful flowers of different colors and for having a long vase life [27]. Available colors are blue, purple, plum, white, pink, and bicolours. It can be used either as cut flowers or as flowering pot plants [17]. It can grow to 50-75 cm in height and produces 20-40 flowers per plant. In nature, Lisianthus initially forms a rosette and grows very slowly during the winter. The stems elongate in the spring, and flowers appear in the summer [32]. However, slow germination and growth Roni al et.[33] make sexual propagation by seeds a complicated and difficult exercise. The clonal propagation of lisianthus, especially through a tissue culture technique, could therefore provide a useful alternative to sexual propagation [18]. Multiplication of selected clones through asexual aseptic culture would be valuable, as it would enable the mass propagation of selected individual plants with desirable characteristics, such as high productivity, a dwarf habit, disease resistance, or a faster growth rate Semeniuk [34] larger blooms in a wide color range, double flowers, heat tolerance, different flower shape and size as well as lack of rosetting etc. [10].

The limited availability of high-quality and uniform seedlings as planting material can be controlled by mass propagation of the plant on a commercial scale that satisfies market demands [2]. A micropropagation is a powerful tool for the large-scale propagation of ornamental plants [7]. However, there are several factors, (including genotype, explant type, source, physiological condition, culture conditions, media, and plant growth regulators), that can determine the success or failure of the micropropagation method [8]. In vitro propagation can be effectively applied for rapid proliferation using different types of explants such as: stems, axillary buds, leaves and meristems in order to accomplish homogenious material for increased high-quality yields in large-scale production [27]. Among various lisianthus plant parts, the use of leaf explants was best for the production callus tissue [30].

The objective of the present study was to optimize the concentration of different plant growth regulators as well as the response of leaf explants in different media for in vitro multiplication of lisianthus plants using direct and indirect organogenesis via callus tissue formation.

MATERIALS AND METHODS

Plant materials preparation

These experiments were conducted at the tissue culture laboratory of the Iraqi Ministry of Agriculture which belongs to the Plant Protection Office. Super magic hybrid (capri blue picotee) seeds provided by the Sinarya Ornamental Seeds Company of Japan. Seeds were initially surface sterilization inside petri dish containing 50% solution of sodium hypochloride (6% NaOCl), with a few drops of Tween-20 for 5 min, followed by triple rinsing with sterile distilled water (SDW) (5 minutes for each rinse). Water was then poured into an empty flask and the seeds were placed on MS medium [21] (HIMEDIA, PT011-25L) free of hormones. The PH of the medium was adjusted to 5.7 [13] before adding agar (7 g. L^{-1}) and then autoclaved at 1.05 kg/cm², 121°c for 20 min. Ten seeds were placed in each jar. Three jars were used for each treatment. The cultures

were incubated at 25±1 °c under an illumination of 1200 Lux during 16/8 h photoperiod obtained from fluorescent lamps. After four weeks the seeds started to germinate. Previously, successful micropropagation of lisianthus was reported using axiallary buds kaviani et al. [14] and shoot tips (7and 24). Also, some studies were on leaf segments [6،28]. Thus, we focused in this paper on leaf segments to evaluate the ability of this explant for mass propagation *in vitro*.

Direct organogenesis experiment

Shoots multiplication

After two months from seeds culture, Young leaves were isolated carefully using forceps and tissue culture blades. The explants (5 mm) were then cultured in MS medium supplemented with different concentrations of BA (0, 2, 3 mg L^{-1}) (13&34) and 30 g. L^{-1} of sucrose. The PH of the media were adjusted to 5.7. The solidification of culture media was done using 7 g. L^{-1} of agar. The number of shoots per explant and the length of the shoots were recorded after one month of culture.

Rooting induction and acclimatization

Proliferated shoots were cut individually (5mm in length) and cultured in rooting media. The rooting media applied in this experiment were MS medium supplemented with various concentrations of IAA (0, 0.5, 1, and 2 mg. L^{-1}) which were chosen on the basis of our own studies done previously, 30 g. L^{-1} of sucrose and solidified with 7 g.L^{-1} of agar. The periodical observation was applied to investigate the root formation process. The number and length of roots were recorded after one month of culture. Well-developed plantlets were removed from the agar medium, washed the roots of plantlets under running tap water to remove agar from them, [35]. Plantlets were then cultured in plastic pots containing a mixture of peat and soil (Local market) (1:1) which was sterilized using an autoclave and acclimated gradually to room temperature. Finally, acclimatized plantlets were transferred to a greenhouse, and watered regularly with a sprinkler for a week using 1/2 MS strength. Further, they were watered twice a day with the help of a water cane, and the survival percentage was recorded after four weeks of transferring to the greenhouse [16].

Indirect organogenesis experiment

Callus initiation and shoots regeneration

For callus induction, Leaf segments at 5 mm were cultured on MS basal medium supplemented with different concentrations of NAA (1-naphthaleneacetic acid) (0, 3, and 4 mg.L^{-1}) which were chosen on the basis of our own studies done previously, at the rate of four pieces in each jar (Fig.1). The media were provided with 30 g.L^{-1} of sucrose and solidified with 7g.L^{-1}. The cultures were incubated for one month. The observation of the growth of callus tissue was recorded. After that, Well-proliferated callus tissue derived from the leaf segments were used for regeneration studies. Green callus tissue was cut into pieces of 10 mm in diameter and transferred into control media (MS free of hormone), (0.5 mg.L^{-1} GA3+1.5 mg.L^{-1}BA) and (0.5 mg.L^{-1}GA3 + 1.5 mg.L^{-1} Kin) (20).

Figure 1: Leaves explants of lisianthus plant that were used for callus induction.

Rooting of plantlets:

The shoots (5mm) that produced from previous experiment were used for rooting. Explants were cultured on two various media (MS free of hormone and MS supplemented with 1 mg.L^{-1} of IBA) [16]. Observations on rooting process were recorded after one month of culture.

STATISTICAL ANALYSIS

One-way experiments were conducted using randomized completely block design (RCBD). (10 replicates, each experiment was repeated three times). The data were statistically analyzed using the statistical program Genstat. Means were compared using the Least Significant Difference (LSD) test at a probability level of 5%.

RESULTS AND DISSCUSSION

Direct organogenesis experiment

Shoots multiplication

The suitability of medium and explant type has an important role in obtaining high morphogenic results in the *in vitro* culture of plants [8]. The ideal concentrations differ from species to species and need to be accomplished accurately in order to achieve effective rates of multiplication [9]. According to our results, cytokinin is able to promote shoot multiplication in Lisianthus. It was observed that shoots regeneration on medium containing BA at both concentrations 2 and 3mg.L^{-1} were superior, and differed significantly from the control (without hormones), as the values of average Shoot numbers were (13.8 and14.2) respectively. These shoots were similar in average length values compared to the control (Fig 2&3). In agreement with our finding, similar results were reported in direct organogenesis of both genotypes of Alstroemeria (Caryophyllaea and Sweet Laura) using aerial explants [3]. The culturing on MS medium supplemented with 2 or 3 mg.L^{-1} of BA was optimal for shoots regeneration of Lisianthus. This result was in contrast with that reported by Xue-hua et al. [37] on micropropagation of Lisianthus, who referred that MS medium supplemented 0.1-0.5 mg. L^{-1} BA along with 0.05 mg. L^{-1} NAA was suitable for adventitious shoot differentiation. In accordance with our finding, Ordogh et al. [23] indicated that reduction of shoot numbers in echo cultivars of Lisianthus occurred when cultured on free-BA medium.

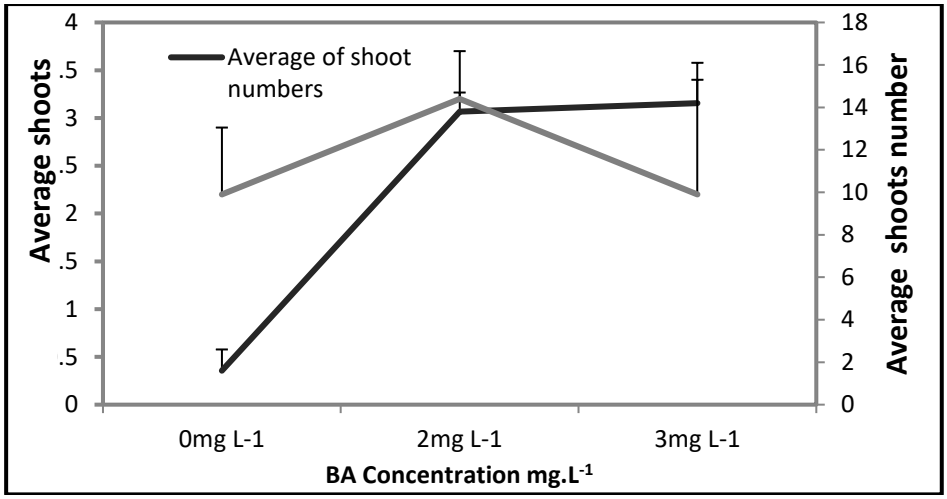

Figure 2: Effect of different concentrations of plant growth regulator on average shoot numbers and lengths. LSD=7.7 for shoot numbers and LSD=2.1 for shoot lengths.

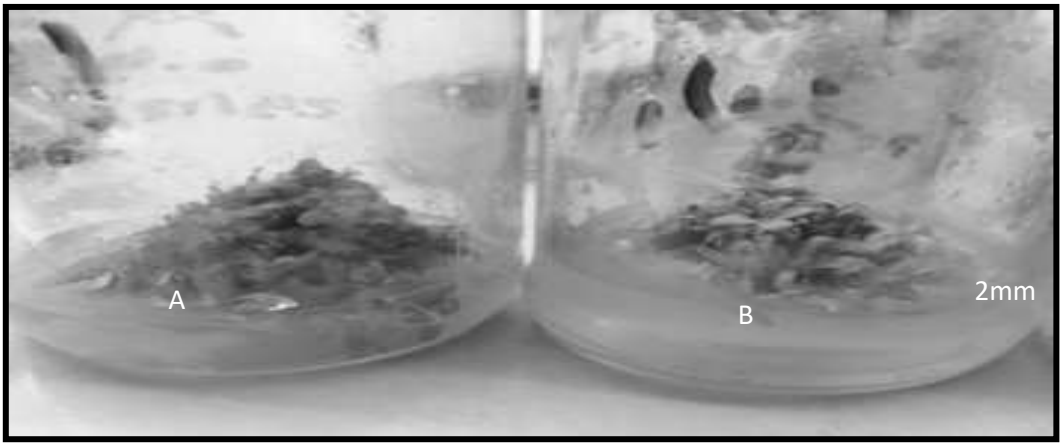

Figure3: Effect of BA concentrations on multiplication of Lisianthus shoots. A) The multiplication of shoots on BA with 2mg. L^{-1}. B) The multiplication of shoots on BA with 3mg. L^{-1}.

Rooting of explants and acclimatization

Auxins promote root induction, germination and seedling growth of many species [12 and 38]. In *Eustoma grandiflorum*, the addition of auxin is necessary for root induction [15]. In this paper, the best root formation was observed on medium supplemented with 2 mg.L^{-1} of IAA. While, observed that the concentrations were raised from 0.5 mg. L^{-1} to 2mg.L^{-1} caused an increase in the number and length of roots (*P*< 0.001) (Fig.4). High average of root numbers (6.4 per plant*let*) and good root lengths (5.8 mm) was observed on media with 2 mg.L^{-1} of IAA (Fig. 5 A). In accordance with our findings, Semeniuk and Griesbach (34) referred the successful *in vitro* rooting of Lisianthus shoots on media provided with IAA at 2 mg.L^{-1} but on half strength of MS. Rahaman [29] reported that the highest percentage of shoots responding to roots is (100%) in medium containing 2 mg.L^{-1} IAA. Contrary to our results, Damiano et al. [5] referred that the best rooting of *in vitro* raised shoots on half strength MS medium containing 1 mg. L^{-1} of IAA. However, MS medium supplemented with 0.1 mg. L^{-1} IAA was reported to be more suitable for rooting of Lisianthus plantlet [6]. Also, optimum rooting was observed by adding IAA or IBA at a

concentration of 10 mg.L^{-1}, IAA induced a smaller number of long roots, whereas a larger number of short roots was recorded on medium with IBA [26].

However, no root formation was recorded on a medium devoid of growth regulator. Acclimatization can be considered a critical stage that is often associated with slow growth and significant plant loss [36]. In the current study, the well-rooted shoots were successfully transferred to *ex vitro* conditions into a mixture of peat and soil, and the survival rate was 80% (Fig.5 B).

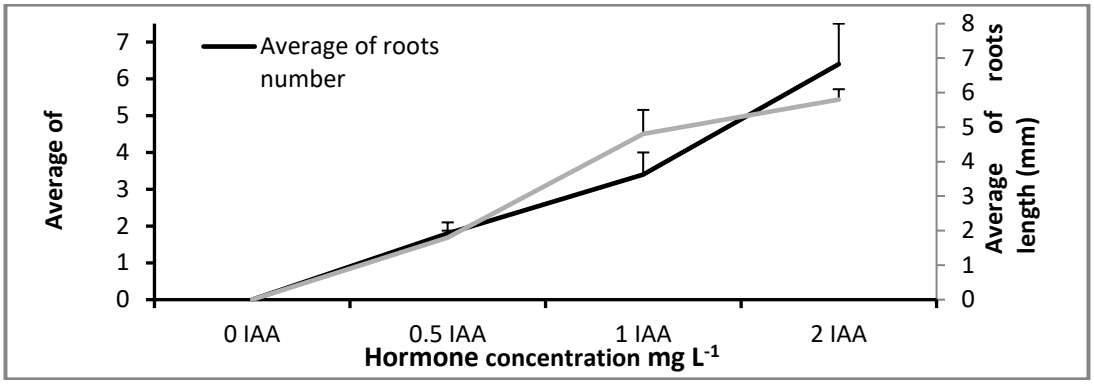

Figure4: Effect of IAA concentrations on average root numbers and lengths. LSD= 2 for root numbers and LSD= 1.2 for root lengths.

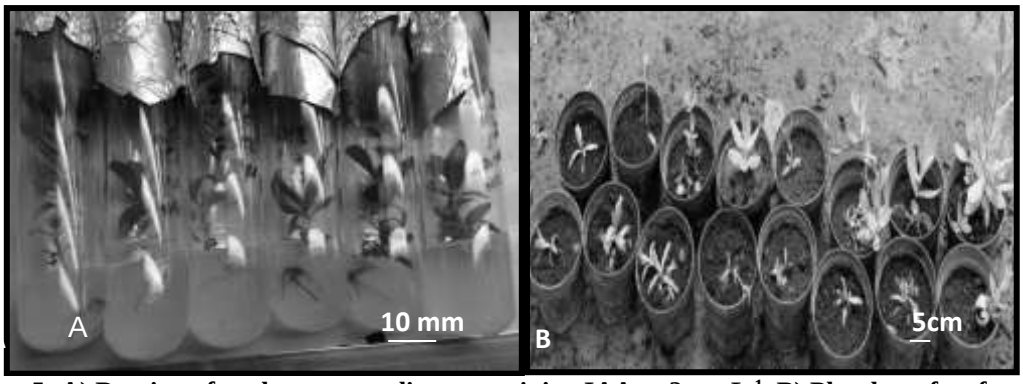

Figure5: A) Rooting of explants on medium containing IAA at 2mg. L^{-1}. B) Plantlets after four weeks which grown in greenhouse.

Indirect organogenesis experiment

Callus induction in Lisianthus

Callus induction and plant proliferation were obtained in lisianthus using various explants like leaves [20]. Auxins are applied to induce cell division, cell elongation, vascular tissue differentiation, rhizogenesis and root formation, embryogenesis and inhibition of axillary shoot growth [4, 8 & 25]. 2, 4-D is commonly used for callus induction but NAA and IAA are also used [8]. Among three applied concentrations of NAA, medium supplemented with 4 mg.L^{-1} was the best for yield of a good callus tissue. The whole surface of the leaf explants was covered with a big mass of callus within four weeks. Based on morphological appearance, callus tissues were friable and green. The highest average of callus diameter (11.2 mm) was recorded on this medium (P<0.001). It was observed that compact and green callus tissue was accomplished on medium with concentration of 3 mg.L^{-1}. In terms of response percentage of explants, the explants on 3 and 4 mg L-1 appeared high percent (94%) compared with the control (without hormone) which was (10.5%) (Fig 6&7). Our findings revealed that using of NAA proved to be

efficient for the callus induction after one month of culture. This result is in accordance with [1, 7, 19 & 20] who referred that most callus formation in lisianthus was on medium containing NAA. Also, [26] revealed that NAA is a strong auxin, callus formation takes place when high concentration was applied. However, it was found that light condition which enhance higher rate of chlorophylls synthesize could be the possible reason behind the green color of callus tissue [39]. The same results were reported in lisianthus plant by [20]. Several studies had been reported regarding the impact of plant growth regulators on callus growth of lisianthus.

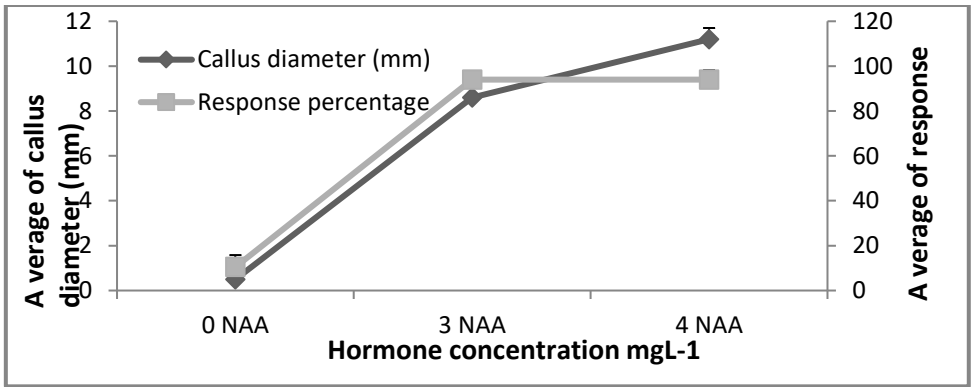

Figure 6: Effect of NAA concentrations on averages of callus diameter and response percentage. LSD= 1.3 for callus diameter and LSD= 12.5 for response percentage.

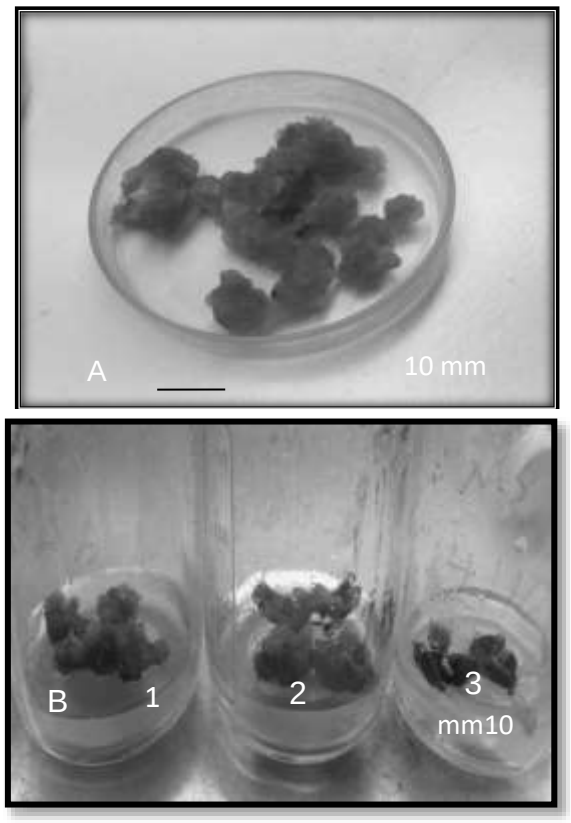

Figure 7: Effect of NAA concentrations on initiation of callus tissue that derived from leave explants in Lisianthus. A. Callus tissue induced on medium with 4 mg.L^{-1}. B. Induction of callus tissue 1. Medium provided with 4mg.L^{-1}. 2. Medium provided with 3 mg.L^{-1}. 3. Medium without hormone.

Shoot regeneration from callus cultures

In current study, regeneration started with the appearance of green dots on one-month-old callus cultures derived from leaf explants, generally produced stem and leaves (Fig.8). Proliferation of shoots occurred in these calli after one month of culture. The results showed that the medium provided with 0.5 mg.L^{-1} of GA3+ 1.5 mg.L^{-1} of BA was most effective for obtaining the highest shoot numbers [23] and shoots length (3.6 mm) compared to medium containing 0.5 mg.L^{-1} of GA3+ 1.5 mg.L^{-1} of Kin which produced average shoot numbers (8.4) and shoot lengths (1.2 mm) and differed significantly (P< 0.001) from the control treatment which produced average of shoot numbers (5.6) and average of shoot lengths (1.6 mm) (Fig.9). However, increasing the concentration of BA and decreasing of apical dominant, was the reason behind of decreasing the length of shoots. On the other hands, GA3 increases the length of shoots [35]. The same concentrations were reported by Mousavi et al. [20] in Lisianthus and led to produce fewer adventitious shoots regenerated (7.6 shoots/ explant). However, our results referred that the verification phenomenon were observed on regenerated shoots as hyperhydric shoots were formed. The same results were obtained in Lisianthus by Paek [24] who reported that using high concentration of BA and Kin resulted in hyperhydric shoots especially BA which produced shoots with high percentages of hyperhy dricity than those on Kin.

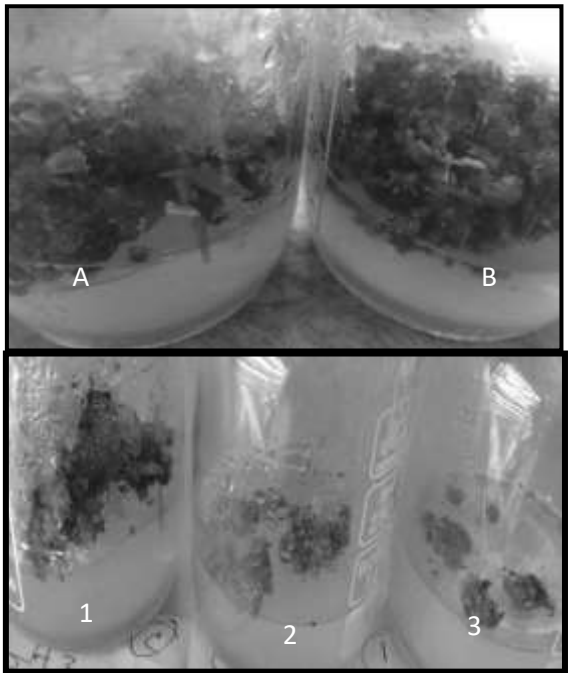

Figure 8: Effect of different combinations of GA3 with (BA and Kin) on proliferation of Lisianthus shoots from callus tissue after one month of culture. A) Proliferation of shoots on medium supplemented with GA3 at 0.5 mg.L^{-1} + BA at 1.5 mg.L^{-1}. B)1. Proliferation of shoots on medium supplemented with GA3 at 0.5 mgL^{-1} + BA at 1.5 mg.L^{-1}. 2. Proliferation of shoots on medium supplemented with GA3 at 0.5 mg.L^{-1} + Kin at 1.5 mg.L^{-1}. 3. Proliferation of shoots on medium without hormone (control).

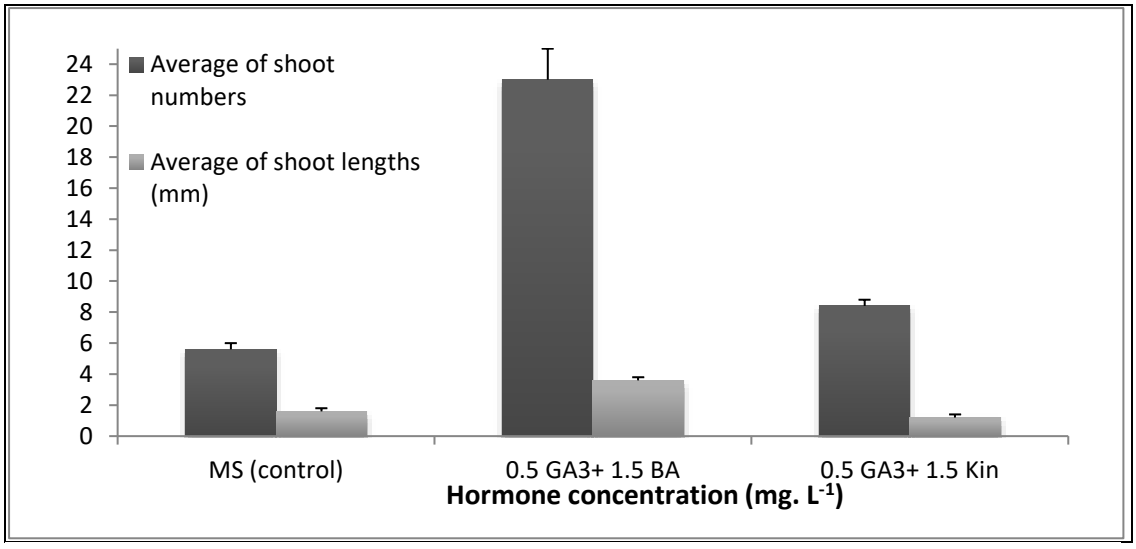

Figure 9: Effect of proliferation media on average shoot numbers and lengths after one month of culture in Lisianthus. LSD= 3.6 for average of shoot numbers and LSD= 0.7 for average of shoot lengths.

Rooting of shoots

The rooting stage prepares the propagated plants for transferring from *in vitro* to *ex vitro* conditions in controlled environment rooms after that, in the glasshouse and later, to their final location in the field. In this stage, including the rooting of shoots as well as the potential for acclimatization and survival during transplanting can be increased [11]. The results indicated that the root formation was observed on MS medium devoid of growth regulator with a percentage of (30%) while no root formation was observed on medium supplemented with 1 mg.L^{-1} of IBA. In this respect, research is still needed to improve the ability of shoots for rooting.

CONCLUSION

These protocols paper incorporated technical, practical, and horticultural aspects relevant to the efficient direct and indirect regeneration from leave explants of lisianthus. Overall findings of the present study are significant in obtaining good shoots proliferation directly from leave explants using BA and the maximum Callogenesis with NAA. We have developed a method for propagation by using GA3 and BA.

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تكوين الاعضاء المباشرة وغير المباشرة من الاجزاء الورقية لنبات Lisianthus [*Eustoma grandiflorum* (Raf.) Shinn.]

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الملخص

تم التوصل الى بروتوكول لإكثار نبات ازهار القطف **Lisianthus** وبكفاءة عالية وذلك عن طريق تكوين الاعضاء بصورة مباشرة وغير مباشرة باستخدام الاجزاء الورقية. في الطريقة المباشرة، تم اتباع إستراتيجية موثوقة للإكثار الخضري الواسع لتكوين الافرع وتضاعفها بعد اختبار تراكيز عديدة من **BA** مع الوسط **MS** التي اكدت ان استخدام وسط **MS** المجهز ب 2 او 3 ملغم/لتر من **BA** اعطت اعلى معدلاً لعدد الافرع (13.8و 14,2على التوالي). جذرت الافرع الناتجة بسهولة على وسط **MS** الحاوي على 2 ملغم/لتر من **IAA** وحققت نسبة بقاء عالية (80%). في الطريقة الثانية التي يتم فيها تكوين الاعضاء بصورة غير مباشرة، زرعت الاجزاء الورقية على وسط **MS** المدعم ب **NAA** وبالتراكيز0, 3 و4 ملغم/لتر لاستحثاث نسيج الكالس. تم تسجيل اعلى معدلاً لتكون نسيج الكالس تم تسجيله بقطر 11.2 ملم على الوسط الحاوي على 4 ملغم/لتر. اما تضاعف الافرع فقد تحقق باستعمال وسط **MS** المجهزب **GA3**عند التركيز 0.5 ملغم / لتر +**BA** عند التركيز 1.5 ملغم/لتر لأنه تميز بإعطاء اعلى معدل لأعداد الافرع (23) واطوال الافرع (3.6ملم) على نسيج الكالس المتكون ولكن لوحظ وجود حالة التزجج على الافرع المتكونة. تحققت عملية التجذير للأفرع على وسط **MS** الخالي من منظمات النمو النباتية. بناءً على نتائج تجاربنا، فان استخدام الطريقة المباشرة ستكون ذات اهمية كبيرة في الاكثار السريع لنبات **Lisianthus**، إذ أمكن الحصول على تضاعف جيد لهذا النبات كماً ونوعاً وانتاج نباتات صحية قادرة على الاستمرار في الحقل لإنتاج واحدة من أجمل ازهار القطف في العالم.

الكلمات الدالة: الاكثار الدقيق، نبات Lisianthus، نسيج الكالس، تكوين الاعضاء.

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