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IN VITRO PROPAGATION OF LISIANTHUS PLANT (*Eustoma grandiflorum*) UTILIZING PHYTOHORMONES AND PERIODICAL SUBCULTURES

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ABSTRACT

Successful proliferation of adventitious shoots via periodical subcultures in Lisianthus plant was achieved. This study showed the essential role of Kinetin (Kin) as a single exogenous plant growth regulator on the micro-propagation of Lisianthus. At the shoots production phase, the highest average of shoot numbers (5.4) was achieved on Murashige and Skoog (MS) medium supplemented with 3 mg.L^{-1} of Kin. During periodical subcultures, shoots multiplication increased gradually up to the fourth subculture. The highest average of shoot numbers (9) was observed at the third subculture and the highest shoots length (6 cm) was recorded at the fourth subculture on MS medium containing 2 mg.L^{-1} of Kin Shoots rooted easily on MS medium containing 1 mg L^{-1}mg L^{-1} of IBA. The plantlets derived from this procedure were successfully acclimatized in a mixture of soil and peat moss (1:1, v/v) with 80% of survival percentage. The reported results have a high practical value in the field of Lisianthus micropropagation.

Keywords: In vitro culture, Lisianthus, Micropropagation, Periodical subcultures, Single growth regulator.

INTRODUCTION

The importance of lisianthus (*Eustoma grandiflorum* (Raf.) Shinn) (Gentianaceae) comes from its garish flowers and excellent post-harvest life [21], which extends from 3 to 6 weeks [4]. This plant has also many other traits desirable features such as, uniform flowering throughout the year, flower color, flower size and form including double flowers [8]. It is a very popular, ornamental plant [25]. Moreover, the plant is tolerant to pathogen, high temperature stresses and soil acidity conditions [24].

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The use of sexual method for propagation of Lisianthus plants is hampered by cross pollination and it is not efficient because of a low rate of seed germination which is estimated 34-39% [2, 17 and 24]. In some cultivars, seed-derived plants, show wide variation because of their heterozygous character and need at least 4.5 months or more to reach the flowering stage [6]. Thus, vegetative propagation has been suggested as an effective alternative method of the sexual approach [16]. Especially through in vitro culture which might provide a useful alternative to seed propagation that results in the production of better-quality planting stock [15] and the production of a large number of plants in a limited place and a short period of time [12]. Micropropagation using shoot tips, internodes stem sections, leaf segments, axillary buds, and root explants of Lisianthus through *in vitro* culture method have been previously applied in modified MS medium [11, 12, 25, and 30]. Tissue culture studies working on the micro-propagation by using different types of plant growth regulators were accomplished [21]. However, there are no many papers dealing with the propagation of Lisianthus using one type of exogenous plant growth regulator. Thus, the objective of the present study was to evaluate the influence of different concentrations of Kin as a single plant growth regulator on the production and regeneration of shoots via periodical subcultures. The current study optimized the whole process of Lisianthus micropropagation including regeneration, multiplication through subsequent culture, rooting and acclimation.

MATERIALS AND METHODS

Explants establishment

The experiment was conducted at the tissue culture laboratory of the Horticulture office of the Iraqi Ministry of Agriculture. Super magic hybrid (capri blue picotee) seeds of were provided by the Sinarya Ornamental Seeds Company (imported from Japan). The seeds were sown in controlled conditions (25±1°c under an illumination of 1200 Lux during 16/8 h photoperiod obtained from fluorescent lamps). Seeds were surface sterilization with 50% solution of sodium hypochloride (6% NaOcl) containing a few drops of Tween-20 for 5 min. This was followed by rinsing them three times in sterile distilled water (5 min for each rinse). The medium for germination was MS (18) (HIMEDIA, PT011-25L) supplemented with 30 g L^{-1} of sucrose and 7g.L^{-1} of agar. The pH media was adjusted to (5.7). One-month-old seedlings were then used for shoots proliferation.

Shoots regeneration

Shoot tip explants (0.5 cm in length) were prepared by cutting the seedlings. The explants were cultured on MS medium enriched with various concentrations (treatments) of Kin (Sigma-Aldrich) (0, 1, 2, 3, 4 and 5 mg.L^{-1}) and supplemented with 30 gL^{-1} of sucrose and 7gL^{-1} of agar (Sigma-Aldrich). Ten replicates per treatment were used. After one month of culture, the data for average shoot numbers and lengths were collected.

Multiplication via subcultures

Shoot tip explants (0.5cm in length) obtained from the previous experiment were planted on MS medium with different concentrations of Kin (0, 2, and 3) mgL^{-1}. The aim was to study the multiplication of explants via subculture periods

for four months (one month for each subculture). One explant was used for each jar with ten replicates for each treatment. Periodical observation was carried out every month to check the response of shoots growth under Kin concentrations. Parameters observed in the current study were averages of shoot heights (cm) and numbers of proliferated shoots per explants.

Rooting of regenerated shoots

Shoots (6 cm in length) were transferred to rooting media, which consisted of MS medium with IBA (0, 0.5, 1, 2 mgL^{-1}). For each treatment, ten replicates were used, with one shoot per jar. Observations of root numbers and lengths were recorded after one month of culture.

Plantlet acclimatization

Well-rooted shoots (10 cm in height with 4-6 leaves) were carefully removed from the culture jars and the remaining agar was washed off gently with tap water. 50 plantlets were soaked with Benlate herbicide, after that placed in peat and soil (Local source) (1:1), then acclimated gradually to room temperature before transfer to a greenhouse. The survival percentage was recorded after one month of culture.

Data analysis

A completely randomized design with ten replicates was applied to examine the effects of culture media and type of explants. Data were analyzed using GenStat software. Test of least significant differences (LSD) at 5% level of probability was used to compare the calculated averages of traits.

RESULTS AND DISCUSSION

Shoot regeneration:

Kin had a significant impact on shoot production (fig. 1). In terms of the shoots number, it was found that the highest average of shoot numbers (5.4) was achieved on medium supplemented with Kin at 3 $mg.L^{-1}$ which differed significantly (P=0.003) from the media with 0, 4 and 5 $mg.L^{-1}$ of Kin but did not differ from media with 1 and 2 mg L^{-1} of Kin as a good averages of shoot numbers were obtained (4.4 and 5 respectively). It was also observed significant differences in the averages of shoot lengths (p<0.001). The highest average of shoot lengths (6.6 cm) was recorded in medium supplemented with a high concentration of Kin at 5 mg L^{-1} which significantly differed from all other treatments (P<0.001) except the medium with 1 $mg.L^{-1}$ Kin. However, Micropropagation is an effective tool for proliferation of ornamental plants in large scale. A propagation approach for Lisianthus using Kin as a single exogenous hormone in the proliferation stage was optimized. Cytokinins are usually applied in the micropropagation media to promote shoots proliferation [7 and 27]. Studies revealed the important role of cytokinins especially Kin on regeneration of other ornamental plants such as orchids (*Vanda coerulea*) and Petunia (*Petunia hybrid*) [9] as well as *Matthiola incana* [10]. Gomes et al. [7] reported that Kin was more effective in inducing shoot growth of *Arbutus unedo* L. than other cytokinins. Also, he referred that the ideal concentration differs from species to species. Thus, it needs to be established accurately to get the effective rates of proliferation. Our findings indicated that the addition of Kin at

3mg.L^{-1} was effective for shoots production from shoot tip explants. It was observed that the average shoot number decreased with the increase of kin concentration. Similarily, Corchete, et.al [3] indicated that via propagation of some plants, the multiplication rate decreases with increasing the concentration of cytokinins in the medium. In terms of shoots length, our data showed that the medium containing 5 mg.L^{-1} of Kin resulted in a good shoot length. Contrary to our finding, Kaviani, et al. [13] showed that in establishment process of lisianthus explants, the most shoot length (2.07 cm per plant) was obtained on medium supplemented with 0.1 mg.L^{-1}of BA. Also, in current study, significant results on shoot length were observed using Kin at 1 mg.L^{-1}. These results were in accordance with Esizad, et al. [5] who referred that the best shoot length in lisianthus plant was achieved using shoot tips media supplemented with Kin at1mg L^{-1}. While, study of Ahmadi, et al. [1] on regeneration of *Matthiola incana* showed that the best shoot length was obtained using Kin at 2 mg.L^{-1}.

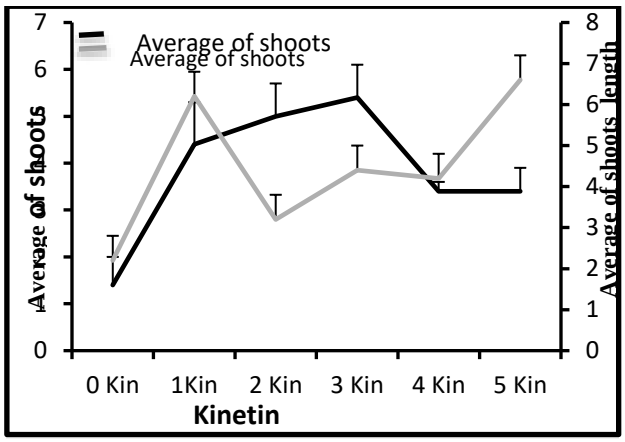

Figure1: Effect of different concentrations of kinetin on the average of shoot numbers and lengths in Lisianthus (LSD=1.9 for shoot numbers and LSD=2 for shoot lengths).

Proliferation of shoots via periodical subcultures:

Multiplication of individual shoot at 4 periodical subcultures on three different Kin concentrations showed that MS medium containing 2 mg.L^{-1} Kin induced shoot proliferation at significant difference in comparison with the other treatments (*P*<0.001) (Fig. 2). The highest average of shoot number [9] and lengths (6 cm) was obtained on MS medium containing 2 mg.L^{-1} Kin (Fig.3). Our results are in accordance with studies of Tatari, et al. [26] on micro propagation of *Gerbera jamesonii* using various plant growth regulators which revealed that most proliferation and plantlets length occurred on medium containing 2 mg.L^{-1} Kin. While other papers referred that the important role of Kin as a single growth regulator in production of stock plants and multiplication of shoots in Lisianthus was using Kin at 1 mg.L^{-1} [5 and 10]. In this paper, it was also observed that the third and fourth subcultures appeared significant impact in terms of average of shoot numbers compared with the first subculture, where the average of shoot lengths that produced from the second, third and fourth subculture differed significantly from the first subculture. Good growth of explants with green leaves at the fourth subculture was achieved (Fig. 4). However, a decrease or an increase in the proliferation potential can be noticed during long-term growth of plants on a medium of the same composition [20]. In current study, increasing in average of shoot numbers and lengths was recorded

with increasing the number of periodical sub cultures reaching to the best results which were recorded during the third and fourth subcultures using 2 mg.L^{-1} of Kin. Studies on some decorative plants showed that the maintenance of a high uniform level of Kin in several subsequent subcultures may lead to the same result [28]. Whatever, on the contrary to our findings, Villafranca, et al. [29] showed that the number of subcultures had an adverse effect on the plantlet vigor of potato plants that propagated *in vitro* for tuberization as the plantlet dry matter in tuberization medium continuously decreased with increasing number of subcultures.

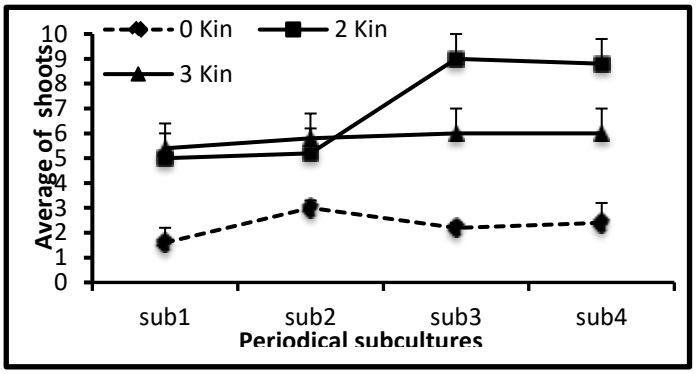

Figure 2: Effect of periodical subcultures and Kin concentrations on average of shoots number. LSD= 1.2 for Kinetin concentration. LSD= 1.4 for subcultures and LSD= 2.5 for the interaction effect.

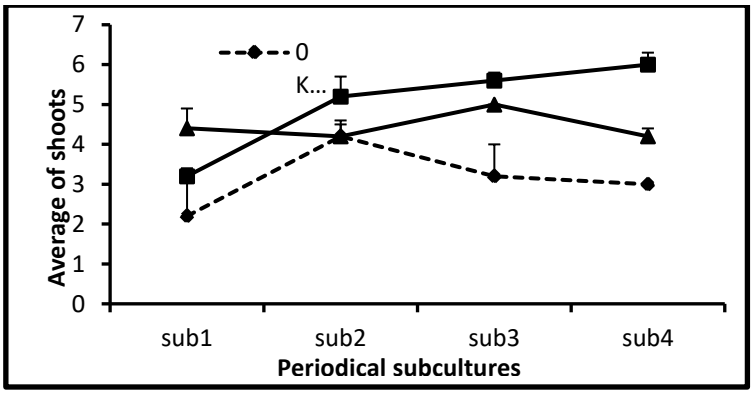

Figure 3: Effect of periodical subcultures and Kin concentrations on average of shoots length. LSD=0.6 for Kinetin concentration. LSD= 0.8 for subcultures and LSD=1.3 for the interaction effect.

Figure 4: Lisianthus explants growth during the fourth subculture on medium containing 2mg. L^{-1} of Kinetin.

Rooting and acclimatization of shoots

The use of 1 mg L^{-1} IBA had a significantly positive impact on both root numbers (P=0.005) and root length (P=0.039). The average of root numbers was (8.6) on MS medium supplemented with 1 mg. L^{-1} IBA and the highest average of root length (10.8mm) was achieved on this medium. A concentration higher than 1 mg/ L^{-1} IBA had negative impacts on both the number and length of the roots (Fig. 5). Rooting is a crucial step in the success of micropropagation. The current research demonstrated that IBA can be used to induce and form roots on Lisianthus explants at concentration of 1mg.L^{-1}. These findings are in agreement with several studies that have showed the positive effect of IBA on rooting in Lisianthus plants at the same concentration [14, 23]. Also, Murayama, et al. [19] observed rooting in IBA-treated shoots of Lisianthus that grew into normal plants. In contrast to our result, Paek and Hahn [22] found that increasing the concentration of IBA and IAA did not promote root formation in Lisianthus plants that proliferated via organogenesis. In this paper, it was noticed that the shoots cultured on MS medium without plant growth regulators led to the lowest values of roots formation and therefore, the addition of plant growth regulators was required for root initiation in Lisianthus.

Well rooted plantlets were transferred to the field after acclimatization inside the laboratory. The survival rate was 80% after one month from culture (Fig. 6). However, after one month of acclimatization, the acclimatized plantlets grew and developed well, increasing in size and height. Thus, the acclimatization of *in vitro* plantlets derived from shoot tip explants with high percent of survival is possible in Lisianthus.

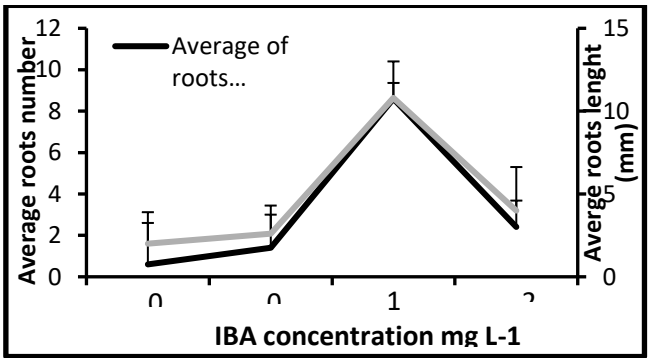

Figure 5: Effect of different concentration of IBA on root numbers and lengths after one month of culture. LSD = 4.3 for root numbers = 6.4 for root lengths.

Figure 6: Lisianthus plants after one month of acclimatization.

CONCLUSION

A full protocol for Lisianthus micro-propagation using two types of plant growth regulators, kin at the regeneration and multiplication stage and IBA at the rooting stage. Moreover, a significant finding of the current study is the possibility of large-scale production of Lisianthus for four subcultures. Further studies on the micropropagation of Lisianthus via periodical subcultures for more than four subcultures are recommended for future studies. The new *in vitro* mass propagation protocol of the Lisianthus using shoot tip explants as explant source was successfully established in this study. New findings of shoot regeneration, proliferation, root formation and acclimatization stages were described.

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الاكثار النسيجي لنبات اللوزيانا (*Eustoma grandiflorum*) باستخدام منظمات النمو واعادة الزراعة الدورية

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الملخص

تمكنت هذه الدراسة من التوصل الى الاكثار الدقيق في نبات اللوزيانا بنجاح وانتاج الافرع العرضية في اثناء مدة اعادة الزراعة الدورية المستخدمة في الزراعة النسيجية. بينت الدراسة الدور الاساس لمنظم النمو الخارجي الكاينتين بشكل منفرد على عملية الاكثار في نبات اللوزيانا. في مرحلة تكوين الافرع، تم الحصول على اعلى معدلاً لعدد الافرع بلغ (5.4) فروع باستخدام وسط MS (Murashige وSkoog) المجهز ب3 ملغم/لتر كاينتين. اما خلال مرحلة اعادة الزراعة الدورية، فان تضاعف الافرع ازداد تدريجياً وصولا الى المرحلة الرابعة لإعادة الزراعة، حيث تحقق اعلى معدلاً لعدد الافرع بلغ (9) فرع في المرحلة الدورية الثالثة لإعادة الزراعة، في حين بلغ اعلى معدلاً لأطوال الافرع (6) سم في المرحلة الدورية الرابعة عند زراعتها على وسط MS الحاوي على 2ملغم/لتر كاينتين. الافرع الناتجة جذرت بسهولة على وسط MSالحاوي على 1ملغم/لترمن IBA. النبيتات المشتقة من هذا البرنامج الاكثاري تمت اقلمتها بنجاح في خليط من التربة والبتموس بنسبة (1:1حجم/حجم) مع الحصول على نسبة بقاء 80%. تعد النتائج المتحققة في هذا البحث ذات اهمية عملية كبيرة في مجال الاكثار الدقيق لنبات اللوزيانا.

الكلمات الدالة: الزراعة خارج الجسم الحي، اللوزيانا، الاكثار الدقيق، إعادة الزراعة الدورية، منظم النمو الفردي.

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