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Seed Germination and In Vitro Micropropagation of *Tulipa Gesneriana*

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RESEARCH ARTICLE

Seed Germination and In Vitro Micropropagation of *Tulipa Gesneriana*

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ABSTRACT

An effective method for the in vitro propagation of *Tulipa gesneriana* was developed using Murashige and Skoog (MS) medium. Seeds were first cultured on hormone-free MS medium, achieving %90 germination after 30 days. Seedlings were obtained, and flowering stem explants excised from these seedlings were used for callus induction. The best callus formation was obtained on MS medium supplemented with thidiazuron (TDZ1.5mg/L) and 2,4-dichlorophenoxyacetic acid (2,4-D1.5mg/L). Shoot proliferation was most effective on MS medium containing 2.0 mg/L benzyladenine (BA) and 0.2 mg/L indole-3-butyric acid (IBA), and bulb formation was promoted by 0.5 mg/L BA, producing 3–4 bulbs per explant, while root induction was achieved with 1.0 mg/L naphthalene acetic acid (NAA), generating up to 5 roots per plantlet. Plantlets with well-developed shoots and roots exhibited survival rates ranging from 70-90% during acclimatization in peat moss and peat moss: soil (1:1) substrates. This study provides a reliable method for large-scale tulip propagation and conservation.

Keywords: Bulb formation, Callus induction, Shoot regeneration, Thidiazuron, Tulip

Introduction

Tulip (*Tulipa gesneriana L.*) is one of the most important ornamental bulbous plants[1] that belongs to the Liliaceae family. Bulbs are used for vegetative multiplication of the tulip plant. Tulips reproduce sexually via flowers and seeds, and asexually through the development of daughter bulbs. Mature tulip bulbs consist of circular arrangements of fleshy scales that store the essential nutrients required to support the future growth of the stem, leaves, and flower.[2,3] Tulips are of significant economic importance, ranking third in the world in terms of sales volume among commercial flowers.[4] When using conventional procedures, the number of tulip bulbs that may be produced from a single bulb is restricted to only two to three bulbs. One effective technique for shoot organogenesis (the regeneration of plant organs such as shoots, roots, or bulbs from cultured tissue) is plant tissue culture. In order to generate genuine plants, researchers turned to tissue culture, which yielded a significant number of bulbs that are free from viruses and increased the content of secondary metabolites. Due to the limited production efficiency of traditional propagation methods for the tulip plants, there is a need to develop more effective strategies based on tissue culture techniques. Organogenesis is one of the most prominent of these techniques, due to its ability to enhance plant regeneration by inducing plant organs under controlled laboratory conditions.[5] Numerous elements, including the explant's size, age, physiological state, and source, have a significant impact on the tissue culture.[6,7] Approximately 20 to 25 years may elapse between the selection of first seedlings and the cultivation of a new cultivar into 10,000 bulbs and its marketing. This time frame can be reduced by employing in vitro propagation techniques.[8] Under controlled conditions, regeneration of plantlets can be achieved either through direct organogenesis or indirectly via callus formation.[9]

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During the 1980s, 1990s, and the early twenty-first century, substantial evidence was produced regarding the immediate induction of stems or tiny bulbs. Explants used at the initiation stage comprised bulb scales, shoot buds, and flowering stems, with the latter exhibiting the greatest ability to regenerate. As the first explants of Tulipa commerce, seedling pieces showed a remarkable regenerative capacity, according to Ma´slanka and Bach.[10] This technique, which is similarly predicated concerning the direct induction of adventitious shoots (shoots arising from non-meristematic tissue, i.e., tissues not originally meant to form shoots), has the potential to effectively replicate wild tulip species, including those that are in danger of going extinct. A comparable use of the in vitro culture technique has recently been reported. We created the tulip Micropropagation (the in vitro production of large numbers of genetically uniform plantlets under controlled laboratory conditions) technique a few years ago. This technique is based on the successive multiplication of adventitious shoots. Higher efficiency is a feature of this technology, and over the course of two to three years, a few bulbs can be converted into about 1,000 micro-bulbs. The process involves regenerating somatic embryos from flower stem fragments, followed by regeneration of adventitious shoots at the tips of stolons derived from the developing embryos, and then cyclically multiplying non-axillary shoots for approximately 2 years on medium containing naphthaleneacetic acid (NAA – an auxin used for root induction), isopentenyl adenosine (iP – a cytokinin used to promote cell division and shoot development), and thidiazuron (TDZ – a cytokinin-like compound highly effective in shoot regeneration and callus induction).[8,11] Flower bulbs are identified based on their unique characteristics and are studied and cultivated with consideration for their potential uses as well as their economic and social value.[12]

One method of plant regeneration that can be applied to clonal proliferation is organogenesis. This method of micropropagation causes adventitious shoots or bulbs to grow in bulbous plants, either directly on explants or indirectly through callus tissue. Organogenesis in geophytes can take place on a variety of explants, including buds. Although some investigations have used non-chilled plant material, in vitro cultures of tulips have primarily been started using chilled bulbs. Inducing organogenesis requires cytokinin (a class of plant hormones that promote cell division and differentiation), which has been shown to influence cell division and differentiation.[13] Thidiazuron (TDZ) is an extremely effective growth regulator in tissue cultures and resembles cytokinin. Auxins (a group of plant hormones that stimulate cell elongation, root induction, and callus formation) are also added to culture media to promote organogenesis.[14]

The current study aims to identify the optimal explant source and concentration of the growth regulator for shoot multiplication, proliferation and the subsequent development of plantlets, as well as bulb formation of tulips using in vitro culture techniques.

Material and methods

Seed sterilization and germination

T. gesnseriana seeds were obtained from a commercial nursery in Istanbul/ Turkey. The seeds were surface-sterilized by immersion in 70% ethanol for 1 min, and then immersed in sodium hypochlorite (NaOCI) solution at a concentration of 1.0% for 10 min. After sterilization, the seeds were rinsed three times with sterile distilled water to remove traces of sterilant. The sterilized seeds were then cultured on Murashige and Skoog (MS) medium.[15] The medium contained 3% sucrose[16] and was solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121˚C for 20 min. No additional sterilization techniques were applied post-autoclaving. Each seed was cultured individually in a 50-ml glass tube containing 10 ml of medium. Cultures were maintained at 25 ± 2˚C under a 16-h photoperiod (light) and 8-h dark cycle. Illumination was provided at a light intensity of approximately 40 μmol $m^{-2}s^{-1}$. Seed germination was recorded after 30 days, with weekly monitoring of germination progress. The final germination percentage reached 90%.

Explant preparation and callus induction

After germination, flowering stem segments (0.5 cm in length) were excised from seedlings and used as explants. Explants were transferred to MS medium supplemented with different concentrations of TDZ (0.0, 0.5, 1.0, 1.5 mg/L) and 2,4-D (0.0, 0.5, 1.0, 1.5 mg/L). Each treatment consisted of three replicates with 10 explants per replicate. Cultures were incubated for 4 weeks, and callus induction percentage and morphology (color, texture) were recorded.

Shoot proliferation

Callus and shoot primordia were subcultured onto MS medium containing BA (1.0, 2.0, 3.0 mg/L) and IBA (0.1, 0.2, 0.3 mg/L). Cultures were incubated for 6 weeks, and the average number of shoots per explant was recorded.

Bulb formation

Shoots (~2–3 cm in length) were transferred onto MS medium containing BA (0.1, 0.5, 1.0 mg/L) or TDZ (0.1, 0.5, 1.0 mg/L). Cultures were maintained for 8 weeks. Observations included bulbing percentage, average number of bulbs per explant, and bulb morphology. Successful bulb formation was defined by scale initiation and basal plate development.

Root induction

Shoots were transferred to MS medium supplemented with NAA (0.0, 0.1, 0.5, 1.0 mg/L). Each treatment included 15 plantlets with three replicates. After 4 weeks, the rooting percentage, average root number, and root length were recorded.

Acclimatization

Rooted plantlets were washed to remove agar residues and transplanted into pots containing three different substrates: peat moss, peat moss: soil mixture (1:1), and soil alone. The plantlets were kept under greenhouse conditions for 4 weeks. Survival percentage was recorded weekly. Criteria for acclimatization success included leaf expansion and continued root growth.

Statistical analysis

The Statistical Analysis System SAS[17] the effects of various factors on the studied parameters was employed to analyze. Mean differences were evaluated using the Least Significant Difference (LSD) test at a probability level of 0.05.

Results and discussion

Seed germination and callus induction

Tulipa gesneriana seeds germinated successfully on hormone-free MS medium after approximately 4 weeks of culture. The germination percentage reached **90%** after 30 days under controlled conditions (16-h photoperiod, 25 ± 2°C). These findings confirm the suitability of hormone-free MS medium for obtaining healthy seedlings that can be used as an explant source Figs. 1 and 2.

As presented in Table 1, callus induction varied depending on the concentration of TDZ and 2,4-D. The highest callus induction percentage (90%) was obtained at 1.5 mg/L TDZ combined with 1.5 mg/L 2,4-D, while lower concentrations (0.5–1.0 mg/L) gave moderate induction (40–70%). These results confirm the strong synergistic effect of auxin and cytokinin-like compounds in callus initiation.

Table 1. Effect of 2,4-D and TDZ (mg/L) and interaction between them on the percentage callus induction from the Flowering plant stem after 4 weeks of culture.

2,4-D \ TDZ	0	0.5	1	1.5	Mean
0	0	10	10	20	10.0
0.5	20	30	40	40	32.5
1	20	60	60	70	52.5
1.5	30	60	70	90	62.5
Mean	17.5	40.0	45.0	55.0	—

LSD value: 2,4-D: 6.91 *, TDZ: 6.91 *, 2,4-D x TDZ: 11.07 *.

At this point, the flowering stem was taken from the seedling and utilized as an explant to induce callus. After four weeks of culture, the explants were employed on MS media containing 2,4-D and TDZ at various concentrations (0, 0.5.1, 1.5) mg/L. *T. gesneriana* in vitro callus cultures were started from flower stem slices on MS medium with varying auxin and cytokinin concentrations. An increase in auxin levels, particularly 2,4-D, results in an explant's intense expansion. Callus formed after four weeks of cultivation. Explants cultured on media supplemented with the plant growth regulators (2,4-D) (1.5 mg/L) and TDZ (1.5 mg/L) developed a yellow, looser-structured nodular callus, as seen in Fig. 3.

In the presence of 2,4-dichlorophenoxyacetic acid and thidiazuron, a granular yellowish-white callus texture developed on the incised surface of the flower stem originating from the vascular bundle tissues. For several plant species, similar callus formation from the cells around the veins has been routinely recorded. It should be mentioned that although we employed a higher concentration of 2,4-D to induce this granular callus, a higher concentration of this auxin was successful in promoting more callus expansion and concurrent embryo development.[8]

The effectiveness of thidiazuron is ascribed to its ability to have a direct effect via interaction with cytokinin receptors or through indirect pathways, enhancing endogenous cytokinins accumulation through the inhibition of cytokinin oxidase/dehydrogenase (CKX)activity. When combined with auxins, this highly effective compound has often been used to induce callus formation in vitro, as well as embryos or adventitious buds in several plant species that are typically thought to be challenging for in vitro regeneration.[8,18]

The strong effect of TDZ on callus induction may be attributed to its cytokinin-like activity, either through direct interaction with cytokinin receptors or by inhibiting cytokinin oxidase, thus increasing

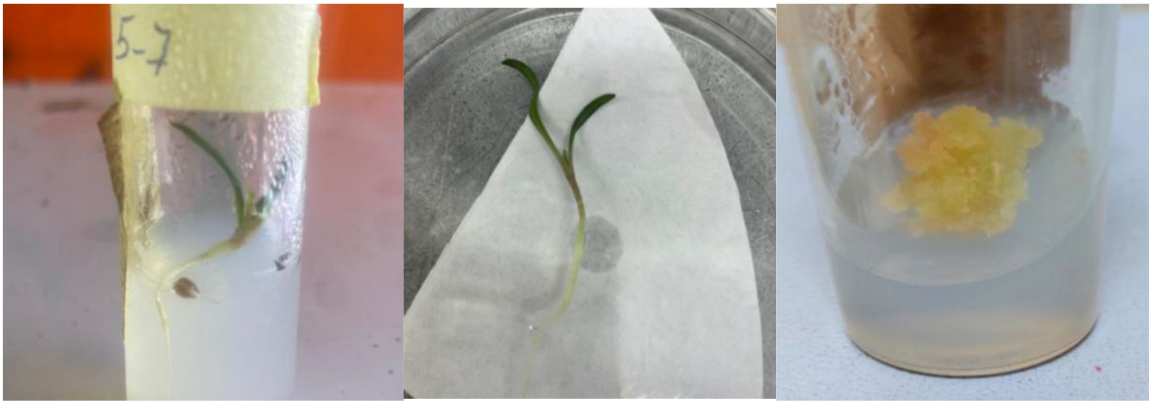

Fig. 1. (A,B) Seedling of *Tulipa gesnseriana* grown on hormone-free MS medium after 30 days (C) callus induction from flowering stem explants of seedling on media with 1.5mg/L TDZ and 1.5mg/L 2,4-D.

endogenous cytokinin levels.[18] When combined with 2,4-D, TDZ enhanced morphogenetic responses by balancing auxin–cytokinin ratios, which is consistent with studies in other bulbous ornamentals.[11,19]

Shoot regeneration and multiplication

Table 2 and Fig. 3 presents the shoots developed in the regeneration and proliferation media. All BA and IBA concentrations examined on *T. gesnseriana* media supplemented with plant regulators generated a greater average number of shoots than the control media. Results obtained indicate that 2mg/L BA and 0.1 IBA are the preferred concentrations for enhanced shoot proliferation. A decline in response was observed with increasing BA concentration to 2 mg/L combined with the addition of IBA to 0.2 mg/L, and an average of 10 branches was achieved. The lowest results were achieved on a medium containing (1) mg/L BA and (0.1) mg/L IBA, which produced 5 branches.

The strong effect of TDZ on callus induction may be attributed to its cytokinin-like activity, either through direct interaction with cytokinin receptors or by inhibiting cytokinin oxidase, thus increasing endogenous cytokinin levels.[20] When combined with 2,4-D, TDZ enhanced morphogenetic responses by balancing auxin–cytokinin ratios, which is consistent with studies in other bulbous ornamentals.[10,21]

The table shows that adding BA to the MS medium induced the growth of vegetative branches to grow faster. This might be because Cytokinin helps with the development of chloroplasts, photosynthesis, expression in leaves, increasing the efficiency of nutrient movement, and hastening the process of cell division and organ development.[20,21] Cytokinin could combine with other growth regulators to enhance the vegetative development of plants and stimulate cell division to enlarge the tissue area and promote plantlet growth. The use of Cytokinin, especially BA, in tissue culture is important and stable because it is durable and highly efficient in disrupting apical dominance, preventing the breakdown of chlorophyll, and stimulating cell division.[20–22]

Table 2. Effect of BA and IBA on shoot regeneration and multiplication.

BA \ IBA	0	0.1	0.2	0.3	Mean
0	0	0	0	0	0.00
1	0	5	5	7	4.25
2	0	12	10	10	8.00
3	0	8	9	8	6.25
Mean	0.00	6.25	6.00	6.25	—
L.S.D. value	BA: 2.942 *, IBA: 2.942 *, BA x IBA: 5.177 *.				

BAP in combination with NAA proved to be more efficient for SE initiation and embryo differentiation into plants in various ornamental geophytes species, like *Hippeastrum hybridum*, *Eucharis grandiflora* and *Crinum asiaticum*.[8,22]

Bulb formation

Bulb formation was significantly influenced by BA and TDZ concentrations. As shown in Table 3, the highest bulbing percentage (80%) was observed at 0.5 mg/L BA, producing an average of 3–4 bulbs per explant. At 1.0 mg/L BA, bulbing reached 70% with 2–3 bulbs per explant, while at 0.1 mg/L BA the bulbing percentage was 55% with 1–2 bulbs per explant. Treatments containing TDZ (0.5–1.0 mg/L) resulted in only 30–40% bulbing, usually producing a single small bulb per explant.

In the discussion, BA was suggested as a possible substitute for TDZ. It should be clarified that this

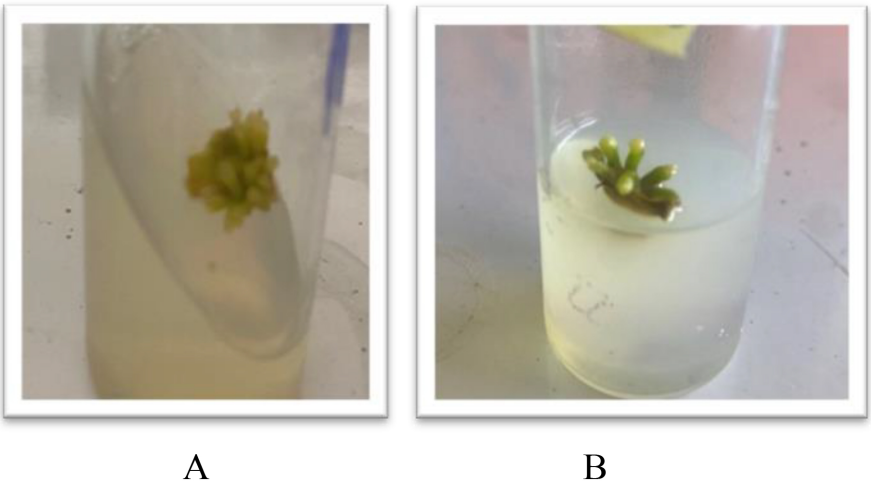

Fig. 2. Shoot regeneration from callus (A) after 4 weeks, (B) after 8 weeks.

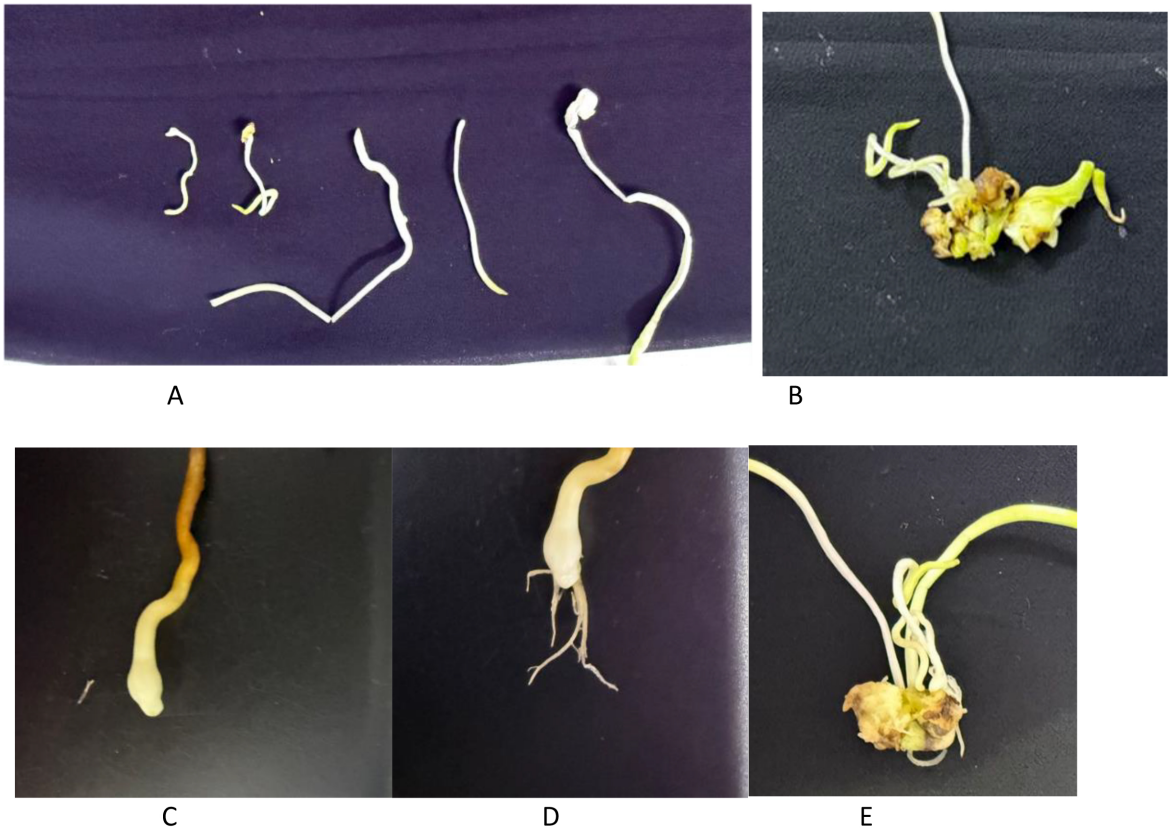

Fig. 3. (A) different stages of preparation for Bulb formation, (B, C) bulb formation after 10 weeks, (D, E) root initiation.

substitution was not directly tested in this study, but is based on conclusions from previous literature.[22] Our results are in agreement with recent findings on tulip micropropagation (5,11), which emphasize the efficiency of BA in bulb induction. All concentrations are expressed in mg/L.

These results align with our observation. The efficiency of Bulbing in tulip shoot cultures was increased by Benzyl adenine instead of Thidiazuron; the hypothesis regarding the substitution of TDZ in the final stage of multiplication subculture could stimulate subsequent bulb development. This conclusion is supported by the findings of,[23] which indicated that medium containing iP stimulates bulb development significantly more than BA-containing medium. These findings strongly suggest that TDZ and BA have

Table 3. Effect of BA and TDZ on percentage of bulb formation.

BA \ TDZ	0	0.1	0.5	1	Mean
0	0	0	0	0	0.00
0.1	70	50	60	40	55.00
0.5	80	50	50	40	55.00
1	70	40	50	40	50.00
Mean	55.00	35.00	40.00	30.00	—

LSD value: BA: 9.026 *, TDZ: 9.026 *, BA x TDZ: 15.961 *.
* ($P \leq 0.05$).

Table 4. Effect of NAA concentrations on root induction in *Tulipa gesneriana*.

Root long (cm)	Number of roots	Rotting (%)	NAA (mg/L)
0	0	0	0
1.2	1	40	0.1
0.8	3	80	0.5
0.8	5	70	1
0.649 *	2.726 *	8.052 *	L.S.D. value

* ($P \leq 0.05$).

sustained effects on the in vitro bulbing of tulips. Modification of MS medium composition and different types of auxin led to significantly divergent results, possibly supporting the confirmation of the importance of selecting the optimal combination of PGRs for each cultivar. This may be linked to the composition of endogenous growth regulators. [24,25]

Root formation

Root formation was strongly affected by NAA concentration Table 4. The highest rooting percentage (80%) was obtained at 0.5 mg/L NAA, producing an average of 3 roots per explant. Increasing NAA to 1.0 mg/L resulted in a greater number of roots (5 roots per explant) but slightly reduced root length. No roots developed on hormone-free media. These results highlight the dual role of auxins in promoting cell division and root primordia initiation, consistent with earlier studies on in vitro root induction. [26,27]

Acclimatization

Plantlets with well-developed shoots and roots were successfully acclimatized under greenhouse conditions Fig. 4 and Table 5 shows that the highest survival rate (90%) was recorded on peat moss, followed by 80% in the peat moss: soil mixture (1:1), whereas only 40% survived in soil alone after 4 weeks. Peat moss was superior due to its high water-holding capacity and aeration, which provided favorable conditions for root development during the critical acclimatization stage. [28]

Table 5. Effect of different substrates on acclimatization of tulip plantlets.

Medium	Survival % (Week 1)	Survival % (Week 2)	Survival % (Week 3)	Survival % (Week 4)
Peat moss	90	90	80	70
Peat moss : Soil (1:1)	90	80	80	70
Soil mixture	70	50	50	40

Fig. 4. Effect of peatmoss and soil mixture on the survival rate of *T. gesnseriana* plantlets at the acclimatization stage after 4 weeks.

Conclusion

This study established an efficient in vitro propagation protocol for *Tulipa gesneriana* using plant tissue culture techniques. Seeds were germinated on hormone-free MS medium, and flowering stem explants were used to induce callus on MS medium containing thidiazuron (TDZ) and 2,4-dichlorophenoxyacetic acid (2,4-D), with the best callus formation at 1.5 mg/L for both. Shoot multiplication was achieved using 2.0 mg/L benzyladenine (BA) and 0.1–0.2 mg/L indole-3-butyric acid (IBA), while bulb formation was most effective with 0.5–1.0 mg/L BA. Rooting was optimized with 1.0 mg/L naphthaleneacetic acid (NAA), resulting in high root number and percentage. The plantlets showed a 90% survival rate during acclimatization in a peat moss and soil mixture (1:1). This protocol provides a reliable method for the mass propagation of tulips.

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Authors' declaration

- Conflicts of Interest: None
- We hereby confirm
- All the Figures in the manuscript are ours. Any Figures and images that are not ours have been included with the necessary permission for re-publication, which is attached to the manuscript
- No animal studies are present in the manuscript.
- No human studies are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at university of Baghdad.

Authors' contributions statement

The study was done by T.H.A who did most of work and wrote this research with this design, modified it, and corrected it. S.F.H the creator of the study project.

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انبات البذور والاكثار الدقيق لنبات التوليب *Tulipa gesnseriana*

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قسم علوم الحياة، كلية العلوم للبنات، جامعة بغداد، بغداد، العراق.

الخلاصة

تضمنت الدراسة استخدام تقنية زراعة الأنسجة النباتية في استخدام أجزاء النبات وتوليفات مختلفة من منظمات نمو النبات. حيث تم زراعة البذور على وسط (MS) الخالي من منظمات النمو ،بعد مرور شهر من الزراعة، اخذت اجزاء من البادرة وهي الحامل الزهري لاستخدامه في استحثاث الكالس، حيث زرع على وسط استحثاث الكالس وهو وسط MS المزود بتراكيز مختلفة من TDZ و D-2,4. فوجد ان أفضل وسط لاستحثاث الكالس TDZ و،D-2,4 وبنفس التركيز (1.5) مجم / لتر. تم استخدام وسطMS المجهزبـ 2 BA مجم / لتر و IBA في 0.2 مجم / لتر لانتاج افضل عدد من الافرع حيث اعتبر وسط مناسب للتضاعف ولتكوين الابصال استخدم وسط MS المضاف له 0.5 مجم / لتر من BA لانتاج ابصال صغيرة ، في حين أن NAA في 1.0 مجم / لتر أعطى نسبة عالية من التجذير واعطى نتيجة جيدة في أعداد الجذر. أظهرت النبتات ذات المجموع الخضري الجذري أعلى نسبة بقاء (90) ٪في مرحلة الاقلمة على الوسط الزرعي المكون من البتموس والتربة المزيجية بنسبة (1: 1).

الكلمات المفتاحية: التوليب، تجديد النمو الخضري، تحفيز الكالس، تكوين الأبصال، ثيديازورون.