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In vitro regeneration and conservation of Ruta chalepensis L. through cell suspension cultures and plating method

Farah Qassim Ali

*Corresponding Author. Department of Biology, College of Education for Pure Sciences, University of Diyala, Iraq. E-mail address: mn76cd@gmail.com

Abstract

Objective

Ruta chalepensis L., commonly known as fringed rue, is a medicinal plant belonging to the Rutaceae family. It is widely recognized for its diverse pharmacological properties, including antimicrobial, antifungal, anti-inflammatory, and antioxidant activities. Rich in bioactive compounds such as alkaloids, flavonoids, coumarins, and essential oils, this plant holds significant value in both traditional medicine and modern pharmaceutical applications. However, natural populations of *R. chalepensis* face threats due to habitat destruction, overharvesting, and low seed viability, necessitating alternative propagation and conservation strategies. Therefore, this study aimed to optimize the in vitro regeneration of *Ruta chalepensis* L. by establishing cell suspension cultures and regenerating plants using a plating method.

Materials and Methods

Callus induction was achieved from *R. chalepensis* stem explants cultured on Murashige and Skoog (MS) medium supplemented with varying concentrations (0.0, 1.0, 2.0, and 3.0 mg/L) of 2,4-Dichlorophenoxyacetic acid (2,4-D) in combination with 0.5 mg/L Benzyl Adenine (BA). The highest fresh callus weight (2.036 g) was observed at 3.0 mg/L 2,4-D + 0.5 mg/L BA after four weeks of culture. The induced callus exhibited a friable texture and was subsequently used to initiate cell suspension cultures in liquid MS medium supplemented with the same concentration of plant growth regulators.

Results

The plating method was employed to culture cell suspensions at different densities in solid induction medium, promoting cell division and the formation of cellular colonies. These colonies progressed to callus primordia, which later developed into small callus segments. The highest

average number of cellular colonies (84.61 per dish) was recorded at a cell density of 20.2 × 10^5 cells/mL, significantly surpassing the initial density of 18.47 colonies per dish. This high-density culture also resulted in an average of 70.24 callus primordia per dish after 27 days. Furthermore, shoot formation was observed, with 60 shoots successfully obtained after seven weeks from callus derived from cell suspensions using the plating method. These shoots were maintained for 30 days on MS medium containing the same concentrations of plant growth regulators. Rooting of regenerated shoots was achieved at a 100% success rate in full-strength MS medium, and the newly developed plants were acclimatized in vitro in pots by the eighth week.

Conclusions

The regenerated shoots exhibited high rooting efficiency and successful acclimatization, validating the effectiveness of this in vitro propagation approach. These findings present a valuable biotechnological method for the conservation and sustainable production of *Ruta chalepensis*, supporting its pharmaceutical and medicinal applications.

Key words: callus, medicinal plant, plating method, somatic embryogenesis

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Introduction

Ruta chalepensis L. is a perennial evergreen shrub belonging to the Rutaceae family and is commonly known as fringed rue (Qahtan et al. 2021). The plant features cymose flowers, each containing 8–10 stamens, 4–5 sepals, 4–5 petals, an ovary, and compound leaves (Khazal 2017). It is widely distributed across Mediterranean regions and other parts of the world, particularly in temperate and tropical zones (Gőnaydin & Savci 2005). This species holds significant medicinal

and pharmaceutical value due to its rich content of secondary metabolites, including essential oils, alkaloids, furanocoumarins, coumarins, terpenes, phenolic compounds, amino acids, sterols, tannins, and saponins, which are primarily found in its leaves and stems (Al-Majmaie et al. 2019; Alotaibi et al. 2018). These bioactive compounds contribute to various biological activities, such as antifungal, anti-inflammatory, antioxidant, and anthelmintic properties (Akkari et al. 2015; Ouerghemmi et al. 2017). *Ruta chalepensis* has been widely used in traditional medicine as an analgesic, antipyretic, and treatment for rheumatism, convulsions, and neuralgia (Jaradat et al. 2017). It is also utilized internally for its antihypertensive and antispasmodic effects (Babu-Kasimala et al. 2014).

The concept of *in vitro* plant cell and tissue culture was introduced by Gottlieb Haberlandt in 1902 through his totipotency hypothesis, which states that each plant cell contains the genetic information necessary to regenerate a complete plant (Fehér 2019). Differentiated plant cells can re-enter the cell cycle, facilitating the regeneration of tissues and organs, ultimately leading to whole-plant development. This remarkable ability is attributed to the developmental plasticity of plant cells (Wójcikowska & Gaj 2017). The totipotent nature of plant cells has been extensively utilized in genetic studies, micropropagation, and germplasm conservation (Sugimoto et al. 2011).

In vitro plant regeneration occurs when explants undergo cell division and differentiation, leading to organogenesis or somatic embryogenesis. This process has played a crucial role in the propagation and conservation of medicinal plants, particularly those facing extinction or exhibiting low reproductive efficiency (Al-Mahdawe & Al-Mallah 2015). Callus and cell suspension cultures have demonstrated significant potential in plant regeneration, particularly for species that are challenging to propagate through conventional methods (Dewanti et al. 2016). These techniques are instrumental in producing high-yield varieties, breeding disease-resistant plants, and ensuring the large-scale propagation of medicinal species within a short period (Dakah et al. 2014; Thorpe, 2013). Furthermore, *in vitro* culture methods enable researchers to study the physiological behavior of isolated plant organs, cells, tissues, and protoplasts under precisely controlled physical and chemical conditions (Shahzad et al. 2017).

Cell suspension cultures offer a viable approach for the regeneration of plants, particularly for species with challenging callus differentiation (Sathish et al. 2018). *In vitro* regeneration through cell suspension culture provides an efficient platform for large-scale propagation of *Ruta chalepensis* and the production of its valuable bioactive compounds. This method ensures a continuous and sustainable supply of medicinal phytochemicals while reducing dependence on natural populations. Additionally, cell suspension cultures serve as an effective system for genetic transformation, secondary metabolite production, and conservation of plant genetic resources. By optimizing culture conditions, the biosynthesis of essential phytochemicals can be enhanced,

benefiting pharmaceutical, cosmetic, and agricultural industries. Furthermore, the utilization of phytobiotics and medicinal plants as natural alternatives to antibiotics in animal feed has been widely recognized for its numerous benefits. These include improvements in zootechnical performance, disease prevention (Amirteymoori et al. 2021; Mohammadabadi et al. 2023), antimicrobial and antioxidant activities (Hajalizadeh et al. 2019; Jafari Ahmadabadi et al. 2023), hypocholesterolemic effects, enhancement of digestive enzymes, and liver function improvement (Safaei et al. 2022; Shokri et al. 2023; Mohammadabadi et al. 2024). Studies have shown that incorporating medicinal plants into animal diets increases feed consumption, feed conversion ratio, and carcass yield (Vahabzadeh et al. 2020; Shokri et al. 2023). Thus, this study focuses on the in vitro regeneration of Ruta chalepensis from cell suspension culture, highlighting its importance in biotechnological applications. The research aims to establish a reliable protocol for plant regeneration, which could serve as a foundation for further studies on metabolite production and genetic improvement of this medicinal species.

Materials and Methods

One-year-old *Ruta chalepensis* plants were obtained from the Agricultural Incubator at the Baghdad Agricultural Directorate and transferred to a plastic greenhouse at the Department of Biology, University of Baghdad. The plants were monitored for all necessary agricultural practices and were taxonomically classified at the Ibn Al-Haitham Herbarium, University of Baghdad, Iraq. The stems of *R. chalepensis* were washed, surface-sterilized, and prepared for culture following the protocol described by Nadir (2021). The sterilized explants were cultured on 20 mL of solid Murashige and Skoog (MS) medium (Murashige and Skoog 1962), supplemented with plant growth regulators (PGRs). The cultures were maintained in a growth room under conditions reported by Ahmad et al. (2020).

The MS medium (Mumbai-400086, India) was prepared by dissolving all components in distilled water, making up the final volume to one liter. Sucrose (30 g/L; Iran Hormone Pharmaceutical Co., Iran) and agar (6.0 g/L; Yekta Shimi Azma Company, Iran) were added to solidify the medium. The pH was adjusted to 5.7-5.8 using 1.0 N NaOH or HCl. This solid MS medium, supplemented with specific PGRs, was used for callus induction and maintenance. A liquid MS medium was prepared for initiating cell suspension cultures derived from callus and for growing cells in liquid nutrient media in 250 mL Erlenmeyer flasks. The media were sterilized by autoclaving at 121°C and 1.04 kg/cm² pressure for 15 minutes (Al-Mahdawe et al. 2018).

Stem segments of *R. chalepensis* measuring 1.0 cm in length were used as explants for callus induction. These were transferred to 100 mL glass jars containing 20 mL of MS medium

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supplemented with 0.0, 1.0, 2.0, and 3.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 0.5 mg/L benzyl adenine (BA). Ten explants were used per treatment, and cultures were maintained in a growth room as described by Ahmad et al. (2020). The explants were observed for morphological changes until they fully developed into callus. Fresh callus weight was recorded after 30 days. Based on the callus induction results, cell suspension cultures were initiated using 5.0 g of friable callus induced on MS medium containing 3.0 mg/L 2,4-D and 0.5 mg/L BA. The callus was suspended in 50 mL of liquid MS medium supplemented with the same PGRs in 250 mL flasks. The cultures were incubated in a shaking incubator at 150 revolutions per minute (rpm) at 25± 2°C under a 16-hour light/8-hour dark photoperiod with a light intensity of 2000 Lux. The cultures were filtered through a sterile micro sieve (46 µm pore size; Plant Genetic Manipulation Lab, Nottingham, UK) to separate individual cells from cell aggregates. The isolated cells were resuspended in fresh nutrient medium with the same PGRs. After allowing cells to settle for 30 minutes, the liquid medium was carefully removed without disturbing the cells (Coleman et al. 2003). The cultures were then returned to the incubator for further growth and subsequent experiments.

To estimate the density of the cell suspension culture, 1.0 mL samples were collected at 0, 24, 48, 72, 96, 120, 144, and 168-hour intervals post-filtration. Cell density was determined using a hemocytometer (Lab W., Germany) by counting the number of cells in 1.0 mL of suspension. The total number of cells in the culture was estimated, and the samples were plated for further experiments. Cell suspension cultures were plated following a modified protocol by Dunstan et al. (1982). Petri dishes (9.0 cm diameter) containing a thin layer of MS solid medium supplemented with the same PGRs as the initial suspension cultures were used. Cells were drawn from the suspension using a sterile 1.0 mL pipette and plated at densities of (5.6, 8.0, 9.0, 10.5, 16.1, 19.0, 20.1, and 20.2) $\times 10^5$ cells/cm^3. The suspension was evenly distributed by gently rotating the Petri dishes on a flat surface before sealing them with parafilm. Cultures were incubated in a growth room at 25± 2°C under a 16-hour light/8-hour dark photoperiod, shielded from direct light using A4 white paper.

Cell division and colony formation were monitored microscopically after 24 hours. The first division and subsequent colony development were recorded until seed colonies formed, eventually developing into callus. The number of cellular colonies and callus primordia was recorded, along with their transition into visible callus pieces. Callus pieces formed from plated cell suspensions were transferred to 100 mL glass jars containing 20 mL of MS medium supplemented with 3.0 mg/L 2,4-D and 0.5 mg/L BA for further growth and proliferation. Descriptive parameters such as texture and color were documented. Additionally, callus primordia from plated suspensions were maintained until the eighth day at a density of 20.2 $\times 10^5$

cells/cm³ in MS medium with 3.0 mg/L 2,4-D and 0.5 mg/L BA to facilitate indirect shoot formation.

Shoots that emerged due to periodic maintenance of callus primordia from cell suspension cultures were excised using a sterile scalpel. Each shoot measured 2.5-3.0 cm in length and contained 3-4 leaves. Shoots were vertically inserted into 30 mL of full-strength solid MS medium and incubated in a growth room. Explants were surface sterilized using 6% sodium hypochlorite (NaOCl) at a 1:9 (v/v) bleach-to-distilled water ratio for 20 minutes. This concentration effectively sterilized explants, ensuring contamination-free callus cultures on solid MS medium supplemented with PGRs. Cultures grown for 30 days were used in subsequent experiments.

All experiments followed a completely randomized design (CRD) with ten replicates per treatment. Mean differences were analyzed using Duncan's multiple range test (DMRT) at a significance level of 0.05 (Loyola-Vargas and Ochoa-Alejo 2018). Statistical analyses were performed using the SPSS software package.

Results and discussion

The results (Table 1) indicate that MS medium supplemented with 2,4-D in combination with BA induced callus formation in 100% of explants at concentrations of 2.0 and 3.0 mg/L 2,4-D + 0.5 mg/L BA. In contrast, callus induction was observed in only 50% of explants at 1.0 mg/L 2,4-D, whereas no callus formation (0%) occurred in the control treatment (Figure 1A). The recorded data on the average fresh weight of callus after 30 days demonstrated a direct correlation with increasing 2,4-D concentrations. The highest average fresh weight (2.036 g) was obtained at 3.0 mg/L 2,4-D, showing a significant increase compared to other concentrations. The fresh weight decreased to 1.520 g and 1.110 g at 2.0 mg/L and 1.0 mg/L 2,4-D, respectively. The control group recorded the lowest average fresh weight of 0.120 g.

Callus induced from stem explants cultured on MS medium supplemented with 2.0 and 3.0 mg/L 2,4-D exhibited a friable texture and a pale green color (Figure 1C, D). After 30 days, these explants completely lost their original structure, forming callus segments. However, explants grown on MS medium containing 1.0 mg/L 2,4-D retained some of their original structure and did not fully transform into callus (Figure 1B).

Callus induction is a fundamental technique in plant tissue culture, relying on the ability of cells to divide and proliferate. This process is commonly assessed by measuring increases in both fresh and dry weight (Mohammad and Abood, 1989). Auxins, particularly 2,4-D, play a crucial role in promoting callus formation by enhancing cellular activity and facilitating the synthesis of essential growth components. Additionally, the interaction between auxins and cytokinins is

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critical in establishing an optimal hormonal balance necessary for efficient callus induction (Trigiano and Gray, 2010).

Table 1. Cultures of callus derived from stem explants of *R. chalepensis* L., and average of their fresh weights on MS medium supplemented with different concentrations of 2,4-D+ 0.5 mg/L BA

Concentration of growth regulators (mg. L^{-1})		Induction (%)	Average fresh weight g. $segment^{-1}$	Size of callus
2,4-D	BA			
0.0	0.5	0	0.120 c	- -
1.0		50	1.110 b	+ +
2.0		100	1.520 b	+ + +
3.0		100	2.036 a	+ + +

10 explant/tractment for callus. 10 callus segments/tractment for calculation of fresh weight.

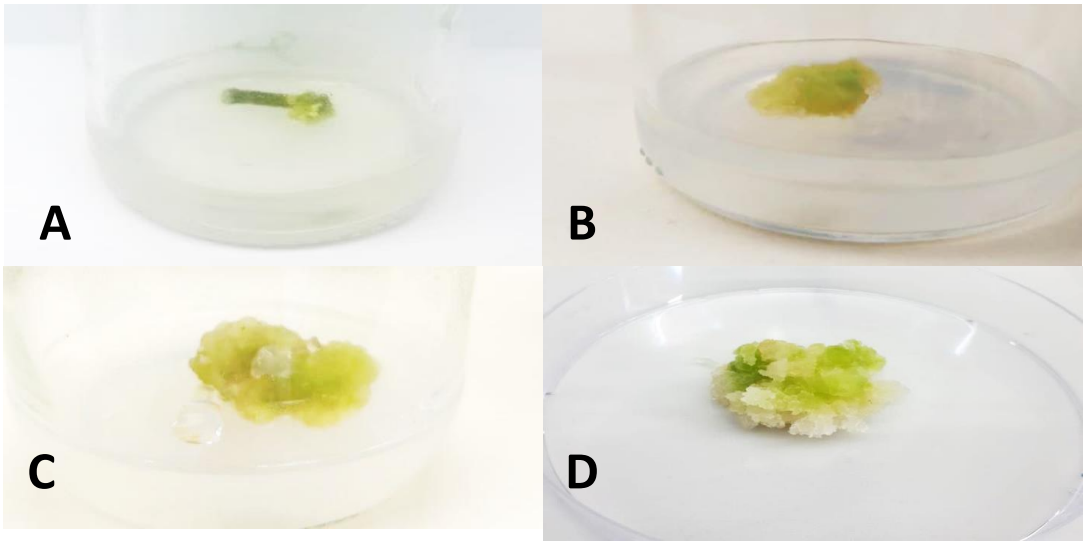

Figure 1. callus induction from stem explant of *R. chalepensis* using different concentrations of the growth regulators 2,4-D, combined with 0.5 mg/L BA. (A): Control treatment. (B): Callus on MS medium + 1.0 mg/L 2,4-D + 0.5 mg/L BA. (C): Callus on MS medium + 2.0 mg/L 2,4-D + 0.5 mg/L BA. (D): Callus on MS medium + 3.0 mg/L 2,4-D + 0.5 mg/L BA

Auxins influence the cell wall by promoting its disintegration and enhancing nucleic acid metabolism, particularly RNA, which plays a crucial role in protein synthesis necessary for cell

division and proliferation. In contrast, cytokinins regulate protein synthesis and carbohydrate metabolism, leading to the stimulation of cell division (Ljung 2013).

Cytokinins contribute to an increase in callus fresh weight by promoting cell division, particularly in parenchymal cells, which dedifferentiate into meristematic cells and facilitate tissue growth. This process occurs in various plant organs, regardless of whether they remain attached to the mother plant or are cultured in sterile nutrient media (Santner et al. 2009; Tahir et al. 2011).

According to Liao et al. (2015), both auxins and cytokinins are required in callus induction media, as cytokinins act as key regulators of cell division in the presence of auxins. However, adenine, a component of cytokinin molecules, may disrupt the optimal hormonal balance, affecting callus development.

Sandhya and Rao (2016) highlighted the significance of 2,4-D in callus induction, as it promotes an increase in callus size by stimulating both cell division and expansion. This expansion is primarily due to increased water uptake, which influences cell elongation and results in a friable callus texture.

The present study confirms that MS liquid medium supplemented with 3.0 mg/L 2,4-D and 0.5 mg/L BA is suitable for establishing cell suspension cultures from friable stem-derived callus. The highest cell proliferation was observed on the eighth day after initiation in MS medium fortified with 2,4-D and BA. The suspension cultures contained individual cells, while cell masses were absent. The medium effectively supported cell growth, with the first division occurring 24 hours after initiation. This was evident from the increase in cell density, which rose from 5.6×10^5 cells/cm³ at the start to 20.2×10^5 cells/cm³ by the eighth day (Figure 2).

When culturing different densities of callus, derived from cell suspensions using the plating method, on MS medium supplemented with 3.0 mg/L 2,4-D and 0.5 mg/L BA, variations were observed in the average number of formed cellular colonies and their subsequent development into callus primordia (Table 2). The highest significant number of cellular colonies was recorded at a culturing density of 20.2×10^5 cells/cm³, reaching an average of 84.61 colonies per dish. In contrast, the initial density yielded only 18.47 colonies per dish. These findings indicate that the development of cellular colonies into callus primordia increased proportionally with culturing density.

The highest significant number of callus primordia (70.24 primordia per dish) was also observed at the density of 20.2×10^5 cells/cm³ (Figure 3D), whereas the initial density of 5.6×10^5 cells/cm³ (Figure 3C) resulted in an average of only 4.51 primordia per dish.

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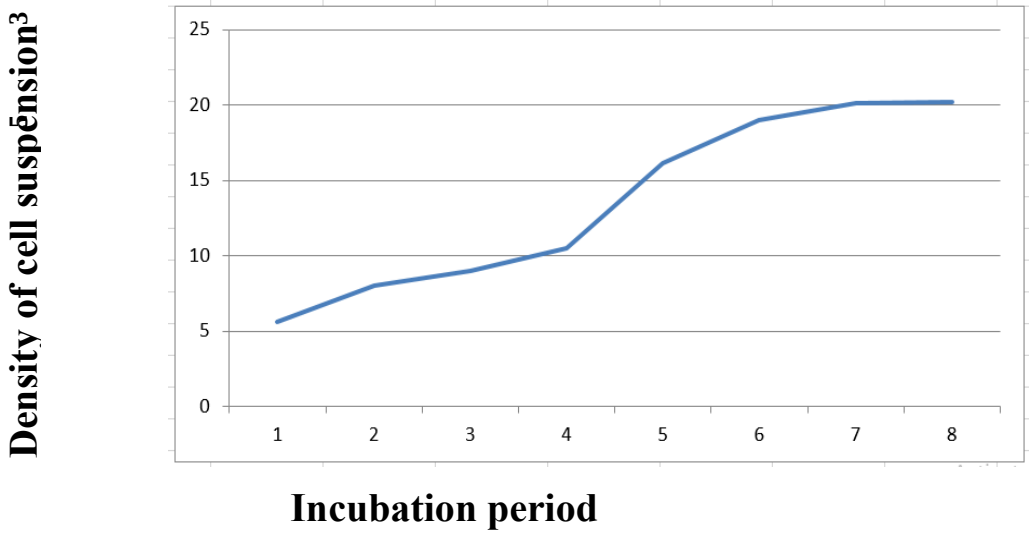

Figure 2. Growth profiles of the cell suspensions derived from stem friable callus of *R. chalepensis* on MS medium enforced with 3.0 mg/L 2,4-D+ 0.5 mg/L BA. Incubation period (day) and density of cell suspension ($\times 10^5$ cell Cm^3)

Light microscopy examination revealed that the cultured cell suspensions initiated their first division within 24 hours, forming daughter cells (Figure 3A). Continuous cell division led to the formation of cellular colonies consisting of numerous cells (Figure 3B). After 12 days, these colonies further developed into callus primordia, which appeared as pale green tissue segments visible to the naked eye on the surface of the medium. The time required for this process ranged from 22 to 27 days, depending on the culturing density.

Callus derived from cell suspensions cultured using the plating method on solid MS medium supplemented with 3.0 mg/L 2,4-D and 0.5 mg/L BA exhibited a friable texture and a green color. Continuous periodic maintenance of callus primordia, formed from stem cell suspensions using the plating method, resulted in a 90% response rate of transferred segments and an average callus primordia weight of 0.59 g after 30 days on the same supplemented medium.

To promote further growth and establish callus cultures, small segments were transferred into glass jars containing 20 mL of the same medium. Additionally, shoot development from small somatic embryos was observed, as shown in Figure 4 (A–E).

Table 2. Effect of culturing different densities of cellular suspensions derived from callus of stem segment of *R. chalepensis* on solid MS medium enriched with 3.0 mg/L 2,4-D+ 0.5 mg/L BA.

Culturing density	Plating method

($\times 10^5$ Cell . cm^3)	Average number of cellular colonies per dish	Average number of callus Primordia per dish
5.6 (initiation density)	18.47 e	4.51 c
8	30.61 de	14.9 bc
9	36.33 cde	15.51 bc
10.5	30.61 de	18.09 bc
16.1	42.04 cd	21.66 bc
19	57.04 bc	28.51 bc
20.1	63.47 b	38.51 ab
20.2	84.61 a	70.24 a

10 repeats/density, Averages with similar letter do not show significant differences.

Cell suspensions represent a significant advancement in plant biotechnology, serving as a valuable system not only for callus induction but also for monitoring individual cell division, growth, and differentiation (Rashid and Qasim 2006).

This study demonstrated that using liquid MS medium supplemented with 3.0 mg/L 2,4-D and 0.5 mg/L BA was highly effective in initiating cell suspensions while maintaining biomass. The success of cell suspension initiation can be attributed to several factors, including the friable texture of the callus, the quantity of callus used, and the continuous agitation of the liquid medium, which facilitated uniform distribution of nutrients and cells. The movement of the liquid medium contributed to the disintegration of the callus, leading to the formation of a well-dispersed cell suspension. Cell division in suspension cultures was observed to be higher during the exponential growth phase compared to callus cultures (Ramawat and Merillon 2019).

In this study, callus primordia were successfully obtained by culturing cell suspensions using the plating method (Dunstan et al., 1982) in MS medium supplemented with 3.0 mg/L 2,4-D and 0.5 mg/L BA. The average number of callus primordia per dish was 70.24, derived from an average of 84.61 colonies per dish by the eighth day at an initial cell density of 20.2 × 10^5 cells/cm^3. In contrast, a lower initiation density of 5.6 × 10^5 cells/cm^3 resulted in an average of 4.51 callus primordia per dish, originating from 18.47 colonies per dish.

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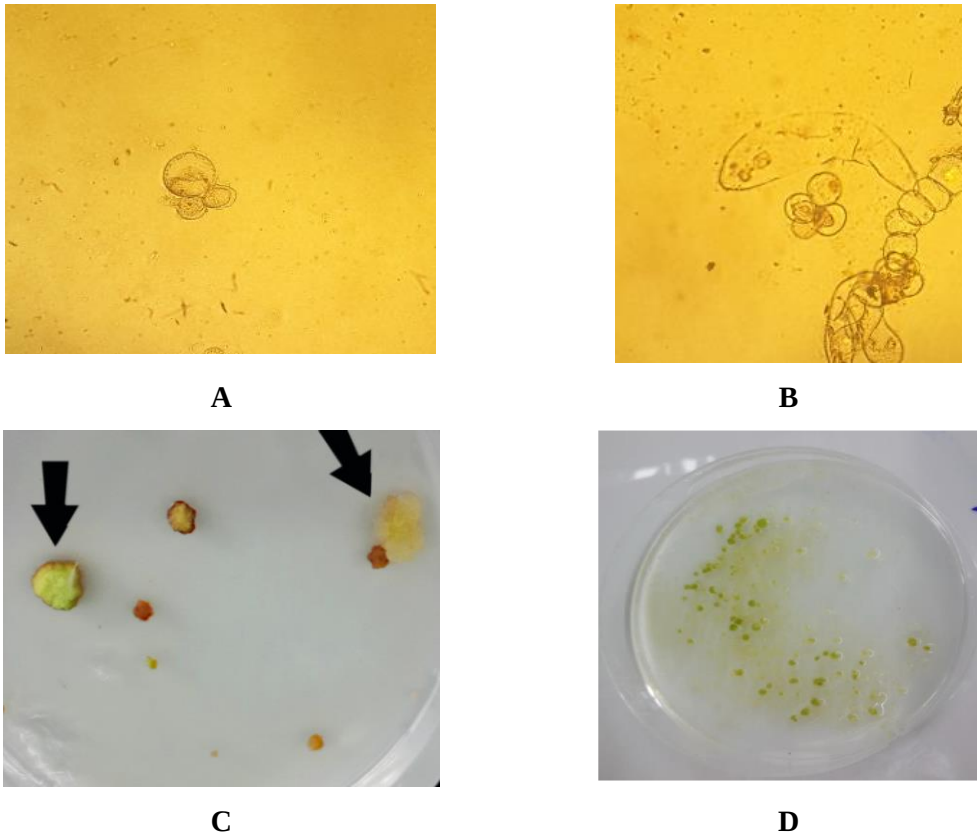

Figure 3. Stages of callus primordia formation from culturing cellular suspensions derived from stem callus of *R. chalepensis* L on solid MS medium enforced with 3.0 mg/L 2,4-D+ 0.5 mg/L BA by plating method. A: First division of some cells of cell suspension distributed on the solid MS medium after 24 h, B: Cellular colony resulted from (A) continued during 12 days, C: Callus Primordia formed from culturing initiation density of 5.6×10^5 cell. cm^3 of cell suspensions after 26 days, D: Callus Primordia formed from culturing density of 20.2×10^5 *c*ell cm^3 of cell suspensions after 25 days

2,4-D is recognized as one of the most effective auxins for stimulating the division of undifferentiated cells. It plays a crucial role in promoting callus induction and growth, with its activity localized within the cell wall, plasma membrane, and nucleic acids (Li et al. 2022). The continuous cell division leading to small callus segment formation can be attributed to the high interaction among individual cells in the culture (Lee and Wetzstein 1988).

Cell density significantly influences the development of cellular colonies into callus primordia. It was observed that low culturing densities inhibited the development of cell colonies into callus primordia, as cells failed to divide in both liquid and solid media. The ability to initiate a new culture depends on the initial cell density, which represents the minimum inoculum quantity required per unit volume of medium. Several factors, including the physiological properties of

the inoculum, duration of culture, environmental conditions, and medium composition, determine the optimal initial cell density (Neumann et al. 2020).

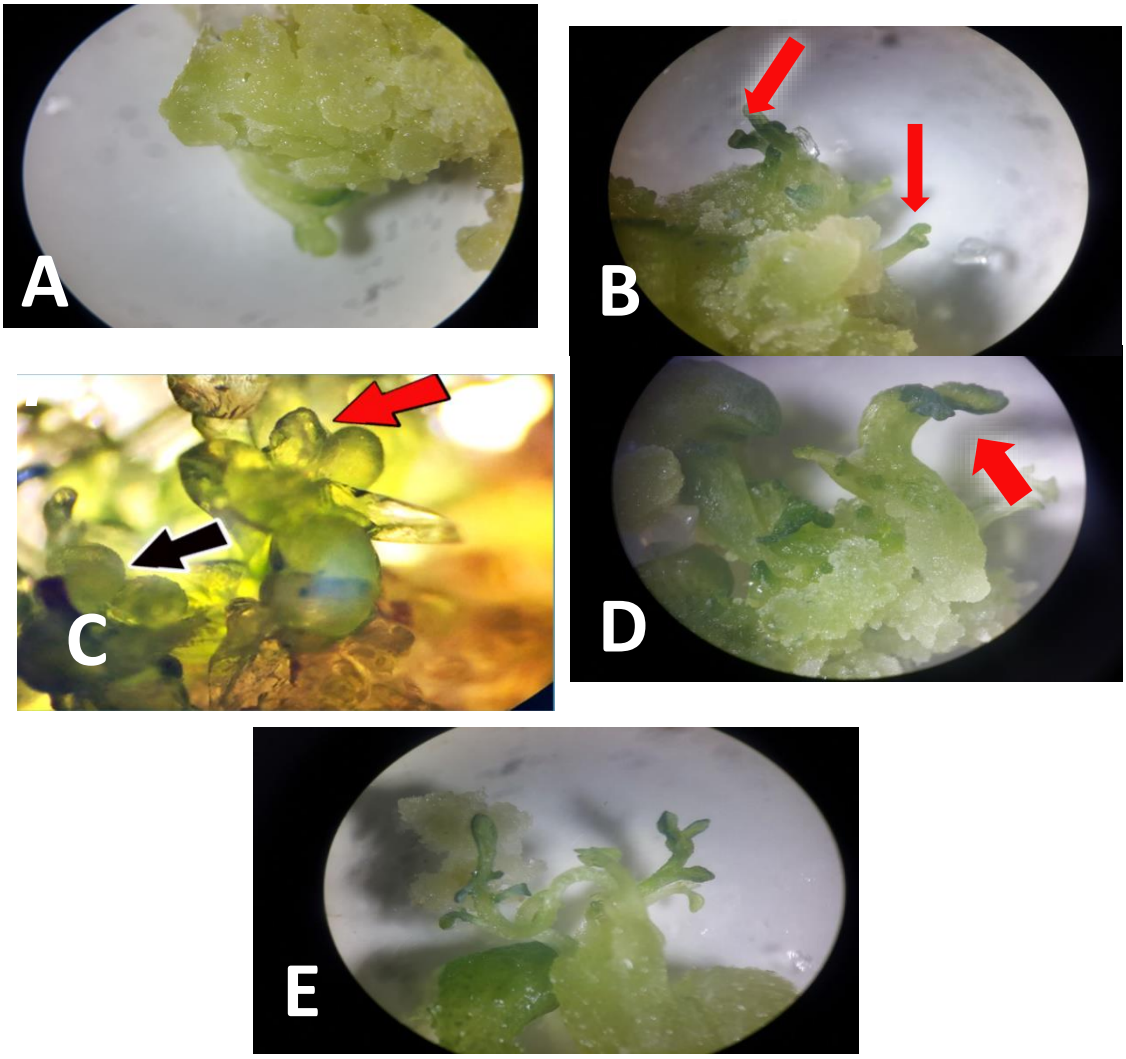

Figure 4. Maintenance of cell suspensions callus on MS medium fortified with 3.0 mg/L 2,4-D+ 0.5 mg/L BA by plating method and regeneration of *R. chalepensis* L. cell suspensions callus after 30 days Maintenance. (A): Emergence of global stage. (B): Torpedo stage. (C): Cardiac stage. (D): Emergence of dicotyledonous leaves. (E): Development of shoot from.

Previous studies have reported that plating cell suspensions of Withania somnifera on MS medium containing 3.0 mg/L 2,4-D and 0.5 mg/L BA successfully induced callus formation, characterized by its friable texture and yellow to pale brown coloration (Alwan 2016). The results of this study also indicated that callus derived from cell suspensions developed more rapidly than that obtained from stem segment cultures. This could be due to differences in nutrient

accessibility, as not all cells in callus tissues are in direct contact with the medium. In contrast, all cells in suspension cultures have direct access to nutrients and precursor compounds, enhancing their development. Consequently, callus derived from explants requires a re-culture period of 4–8 weeks, whereas callus originating from cell suspensions develops within 7–21 days (Ramawat and Merillon 2019). Furthermore, callus derived from suspension cultures is likely initiated from individual or undifferentiated cell masses, which differ from the differentiated cells of explants. This characteristic may contribute to the resilience and adaptability of the callus (Kirakosyan and Kaufman 2009).

The emergence of somatic embryo stages in this study can be linked to genetic factors, tissue type, developmental stage, plant growth regulators, and their concentrations in the medium. 2,4-D is the most commonly used auxin for inducing somatic embryo formation, potentially due to its role in ethylene accumulation, which contributes to embryo initiation. Additionally, the callus from which the embryo develops may have originated from pro-embryogenic masses, characterized by their ability to divide in response to organic compounds such as the auxin-to-cytokinin ratio (Tameshige et al. 2015). Moreover, the spontaneous shoot formation observed in this study may be attributed to the maintenance medium used for callus primordia of *R. chelepensis* L., which demonstrated the totipotency of the plant's cells. This was further supported by endogenous growth regulators present within the callus (Kumari et al. 2020). These findings align with the results of Zuraida et al. (2015), who reported successful in vitro regeneration of *Pelargonium radula* plantlets on MS medium containing 0.2 mg/L 2,4-D.

Additionally, the study found that shoots derived from the maintenance of cell suspension callus, when separated and vertically transferred to full-strength MS medium, readily developed roots (Table 3).

Table 3. Rooting of shoot of *R. chalepensis* L. on full strength solid MS medium

Planted shoots rooted	Average root number. branch^{-1}	Average root length. branch^{-1} (cm)	Rooting (%)	Rooting period (day)
60/60	9.68	6.20	100	16

The full-strength solid MS medium resulted in 100% rooting of the planted shoots, which developed into complete plants within 16 days. The newly formed branches continued to grow, producing an interconnected root system with a branched, thread-like structure after 24 days.

Healthy rooted plantlets were carefully removed, washed with tap water to eliminate residual agar from the roots, and transferred to pots containing a mixture of soil and fertilizer.

The successful rooting of shoots in full-strength MS medium without plant growth regulators may be attributed to the presence of endogenous auxins within the plant segments at levels sufficient to induce root formation. Additionally, the complete composition of the MS medium may contribute to this process (Al-Nema and Al-Mallah 2014; Ravanfar et al. 2014). Abdullah et al. (2021) reported that spontaneously developed shoots from stem callus of *Brassica oleracea* var. *italica* exhibited 100% rooting in full-strength MS medium, with continuous growth and the formation of well-developed root systems.

Conclusions: This study successfully established an efficient in vitro regeneration protocol for *Ruta chalepensis L.* through somatic embryogenesis from callus derived via cell suspension cultures using the plating method. The optimized conditions for callus induction and cell suspension culture resulted in a significant increase in cellular colony formation and callus primordia development, demonstrating the potential of this technique for large-scale propagation. Moreover, the regenerated shoots exhibited a high rooting percentage and successful acclimatization, confirming the effectiveness of the proposed method. These findings provide a valuable biotechnological approach for the conservation and sustainable production of *Ruta chalepensis*, facilitating its pharmaceutical and medicinal applications.

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References

Abdullah RM, Salih SM, Al-Nema QS (2021) Plant regeneration from transformed tissues of Broccoli (Brassica oleracea var. italica) by Agrobacterium rhizogenes ATCC13332 and their retention of rol-Genes. Eur Acad Res 9(9), 6006-6017.

Ahmad TA, Al-Mahdawe MM, Nadir DS (2020) Effect of methyl jasmonate on the production of furanocoumarins in cell suspension cultures of Ruta graveolens L. Plant Cell Tiss Organ Cult 143(3), 565-571.

Akkari H, Ezzine O, Dhahri SB, et al. (2015) Chemical composition, insecticidal, and in vitro antihelmintic activities of Ruta chalepensis (Rutaceae) essential oil. Ind Crop Prod 74, 745-751.

Al-Mahdawe M, Al-Mallah M (2015) Regeneration of the medicinal plant Ruta graveolens L. from hypocotyl. Eur Acad Res 2, 16256-16263.

Al-Mahdawe MM, Ahmad TA, Nadir DS (2018) Effect of different cell suspension culture densities of Physalis angulata L. by using plating or embedding methods on callus formation. J Biotech Res Cent 12(1), 25-32.

Al-Majmaie S, Nahar L, Sharples GP, et al. (2019) Isolation and antimicrobial activity of rutin and its derivatives from Ruta chalepensis (Rutaceae) growing in Iraq. Rec Nat Prod 13, 64-70.

Al-Nema QS, Al-Mallah MK (2014) Establishment of rapid propagation cultures of Agrobacterium rhizogenes-mediated transgenic hairy roots in sugar beet. Eur Acad Res 3(12), 12673-12682.

Alotaibi SM, Saleem MS, Al-Humaidi JG (2018) Phytochemical contents and biological evaluation of Ruta chalepensis L. growing in Saudi Arabia. J Saudi Pharm 26, 504-508.

Alwan BM (2016) Comparison between planting and landfill methods in steroid withanolide production in cellular suspensions of Withania somnifera. MSc Dissertation, College of Education for Pure Sciences, University of Diyala.

Amirteymoori E, Khezri A, Dayani O, et al. (2021) Effects of linseed processing method (ground versus extruded) and dietary crude protein content on performance, digestibility, ruminal fermentation pattern, and rumen protozoa. Ital J Anim Sci 20(1), 1506-1517.

Babu-Kasimala M, Tukue M, Ermias R (2014) Phytochemical screening and antibacterial activity of two common terrestrial medicinal plants Ruta chalepensis and Rumex nervosus. Bali Med J 3, 116-121.

Coleman J, Evans D, Kearns A (2003) Plant Cell Cultures. 1st ed. Taylor and Francis. pp. 208.

Dakah A, Zaid S, Suleiman M, Abbas S, Wink M (2014) In vitro propagation of the medicinal plant Ziziphora tenuior L. and evaluation of its antioxidant activity. Saudi J Biol Sci 21, 317-323.

Dewanti P, Widuri LI, Alfian FN, et al. (2016) Rapid propagation of virus-free sugarcane (Saccharum officinarum) by somatic embryogenesis. Agric Sci Procedia 9, 456-461.

Dunstan DI, Short KC, Merrik MA, Collin HA (1982) Origin and early growth of celery embryos. New Phytol 91, 121-128.

Feher A (2019) Callus, dedifferentiation, totipotency, somatic embryogenesis: What these terms mean in the era of molecular plant biology? Front Plant Sci 10, e536.

Gűnaydin K, Savci S (2005) Phytochemical studies on Ruta chalepensis (LAM) Lamarck. Nat Prod Res 19, 203-210.

Hajalizadeh Z, Dayani O, Khezri A, et al. (2019) The effect of adding fennel (Foeniculum vulgare) seed powder to the diet of fattening lambs on performance, carcass characteristics and liver enzymes. Small Rumin Res 175, 72-77.

Jafari Ahmadabadi SAA, Askari-Hemmat H, Mohammadabadi M, et al. (2023) The effect of Cannabis seed on DLK1 gene expression in heart tissue of Kermani lambs. Agric Biotechnol J 15 (1), 217-234.

Jaradat N, Adwan L, Kaibni S, et al. (2017) Variability of chemical composition and antimicrobial and antioxidant activities of Ruta chalepensis leaf essential oils from three Palestinian regions. Biomed Res Int 9, 267-268.

Khazal RM (2017) Impact of Ruta chalepensis ethanol extract in ochratoxin-treated male rats: a biochemical and histopathological study. Asian J Appl Sci 5(3), 566-572.

Kirakosyan A, Kaufman PB (2009) Recent Advances in Plant Biotechnology. New York, pp. 404.

Kumari SMP, Saravanan S, Pillai MA (2020) In vitro propagation of medicinally valuable traditional banana cultivar, Musa acuminata cv. Matti by shoot tip culture. Int J Curr Microbiol App Sci 9(8), 2240-2250.

Lee N, Wetzstein HY (1988) Protoplast isolation and callus production from leaves of tissue-cultured Vitis spp. Plant Cell Rep 7, 531-534.

Li W, Keynia S, Belteton SA, et al. (2022) Protocol for mapping the variability in cell wall mechanical bending behavior in living leaf pavement cells. Plant Physiol 188(3), 1435–1449.

Liao CY, Smet W, Brunoud G, et al. (2015) Reporters for sensitive and quantitative measurement of auxin response. Nat Methods 12(3), 207–210.

Ljung K (2013) Auxin metabolism and homeostasis during plant development. Development 140, 943–950.

Loyola-Vargas VM, Ochoa-Alejo N (2018) Plant Cell Culture Protocols. 4th ed. Humana Press, New York, pp. 507.

Mohammad AMS, Abood SA (1989) Propagation of lettuce (Lactuca sativa L. cv. longiflora) by tissue culture. Agric Biol Chem 51, 1320-1324.

Mohammadabadi M, Babenko O, Borshch OO, et al. (2024) Measurement of the relative expression pattern of the UCP2 gene in different tissues of the Raini Cashmere goat. Agric Biotechnol J 16(3), 317-332.

Mohammadabadi M, Golkar A, Askari Hesni M (2023) The effect of fennel (Foeniculum vulgare) on insulin-like growth factor 1 gene expression in the rumen tissue of Kermani sheep. Agric Biotechnol J 15(4), 239-256.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant 15, 473-497.

Nadir DS, Ibrahem MM, Ahmad TA (2021) Effect of methyl jasmonate on the production of furanocoumarins in callus cultures of Ruta graveolens L. Indian J Ecol 48(3), 950-954.

Neumann KH, Kumar A, Imani J (2020) Plant Cell and Tissue Culture—A Tool in Biotechnology: Basics and Applications. 2nd ed. Springer, pp. 650.

Ouerghemmi I, Bettaieb Rebey I, Rahali FZ, et al. (2017) Antioxidant and antimicrobial phenolic compounds from extracts of cultivated and wild-grown Tunisian Ruta chalepensis. J Food Drug Anal 25, 350-359.

Qahtan AA, Faisal M, Alatar AA, Abdel-Salam EM (2021) High-frequency plant regeneration, genetic uniformity, and flow cytometric analysis of regenerants in Ruta chalepensis L. J Plant 10(2820), 1-20.

Ramawat KG, Merillon JM (2019) Biotechnology, Secondary Metabolites. 2nd ed. CRC Press, pp. 586.

Rashid GH, Qasim SW (2006) The role of thermal treatment in the preparation of cellular division and callus formation from cellular suspensions of sunflower (Helianthus annuus) in multiple agar drops. J Al-Rafidin Agric 24(2).

Ravanfar SA, Aziz MA, Rashid AA, Salim S (2014) In vitro adventitious shoot regeneration from cotyledon explants of Brassica oleracea subsp. italica and Brassica oleracea subsp. capitata using TDZ and NAA. Pak J Bot 46, 329–335.

Safaei SMH, Dadpasand M, Mohammadabadi M, et al. (2022) An Origanum majorana Leaf Diet Influences Myogenin Gene Expression, Performance, and Carcass Characteristics in Lambs. Animals 13(1), e14.

Sandhya H, Rao S (2016) Role of growth regulators on in vitro callus regeneration in Physalis minima L. Int Lett Nat Sci 44, 38-44.

Santner A, Calderon-Villalobos LIA, Estelle M (2009) Plant hormones are versatile chemical regulators of plant growth. Nat Chem Biol 5, 301-307.

Sathish S, Venkatesh R, Safia N, Sathishkumar R (2018) Studies on growth dynamics of embryogenic cell suspension cultures of commercially important Indica rice cultivars ASD16 and Pusa basmati. 3 Biotech 8(4), 194.

Shahsavari M, Mohammadabadi M, Khezri A, et al. (2022) Effect of Fennel (Foeniculum Vulgare) Seed Powder Consumption on Insulin-like Growth Factor 1 Gene Expression in the Liver Tissue of Growing Lambs. Gene Express 21(2), 21-26.

Shahzad A, Sharma S, Parveen S, et al. (2017) Historical perspective and basic principles of plant tissue culture. In: Plant Biotechnology: Principles and Applications; edited by Abdin MZ, Kiran U, Kamaluddin A. Springer, Singapore, pp. 1-36.

Shokri S, Khezri A, Mohammadabadi M, Kheyrodin H (2023) The expression of MYH7 gene in femur, humeral muscle and back muscle tissues of fattening lambs of the Kermani breed. Agric Biotechnol J 15(2), 217-236.

Sugimoto K, Gordon SP, Meyerowitz EM (2011) Regeneration in plants and animals: Dedifferentiation, transdifferentiation, or just differentiation? Trends Cell Biol 21, 212-218.

Tahir SM, Victor K, Abdulkadir S (2011) The effect of 2,4-Dichlorophenoxyacetic acid (2,4-D) concentration on callus induction in sugarcane (Saccharum officinarum). Niger J Basic Appl Sci 19(2), 213-217.

Tameshige T, Hirakawa Y, Torii KU, Uchida N (2015) Cell walls as a stage for intercellular communication regulating shoot meristem development. Front Plant Sci 6, 324.

Thorpe TA (2013) History of plant cell culture. In: Plant Tissue Culture, 3rd ed.; edited by Smith RH. Academic Press, San Diego, CA, USA, pp. 1-22.

Trigiano RN, Gray DJ (2010) Plant Tissue Culture, Development and Biotechnology. 1st ed. CRC Press, pp. 608.

Vahabzadeh M, Chamani M, Dayani O, et al. (2020) Effect of Origanum majorana leaf (Sweet marjoram) feeding on lamb's growth, carcass characteristics and blood biochemical parameters. Small Rumin Res 192, e106233.

Wójcikowska B, Gaj MD (2017) Expression profiling of Auxin response factor genes during somatic embryogenesis induction in Arabidopsis. Plant Cell Rep 36, 843–858.

Zuraida AR, Shukri MAM, Sabrina MNE, Ayu Nazreena OA (2015) Improvement of regeneration of Pelargonium radula via somatic embryogenesis. Br Biotechnol J 5(4), 166-173.

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بازسازی و حفاظت Ruta chalepensis L. در شرایط آزمایشگاهی از طریق کشت سوسپانسیون سلولی و روش plating

فرح قاسم علی

*نویسنده مسئول: گروه زیست شناسی، دانشکده آموزش علوم محض، دانشگاه دیالی، عراق. آدرس پست الکترونیکی: mn76cd@gmail.com

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چکیده

هدف: Ruta chalepensis L.، که معمولاً به نام سرمه‌ای حاشیه‌دار شناخته می‌شود، یک گیاه دارویی از خانواده Rutaceae است. به طور گسترده‌ای به دلیل خواص دارویی متنوع خود از جمله فعالیت‌های ضد میکروبی، ضد قارچی، ضد التهابی و آنتی اکسیدانی شناخته شده است. این گیاه سرشار از ترکیبات فعال زیستی مانند آلکالوئیدها، فلاونوئیدها، کومارین‌ها و اسانس‌ها است که هم در طب سنتی و هم در کاربردهای دارویی مدرن ارزش قابل توجهی دارد. با این حال، جمعیت های طبیعی R. chalepensis به دلیل تخریب زیستگاه، برداشت بیش از حد، و زنده ماندن کم بذر با تهدیداتی روبرو هستند که نیاز به تکثیر و استراتژی‌های حفاظتی جایگزین دارد. بنابراین، این مطالعه با هدف بهینه‌سازی باززایی Ruta chalepensis L. در شرایط آزمایشگاهی با ایجاد کشت‌های سوسپانسیون سلولی و باززایی گیاهان با استفاده از روش plating انجام شد.

مواد و روش‌ها: القای پینه از ریزنمونه های ساقه R. chalepensis کشت شده بر روی محیط کشت Murashige و Skoog (MS) همراه با غلظت های مختلف (۰.۰، ۱.۰، ۲.۰ و ۳.۰ میلی گرم در لیتر) اسید ۲،۴–دیکلروفنوکسی استیک (2.4-D) در ترکیب با ۰/۵ mg/L بنزیل آدلیل (BA) به دست آمد. بالاترین وزن کالوس تازه (۲/۰۳۶ گرم) در ۳/۰ میلی گرم در لیتر 2.4-D +۰/۵ میلی‌گرم در لیتر BA پس از چهار هفته کشت مشاهده شد. کالوس القا شده بافتی شکننده نشان داد و متعاقباً برای شروع کشت سوسپانسیون سلولی در محیط MS مایع همراه با همان غلظت تنظیم‌کننده‌های رشد گیاه مورد استفاده قرار گرفت.

نتایج: روش plating برای کشت سوسپانسیون‌های سلولی در تراکم‌های مختلف در محیط القایی جامد، ترویج تقسیم سلولی و تشکیل کلنی‌های سلولی استفاده شد. این کلنی‌ها به کالوس پریموردیا تبدیل شدند که بعداً به قطعات کوچک کالوس تبدیل شدند. بیشترین میانگین تعداد کلنی‌های سلولی (۸۴.۶۱ در هر ظرف) در تراکم سلولی 10^5 × 20.2 سلول در میلی لیتر ثبت شد که به

طور قابل توجهی از تراکم اولیه ۱۸.۴۷ کلنی در هر ظرف فراتر رفت. این کشت با چگالی بالا همچنین به طور متوسط ۷۰.۲۴ کالوس پریموردیا در هر ظرف پس از ۲۷ روز منجر شد. علاوه بر این، تشکیل شاخساره مشاهده شد که ۶۰ شاخه با موفقیت پس از هفت هفته از کالوس مشتق شده از سوسپانسیون سلولی با استفاده از روش plating به دست آمد. این شاخساره‌ها به مدت ۳۰ روز در محیط کشت MS حاوی همان غلظت تنظیم کننده‌های رشد گیاه نگهداری شدند. ریشه زایی شاخه‌های بازسازی شده با میزان موفقیت ۱۰۰ درصد در محیط کشت MS با قدرت کامل به دست آمد و گیاهان تازه توسعه یافته تا هفته هشتم در گلدان در شرایط آزمایشگاهی سازگار شدند.

نتیجه‌گیری: شاخساره‌های بازسازی‌شده راندمان ریشه‌زایی بالا و سازگاری موفقیت‌آمیز را نشان دادند که اثربخشی این روش تکثیر در شرایط آزمایشگاهی را تأیید می‌کند. این یافته‌ها یک روش بیوتکنولوژیکی ارزشمند را برای حفاظت و تولید پایدار Ruta chalepensis ارائه می‌کند که از کاربردهای دارویی و درمانی آن پشتیبانی می‌کند.

واژه‌های کلیدی: پینه، جنین زایی جسمی، روش plating، گیاه دارویی

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