



Micro-propagation of a New Strain of Date Palm via Inflorescences Culture

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Abstract

The date palm (*Phoenix dactylifera* L.) is a fruit-bearing tree that demonstrates resilience to the harsh climatic conditions commonly found in the hot arid regions of the North Africa and Middle East. Inflorescence explants derived from date palm have demonstrated significant potential as a means for the micro-propagation of elite cultivars, as well as rare male and female specimens, due to the successful achievement of organogenesis and somatic embryogenesis. These plant materials are readily available each year, making them an economical and effective source of explants.

The objective of this research was to evaluate the effects of Picloram and Thidiazuron on optimizing callus formation and the induction of somatic embryos in date palm using inflorescence explants. The impact of various concentrations of Picloram and TDZ was analyzed concerning callus induction, browning rates, and the formation of embryogenic callus. The findings indicated that a concentration of 10.0 mg/l Picloram significantly enhanced the percentage of explants that induced callus formation (90.2%) while simultaneously reducing the degree of browning (1.0). The optimal induction of somatic embryos from floral parts was observed with a combination of 1.0 mg/l Picloram and 2.0 mg/l Thidiazuron, along with 0.1 mg/l ABA. In contrast, the highest percentage of embryo germination (54.0%) and the most significant number of embryos (26 embryos/culture) were recorded with a $\frac{3}{4}$ MS medium supplemented with 0.1 mg/l NAA, 0.25 mg/l BA, and an additional 0.2 mg/l ABA. Furthermore, the combination of 3.0 mg/l TDZ and 1.0 mg/l BA with 0.5 mg/l NAA was crucial in enhancing and regulating shoot regeneration. IBA is



recognized as a superior hormone for promoting root development. The acclimatization success rate for the plantlets that were cultivated reached 75%.

Keywords: Date palm, Inflorescences, *In vitro*, Picloram, Thidiazuron, Callus Formation, Somatic embryos and RAPD-based genetic relationships.

1. Introduction

The advancement of date palm farming in Egypt has been notably remarkable in recent years. Egypt ranks among the leading nations in the global export and production of dates. As noted by **Bayoumi et al. (2022)**, the area dedicated to date palm cultivation continues to grow annually. Recently cultivated regions such as Beheira, Nubaria, Kafr El-Shikh, Giza, Aswan, New Valley, Matrouh, North Sinai, and Wahat El-Baharya have contributed to the overall production volume. Projections indicate that Egypt is expected to account for approximately 6.02% of the high-quality date market during the period from 2022 to 2034, compared to the current global share of 1.9% (**Mahmoued et al., 2022**). Egypt holds the title of the world's leading date producer, with its dates representing 17.7% of total global production (**FAO, 2020**).

The date palm is recognized as one of the most significant fruit crops globally, valued for both its fruit and ornamental attributes (**Al-Mayahi, 2022**). The date palm, a perennial dioecious monocot belonging to the *Arecaceae* family, is often referred to as the "tree of life." It was among the earliest fruit crops extensively cultivated in North Africa and the Middle East. Additionally, it holds considerable economic importance in arid and warm regions (**Johnson, 2011**).

The new strain of date palm, known as Nag Hamadi 2, is approximately 25 years old and cultivated in Sheikh Ali Village, located in the Nag Hamadi City of Qena governorate, Egypt. Its favorable characteristics render it suitable for export, and it is regarded as one of the promising varieties of Egyptian date palms. The yield of dates from this palm is 225 kg per tree. Notably, this palm has not produced any offshoots. It was planted in unamended soil without any fertilization or irrigation, indicating a lack of care. The fruit is sizable, measuring 5.6 cm in length and 2.53 cm in diameter, with a weight of 30 g. When fully grown, the fruits become brownish-black color. The dates are composed of carbohydrates and proteins, quantified at 76.2 g/100g and 3.46 g/100g, respectively. The ash content and crude fiber levels were determined to be 0.87 g/100g and 6 g/100g, respectively. Analysis revealed that the carbohydrate composition is predominantly glucose and fructose, with no sucrose present (**Rasha et al., 2022**).

The date palm serves as a vital crop in arid and semi-arid regions, offering significant economic and nutritional advantages. Nevertheless, traditional propagation methods using seeds are often inefficient and unreliable. The reliance on seeds results in considerable genetic variation and an extended juvenile phase before fruiting, complicating the preservation of desirable traits



from the parent tree. The vegetative propagation of date palms typically involves offshoots, which are limited by low multiplication rates, genetic diversity, and vulnerability to both biotic and abiotic stresses. Tissue culture, especially somatic embryogenesis, presents an effective alternative for large-scale propagation, genetic enhancement, and the conservation of superior date palm genotypes (**Jaradat, 2016**).

Micro-propagation is the leading technological method employed to generate a significant number of genetically identical plants from a small tissue sample (such as a shoot tip, floral bud or calli) within a sterile and controlled environment (**Al-Qatrani et al., 2021**). This method has been widely employed for the large-scale cultivation of a range of plants, such as fruit-bearing trees, ornamental species, and timber-producing trees. The micro-propagation of date palms has been effectively accomplished through several tissue culture methods, including somatic embryogenesis, organogenesis, and callus culture (**Taha et al., 2021**). This approach has demonstrated its effectiveness in generating high-quality, disease-free and genetically uniform plantlets. Furthermore, it enables the rapid production of a large number of plantlets, which reduces the time required for the propagation of desirable traits and the establishment of new plantations (**Maher et al., 2021**). The techniques of micro-propagation have been established as an alternative method for the propagation of date palms (**Solangi et al., 2023**). As highlighted in a recent review by **Nimavat and Parikh (2024)**, the implementation of effective micro-propagation protocols is essential for the successful conservation and utilization of valuable date palm genotypes. They performed a comprehensive analysis of the *in vitro* culture protocols for date palms and investigated the factors influencing the development of multiple shoots, roots, and organs. Detailed information was presented concerning the optimal selection of explants, media formulations and the combinations of growth regulators required for successful propagation.

The micro-propagation of date palm from either the terminal apex or lateral buds has been practiced for many years. Recently, the flowering inflorescence has emerged as a viable alternative explant (**Taha et al., 2016**). The potential of explant inflorescences for culture was evaluated to facilitate the development of indirect somatic embryos of date palm (**Ribeiro and Teixeira, 2017**). This method is commonly utilized to generate normal plantlets from various other plant species (**Chen and Chang, 2002**). **Mirani et al. (2022)** noted that *in vitro* propagation using inflorescence explants yields a low percentage of variations or none at all during the fruiting stage. Considering factors such as genotype, age of the explant, and responses to auxin-cytokinin, the established protocols can be adapted for other cultivars (**Abul-Soad and Al-Khayri, 2018**).

The success of tissue culture is significantly influenced by plant growth regulators, as they control cell division and the differentiation of tissues and organs (**Jennifer et al., 2010**). Most



research in tissue culture has concentrated on the impact of various auxins and their concentrations on date palm explant cultures (**Othmani et al., 2009**). Plant growth regulators are crucial in the micro-propagation of date palm. Research on callus induction in the Barhee cultivar indicated that a concentration of 0.5 mg/l TDZ was optimal for all traits assessed (**Al-Asadi et al., 2019**).

The *in vitro* growth, differentiation, and regeneration of date palm are heavily reliant on the nutrient medium and the external application of plant growth regulators (**Hapsoro et al., 2020**). High concentrations of 2,4-D have been linked to the potential for somaclonal variation in regenerates (**Fki et al., 2011**). Consequently, utilizing alternative auxins like picloram at lower levels may serve as a suitable option to promote somatic embryogenesis without adverse effects on the regenerated plants (**Abahmane, 2010**). Picloram has also been employed for the induction of peach palm (**Steinmacher et al., 2007**). In the case of date palm, picloram has been utilized to induce somatic embryogenesis from inflorescence explants.

Thus, the primary objective of this research was to identify the optimal combination of plant growth regulators and cultural growth conditions to promote the initiation and regeneration of somatic embryos from mature female inflorescences, ultimately leading to plant production. Investigation of the genetic similarities between the leaves of regenerated shoots produced *in vitro* from inflorescence culture and spikelets from the mother palm of the new strain, known as Nag Hamadi 2, was examined at the molecular level using the RAPD technique.

2. Materials and Methods

This study was conducted in the Central Lab of Date Palm for Researches and Development - Agricultural Research Center, Egypt during the period from 2023 to 2025.

2.1- Plant material

The mature inflorescences were collected in March 2023 from an adult female mother palm located in Upper Egypt (Nag Hamadi city), **Fig. (1 and 2)**, which had been previously assessed (**Rasha et al., 2022**) for use as an explant in *in vitro* culture within the laboratory.

The spathes of different sizes (avg. 37 and 50 cm) were excised from new strain of date palm trees grown in Nag Hamadi City of Qena governorate, Egypt. The removed spathes were transferred to the laboratory for the purposes of sterilization and culturing.



Fig. 1. The date palm tree unknown in NagHamady City.



Fig. 2. The new strain of date palm fruits, NagHamady 2.

2.2- Explant preparation

The flowers were collected in March (**Fig. 3**). After the first 50 cm of flower appeared from the base of the leaves, they were separated and transferred to the laboratory. In a laminar air flow cabinet, the outer surfaces of the spathe and the cut surface were disinfected by soaking in 70% ethanol for 15 minutes. The spathes were then longitudinally dissected from both sides, gradually reaching the inflorescence bunch, which allowed for the complete removal of the outer hard cover of the spathe under sterile conditions. Subsequently, the intact inflorescence bunch was extracted from the spathe, and the inflorescences were sterilized using a 0.5% mercuric chloride solution for 30 minutes, followed by thorough rinsing three times with sterile distilled water. The spikelets were then cut into segments of 2–3 cm, each containing 3–5 flowers (**Fig. 4**), and placed horizontally as primary explants on the initiation media to enhance contact.





Fig. 3. The mature floral buds



Fig. 4. The spikelets of floral buds.

2.3- Media preparation and culture conditions

MS (Murashige and Skoog, 1962) medium comprising of various treatments of plant growth regulators specified to callus formation, somatic embryogenesis, germination and shoots formation.

The initiation medium consisted of $\frac{3}{4}$ MS medium combined with 0.1 mg/L pyridoxine, 0.5 mg/l nicotinic acid, 100 mg/l myo-Inositol, 0.1 mg/l Thiamine-HCl, 2.0 glycine, 2.0 biotin, 200 glutamine; 170 mg/l $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, Ca-pantothenate (0.2 g/l), 40 mg/l adenine sulphate, 2.0



g/l polyvinylpyrrolidone (PVP), 50 g/l sucrose, 6 g/l agar and 1.5 g/l activated charcoal. The pH was gauged at 5.7 ± 0.1 and charcoal and agar were added after measuring pH. The medium was sterilized in autoclave for a period of 20 minutes, at a temperature of $\pm 121^\circ\text{C}$ and pressure of 1 kgf cm^{-2} . After preparation, the nutritious medium was distributed in small jars (250 ml) at 40 ml per jar, covered with polypropylene caps, and these cultures were incubated in darkness at a constant temperature of $28 \pm 2^\circ\text{C}$.

2.4- Callus induction

The experiment used 6 different formulations of callus induction medium. The media contained varying concentrations of 4-amino-3,5,6-trichloropicolinic acid (Picloram) at 0.0, 0.5, 2.0, 5.0 and 10.0 mg/l and Thidiazouron (TDZ) at 0.5, 1, 2 and 3 mg/l, combined as treatments. The pH of the media was adjusted to 5.8 and the media were sterilized by autoclaving at 121°C for 20 min. The cultures were stored in a dark room at $26 \pm 2^\circ\text{C}$ until callus formation was observed. Each treatment included 9 replicates. The subcultures were performed every four weeks on fresh medium of the same composition for a total of five months. After this period, the flower bud swelling and callus formation were considered explant reactions. Swelling degree of explants, browning degree, percentage of response (number of explant induce callus formation) and culture fresh weight (g) were recorded after five months.

Browning and swelling were determined by degrees according to **Pottino (1981)** as followed: low (1), below average (2), average (3), good (4) and excellent (5).

2.5- Somatic embryos induction medium

When embryonic callus formation was observed, the callus were transferred to somatic embryos induction medium consisting of $\frac{3}{4}$ MS salts and vitamins, 40 mg/L adenine sulphate, 2.0 mg/l glycine, 2.0 mg/l biotin, 200 mg/l glutamine, 30 g/l sucrose, and 6.0 g/l agar supplemented with 0.05, 0.1, 0.5 and 1.0 mg/l picloram, 0.1, 0.5, 1.0 and 2.0 mg/l TDZ and 0.1 ABA to promote somatic embryos induction. The control (medium without growth regulators) was also used. All cultures were maintained under complete darkness for four months. The subcultures were performed every four weeks on fresh medium of the same composition for a total of four months.

Each treatment included 9 replicates having about 0.5 g embryogenic calli/jar. The percentage of calli differentiation, the number and length of somatic embryos induction were recorded after 16 weeks.

2.6- Germination medium

When somatic embryos formation was observed, the clusters of embryos (each one contains 3–4 embryos) were cultured on maturation medium consisting of $\frac{3}{4}$ MS salts and



vitamins, 30 g/l sucrose, 40 mg/l adenine sulphate, 2.0 mg/l glycine, 2.0 mg/l biotin, 200 mg/l glutamine, 6.0 g/l agar and 0.5 g/l activated charcoal supplemented with different concentrations of NAA at 0.05, 0.1, 0.5 and 1.0 mg/l with added different concentrations of BA at 0.1, 0.25, 0.5 and 1.0 mg/l + 0.2 mg/l ABA and then dispensed at 30 ml in small jars for 16 weeks to promote shoots regeneration. The cultured jars were kept for four re-cultures at four-week intervals, the cultures were then maintained in a controlled condition at a temperature of 28 ± 2 °C under low light intensity (500 lux). Nine replicates were used for each treatment. The germination percentage, number of somatic embryos, number of leaves and their length was recorded after 12 weeks in culture. After that, shoots were transferred to larger jars to complete growth and development.

2.7- Multiplication medium

Clusters of developed shoot (3–5 shoots) which resulted from maturation stage were transferred to liquid culture media to obtain healthy individual shoots for rooting stage. Liquid culture medium consists of $\frac{3}{4}$ MS medium supplemented with different concentrations of TDZ at (0.5, 1.0, 2.0 and 3.0 mg/l) and BA at (0.05, 0.1, 0.5 and 1.0 mg/l) with added 0.5 mg/l NAA, 35 g/l sucrose and 2.0 mg/l Ca-pentothionate with 0.2 g/l activated charcoal and dispensed into big jars (375 ml) at 45 ml/jar. All cultures were continued in liquid medium for 8 weeks (4-week interval) and incubated at 27 ± 2 °C with light intensity of 3000 lux. After that the cultures transferred to the control medium without any plant growth regulators to one month. The number of shoots, number of leaves and their length was recorded after 12 weeks in culture.

2.8- Elongation of shoots

All shoots from the previous treatments were transferred to elongation medium consist of $\frac{3}{4}$ MS medium with addition of 1.0 mg/l BA, 0.5 mg/l 2ip and 1.0 mg/l gibberellic acid (GA₃), 30 g/l sucrose, 5.0 mg/l thiamine HCL, 1.0 mg/l biotin, 100 mg/l glutamine and 0.5 mg/l activated charcoal, to increase the shoot length **Tamer et al. (2022)**. The cultures underwent sub-culturing every four weeks for a total of two subcultures. All cultures were maintained at an incubation temperature of 27 ± 2 °C and a light intensity of 2000 lux to facilitate the transfer to rooting media.

2.9- Rooting stage

Following the elongation phase, individual healthy shoots of appropriate length (3 to 4 cm) were isolated for cultivation on a rooting culture medium. This rooting medium consist of solid half-strength MS medium contained with 30 g/l sucrose, 2.0 mg/l glycine, 5.0 mg/l thiamine-HCl and 1.0 mg/l biotin, along with 0.5 g/l activated charcoal and 0.1 mg/l paclobutrazol. Additionally, varying concentrations of indole-3-butyric acid (IBA) at (1.0, 2.0 and 3.0 mg/l) were included, along with 0.5 mg/l BA, and different concentrations of naphthalene acetic acid (NAA) at (1.0, 2.0 and 3.0 mg/l) with 0.5 mg/l BA to evaluate their effects on root development. The medium,



solidified with 6.0 g/l of agar, was dispensed into large culture jars at a volume of 40 ml per jar, two shoot for each jar. The shoots were then incubated for 18 weeks (with refreshment every 6 weeks) to facilitate root formation at a temperature of $27\text{ }^{\circ}\text{C}\pm 2\text{ }^{\circ}\text{C}$ and a light intensity of 4000 lux (Hassan et al., 2021). The outcomes of rooting, including the number of roots, root length and the number of root hairs per root, were documented after a duration of 4 months.

2.10- Acclimatization

It is the final and most important step in the tissue culture process and the basis for effective plant propagation (Hazarika, 2003). For transplantation, healthy rooted plantlets were chosen, each having 1 to 3 leaves measuring approximately 10 to 12 cm in length. The healthy plantlets were carefully extracted from the jars, and any residual agar on the roots was thoroughly rinsed off under running tap water.

Next, the plantlets were soaked in a 0.5% (w/v) fungicide solution (Benlate 2 g/l) for 5 minutes. Following this, the plantlets were transplanted into plastic pots (5 cm in diameter and 18 cm in length) filled with a sterilized soil mixture of peat moss and perlite in a 2:1 (v/v) ratio. The pots were then placed under a plastic-sheet tunnel within the greenhouse.

Over a period of 8 weeks, the seedlings were kept at a natural daylight temperature of $27\pm 2\text{ }^{\circ}\text{C}$ and high relative humidity levels of 90-95%, achieved by using white, transparent polyethylene sheets (Mona et al., 2014). After one week, the polyethylene sheets were opened for ventilation for 10 to 15 minutes, and insecticide was applied; the openings were gradually enlarged over the following two weeks. Subsequently, the polyethylene sheets were slowly removed to help the plants acclimate to the greenhouse environment. The plantlets were watered weekly and treated with fungicide as necessary. After five months in *ex vitro* conditions, the acclimatized plants developed new leaves and roots. These plants were then moved to a shade house for further hardening for 6 to 8 months before being transferred to an open field.

Plant survival, defined as the percentage of plants that successfully transitioned from *in vitro* culture to *ex vitro* conditions, was assessed after nine months.

2.11- Total DNA extraction

To investigate the genetic similarities, the leaves of regenerated shoots and spikelets from the mother palm of the new strain, known as Nag Hamadi 2 were examined at the molecular level utilizing the RAPD technique, **Table (1)**.

Total DNA (deoxyribonucleic acid) was isolated from female flowers and *in vitro* leaves of shoots utilizing the Wizard Genomic DNA Purification Kit (Promega). The procedure was as follows: - around 50 mg of flowers were ground in liquid nitrogen, followed by the addition of 600



μ l of Nuclei Lysis solution, which was incubated at 65°C for 15 minutes. Subsequently, 3 μ l of RNase solution was introduced and incubated at 37°C for another 15 minutes, after which the sample was allowed to cool to room temperature for 5 minutes. A protein precipitation solution was subsequently introduced and incubated on ice for a duration of 5 minutes. Centrifugation was conducted at 16,000 g for 3 minutes. The supernatant was meticulously transferred to a clean tube containing isopropanol at room temperature, followed by mixing through inversion and centrifugation at 16,000 g for 1 minute. The supernatant was discarded, and 70% ethanol at room temperature was added prior to centrifugation at 16,000 g. The ethanol was aspirated, and the pellet was permitted to air-dry. A DNA rehydration solution was introduced, and rehydration took place at 65°C for 1 hour or overnight at 4°C. Total DNA was extracted from shoot leaves and female flowers as described by **Dellaporta *et al.* (1983)**. The stock of DNA samples was diluted in sterile distilled water to achieve a working concentration of 10 ng. μ l⁻¹ for application in PCR analysis.

2.11-1. DNA analysis utilizing the RAPD technique

This study employed eight microsatellite RAPD primers. The reaction mixture, totaling 20 μ l, contained 10 ng of DNA, 200 μ M deoxynucleotide triphosphates (dNTPs), 1 μ M of primer, 0.5 units of Red Hot Taq polymerase (AB-gene Housse, UK), and 10-X Taq polymerase buffer (AB-gene Housse, UK). Amplifications were conducted on total DNA using a 2700 thermo cycler (Applied Biosystems).

The resulting amplification products were analyzed via 6% polyacrylamide gel electrophoresis with TBE 1X buffer (**Sambrook *et al.*, 1989**). DNA bands were visualized by staining with ethidium bromide (1 μ g.ml/l). The reproducibility of the DNA profiles was evaluated by repeating all RAPD reactions at least three times with DNA markers. The primers were assessed through wise pair comparison to ascertain the proportion of shared bands that were amplified (**Nei, 1978**). The similarity coefficient was calculated using the statistical software package STATISTICA-SPSS (Stat Soft Inc.).

Table 1. RAPD primers with their sequences were used to assess genetic accuracy.

Primers	Sequences
OPA-01	TCGGCGATAG
OPA-02	TGCCGAGCTC
OPA-03	TGCGCCCTTC



Primers	Sequences
OPB-05	AGTCAGCCAC
OPB-06	AATCGGGCTG
OPB-08	ACCCGACCTG
OPE-01	GAAACGGGTG
OPE-03	GTGACGTAGG

2.12- Statistical analysis

The experiment followed a completely randomized block design as described by **Gomez and Gomez (1984)**. Data were analyzed using the MSTAT computer program (**MSTAT Development Team, 1989**). Treatment means were compared using Duncan's Multiple Range Test (**Duncan, 1955**) to identify significant differences.

3. Results

3.1- Callus induction

The media contained varying concentrations of picloram and TDZ were significant effect on swelling degree, response percentage, browning degree and culture fresh weight. Specifically, the swelling degree was maximized by the addition of picloram at 10.0 mg/l in combination with TDZ at concentration of 3.0 mg/l to the initiation medium, **Table (2)** and **Fig. (5)**. However, flowers that did not participate in callus initiation were less swelling, the swelling degree decreased significantly when the spikelets were cultivated on the initiation medium containing picloram at 0.5 mg/l with 0.5 mg/l of TDZ.

Table 2: Effect of combination between Picloram and TDZ on callus formation from inflorescences of date palm after five subcultures.

Treatments (mg/l)		Swelling degree of explants	Browning degree	Percentage explant induce callus formation	Culture fresh weight (g)
Picloram	TDZ				
0.0	0.0	0.5 e	3.2 a	10.3 e	0.6 e
0.5	0.5	1.3 d	2.5 b	28.2 d	1.2 d



2.0	1.0	2.6 c	2.0 c	40.5 c	2.3 c
5.0	2.0	4.3 ab	1.7 c	71.4 b	3.4 b
10.0	3.0	5.0 a	1.0 d	90.2 a	4.6 a

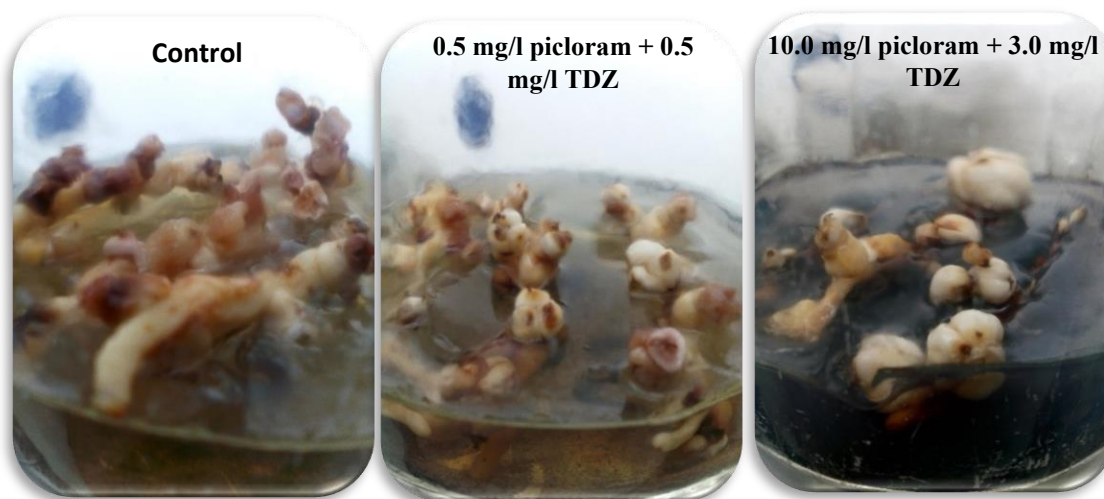


Fig. 5. Effect of combination between Picloram and TDZ on the swelling degree of floral buds.

The browning of the medium, attributed to phenolic compounds, occurs in inflorescence explants and is mitigated by transferring spikelets to fresh media every four weeks. In this context, it is essential to relocate explants to fresh media to prevent the adverse effects of oxidized phenolic compounds that appear from prolonged subculture periods, which lead to the blacken of the media.

The impact of varying concentrations of Picloram and TDZ on the degree of browning is presented in **Table (2)**. The data regarding the browning degree indicates significant differences among the various concentrations of Picloram treatments in combination with TDZ. The highest browning degree value (2.5%) was observed in mature flower cultures on media containing a low concentration of Picloram (0.5 mg/l) combined with 0.5 mg/l of TDZ. Conversely, the browning degree diminished with the application of 10.0 mg/l of Picloram and 3.0 mg/l of TDZ, resulting in the lowest browning level (1.0%). This finding implies that Picloram not only facilitates callus induction but also alleviates oxidative stress in tissue culture.

The highest response percentage of the explants 90.2% was obtained using MS medium supplemented with 10.0 mg/l picloram and 3.0 mg/l TDZ, supplementation with 5.0 mg/l picloram and 2.0 mg/l TDZ was the next most effective condition. Whereas the lowest values 28.2% were



achieved with 0.5 mg/l picloram and 0.5 mg/l TDZ. The concentrations of auxin and cytokinin had a demonstrable effect on the initiation stage. The information presented in **Table (2) and Fig (6)** suggests that the MS medium supplemented with a mixture of 10.0 mg/l picloram and 3.0 mg/l TDZ proved to be the most effective medium for the initiation phase.

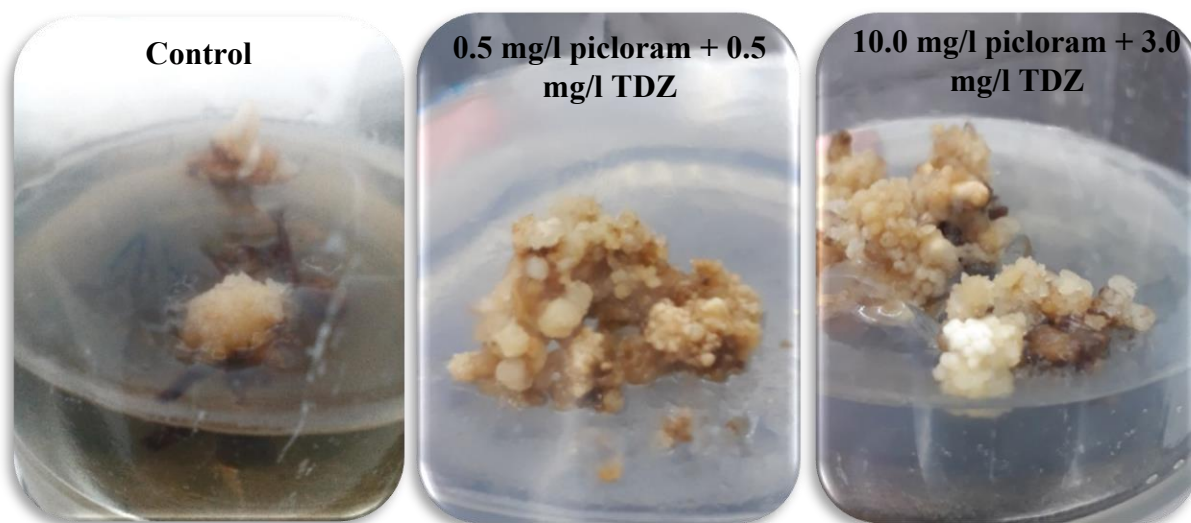


Fig. 6. Impact of combination between Picloram and TDZ on the callus induction from floral bud after five subcultures.

Similar conditions also maximized the culture fresh weight resulted in MS medium supplemented with 10.0 mg/l picloram and 3.0 mg/l TDZ which produced better results than other treatments, the highest culture fresh weight was 4.6 g/jar, whereas the lowest culture fresh weight 1.2 g/jar was achieved with 0.5 mg/l picloram and 0.5 mg/l TDZ.

Results clearly showed that Successful in vitro regeneration from mature female date palm inflorescences has been achieved. The efficiency of converting floral buds into vegetative shoots is influenced by several factors, including the developmental stage of the floral buds at the time of explant collection (**Zayed et al., 2016**) and the type and concentration of plant growth regulators present in the culture medium (**George et al., 2007**), and the specific physicochemical conditions applied during sub-culturing (**Kriaâ et al., 2012**).

Solangi et al. (2022) an extensive investigation was conducted on the micro-propagation of date palm utilizing inflorescence explants. The inflorescences are capable of used as explants, as indicated in the organogenesis of the Medjool, Barhee and Selmi varieties (**Taha et al., 2021**). The formation of callus on initial explants is affected by a variety of factors, such as the type of



explant, genotype, duration of culture and the type and concentration of plant growth regulators, as outlined by **Abul-Soad and Al-Khayri (2018)**. Furthermore, **Solangi et al. (2023)** emphasized that the presence of auxins in the media is crucial for the initiation of callus in initial explants.

Callus induction has been reported from nearly all categories of date palm tissues across different media formulations, which generally consist of a blend of an auxin and a cytokinin (**Al-Mayahi, 2013**).

While the auxin 2,4-D is frequently utilized for callus induction in palm trees, with a common concentration of 100 mg/l for date palm (**Al-Khayri, 2012**), higher concentrations may lead to somaclonal variation (**Fki et al., 2011**). Nevertheless, some research has indicated that lower concentrations can be more effective (**Saptari and Sumaryono, 2018**). Therefore, employing different types of auxins at varying levels to promote somatic embryogenesis in date palm cultivars could be advantageous. **Abahmane (2010)** reported that picloram could serve as alternative auxin in the *in vitro* culture of certain species without causing somaclonal variation in the regenerates.

Scherwinski-Pereira et al. (2010) discovered that applying Picloram at a concentration of 108.68 ppm effectively induced the formation of embryogenic callus on explants taken from the basal region of young plants. **Jayanthi et al. (2015)** achieved the highest callus induction rate of 82% by culturing immature male inflorescence explants on a medium containing 72.45 ppm of Picloram.

In the cultivation of date palm, Picloram has been employed to enhance callus culture and somatic embryogenesis, utilizing a concentration that range from 10.8 mg/l (for shoot tips) to 50 mg/l (for leaf explants) (**Mazri et al., 2018**). It was confirmed that the concentration of Picloram in the culture medium plays a vital role in the proliferation of callus on immature inflorescence explants of oil palm. Callus proliferation was first observed at the edges, with tissue swelling occurring at all concentrations of Picloram. Different concentrations of Picloram resulted in varying levels of callus proliferation. Notably, the use of 50 ppm Picloram raised the percentage of explants developing callus to 77.78% of the total cultured explants (**Kartika et al., 2019**).

TDZ emerged as the most effective growth regulator for inducing embryogenic callus, utilized at an effective concentration of 5 mg/l, in combination with various concentrations of 2,4-D and NAA to determine if TDZ alone suffices for embryogenic callus induction or direct somatic embryogenesis (**Al-Najm et al., 2018**). The callus derived from inflorescence in the TDZ medium exhibited a white color and granular texture, leading to the subsequent formation of globular opaque bodies. This type of callus is classified as primary embryogenic callus (**Jasim, 1999**).



The browning observed in plant tissues can be linked to the oxidation of various phenolic compounds present in these tissues, which convert to highly toxic quinones under the action of oxidation enzymes like peroxidase and polyphenol oxidase **Meziani et al. (2016)**. The browning of the medium caused by phenolic compounds in inflorescence explants can be minimized by transferring spikelets to fresh media every four weeks. It is essential to move the explants to new media to prevent the adverse effects of oxidized phenolic compounds that develop during prolonged subculture periods, which lead to media blackening. Additionally, calli proliferated on media devoid of antioxidants due to the lower levels of phenolic compounds in spikelet explants compared to shoot tip explants, which have higher phenolic content and result in significant browning (**Solangi et al., 2020**).

This research emphasizes the synergistic effects of Picloram and TDZ on the induction of embryogenic callus from mature inflorescence explants of date palm. The results emphasize the critical importance of growth regulators in solving the problems in date palm tissue culture, such as browning and embryogenesis potential. Function of picloram in promoting callus formation and reducing browning: Picloram has been identified as a vital auxin hormone for callus stimulation, with a concentration of 5.0 mg/l producing the highest callus formation rate (92.8%) and the lowest browning rate (20.3%) (**Nakum et al., 2025**). The reduced browning observed at higher picloram concentrations could be attributed to its antioxidant properties, which mitigate the effects of phenolic oxidation, a major contributor to tissue browning *in vitro* (**Baskaran et al., 2015**). These results are consistent with studies on other monocotyledonous species, including oil palm and banana, where picloram successfully reduced browning while enhancing callus vigor (**Bhattacharya, 2022**).

The findings of this study provide valuable insights for the large-scale propagation of mature inflorescences from a new strain of date palms. The optimized mixture of 10.0 mg/l picloram and 3.0 mg/l TDZ can be incorporated into commercial tissue culture protocols to improve efficiency.

3.2- Somatic embryos induction medium

The addition of TDZ with the picloram were significantly enhanced the somatic embryos induction. Callus induction in spikelet explants influenced significantly by different auxin-cytokinin combinations. Lower TDZ concentration (0.1 mg/l) showed the moderate improvements while higher concentration (2.0 mg/l) had more additional benefits. Data in **Table (3)** revealed with significantly the highest percentage of calli differentiation (72.8%) occurred on medium comprising of 1.0 mg/l picloram and 2.0 mg/l TDZ (**Fig. 7**). Medium comprising of 0.5 mg/l picloram, 1.0 mg/l TDZ similarly induced significantly high percentage of calli differentiation



(46.9%) than rest of treatments. Contrary, the significantly lowest percentage of calli differentiation (16.2%) observed on medium comprising of 0.05 mg/l picloram and 0.1 mg/l TDZ.

Table 3: Effect of different concentrations of Picloram and TDZ with 0.1 ABA on somatic embryogenesis induction from inflorescences of date palm after four subcultures.

Treatments (mg/l)		Calli differentiation (%)	Number of somatic embryos	Length of somatic embryos (cm)
Picloram	TDZ			
0.0	0.0	7.0 e	5 e	1.5 a
0.05	0.1	16.2 d	16 cd	1.2 ab
0.1	0.5	28.6 c	22 c	1.0 b
0.5	1.0	46.9 b	30 b	1.0 b
1.0	2.0	72.8 a	45 a	0.7 c

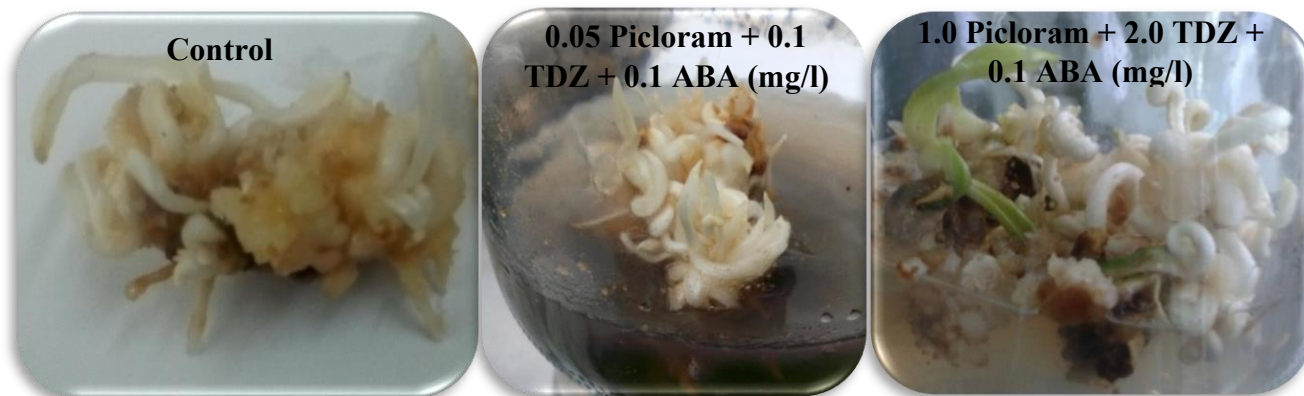


Fig. 7. The somatic embryogenesis induction from inflorescences of date palm after four subcultures on MS medium containing different concentrations of Picloram and TDZ with 0.1 mg/l ABA.

Results shown in **Table (3)** and **Fig. 7** showed that after 4 months of incubation, the calli which cultured on MS medium containing 0.05 mg/l picloram and 0.1 mg/l TDZ scored the lowest significant number of somatic embryos induction (16 embryos/explant) with the longest length 1.2 cm. Moreover, TDZ at increased concentration (2.0 mg/l) with 1.0 mg/l of picloram had a significant reversible influence on all development parameters. On the other hand; the number of somatic embryos induction was higher (30 embryos/explant) on MS medium supplemented with 0.5 mg/l picloram and 1.0 mg/l TDZ following by the medium consist of 1.0 mg/l picloram and



2.0 mg/l TDZ which recorded the highest number of somatic embryos induction (45 embryos/explant) with the lowest length (0.7 cm) in comparison to the other studied concentrations.

These observations support those of different authors, the process of somatic embryogenesis encompasses several stages, beginning with the induction of embryonic calli, followed by maturation, germination of somatic embryos, and ultimately the formation of plantlets (**Thuzar et al., 2011**). To generate embryogenic callus, modifications to environmental factors, particularly the culture medium composition, can be implemented, as somatic embryogenesis is chemically regulated by various growth regulators (**Tapingkae et al., 2012**). The significance of plant growth regulators in somatic embryogenesis has been thoroughly reviewed by (**Jiménez, 2005**) and **Fehér (2008)**. Among these growth regulators, auxins and cytokinins play a crucial role in influencing the embryogenic response, likely due to their involvement in cell cycling, division and differentiation.

The combination of cytokinins and auxins has been extensively utilized for organogenic differentiation in various protocols established for date palm (**AL-Mayahi, 2012**). It has been suggested that the addition of auxins to the culture medium can disrupt the levels of endogenous hormones in explants, potentially creating stress conditions that promote the embryogenic transition in somatic cells **Fehér (2005)**. Consequently, employing auxin at low concentrations to stimulate somatic embryogenesis in date palm is of significant interest. The picloram may serve as an alternative auxin, as it was subsequently used for the induction of somatic embryos in peach palm (**Steinmacher et al., 2007**) and macauba palm (**Moura et al., 2008**).

Auxins, such as picloram, facilitate cellular de-differentiation and proliferation by enhancing the expression of genes associated with cell division (**Baskaran et al., 2015**). For date palm, **Mazri et al. (2017)** discovered that incorporating 45 μ M picloram into the culture medium improved somatic embryogenesis in the cv. Najda. Recently, **Hassan et al. (2021)** successfully induced somatic embryogenesis from immature inflorescence explants of the Medjool cv. using picloram at a concentration of 2 mg/l.

The MS medium supplemented with 10.8 mg/l picloram and 1.0 mg/l 2iP proved to be the most effective medium for inducing somatic embryogenesis, achieving a rate of 70%. The optimal conditions for somatic embryogenesis in *Picea abies* were attained by adding picloram into the initiation medium. Additionally, the inclusion of BA in the picloram-supplemented medium enhanced the response (**Hazubska-Przybył et al., 2020**).

Thidiazuron not only substituted the auxin-cytokinin requirement for the induction process in this investigation but also enhanced the number of somatic embryos and improved the



embryogenic response of floral explants, thereby promoted normal growth and development. **Saxena et al. (1992)** suggested that TDZ contributes to establishing the optimal internal balance between cytokinin and auxin that is essential for the induction and development of somatic embryogenesis in *Solanum melongena*.

The synergistic effects of picloram and TDZ on somatic embryogenesis were apparent: the research demonstrated that TDZ significantly enhances the embryogenic potential of callus when combined with picloram. The highest embryogenic rate (75.5%) was observed in IF-85 medium containing 5.0 mg/l picloram and 1.0 mg/l TDZ (**Nakum et al., 2025**), a cytokinin-like growth regulator recognized for stimulating secondary metabolite production and promoting cellular differentiation. Its mechanism likely complements picloram by regulating the hormonal interactions between auxin and cytokinin pathways, thus creating a conducive environment for embryogenesis. Previous studies have indicated related synergistic effects in *Cocos nucifera* and *Zea mays*, where TDZ facilitated auxin-induced somatic embryogenesis (**Khatri and Joshee, 2024**). The composition of the media and nutrient-driven interactions: The results underscored the significant interactions between media composition, Picloram and TDZ.

Media such as IF-85 promoted high embryogenic rates due to a well-balanced nutrient profile that likely enhanced the effectiveness of growth regulators. These results underscore the necessity of optimizing both hormonal and nutritional factors to achieve consistent and high embryogenic results (**Swain et al., 2016**).

The addition of abscisic acid (ABA) was found to be beneficial for the generation of healthy embryos. Furthermore, ABA enhanced the count of individual embryos when used alongside picloram, as well as the production of multiple embryos in combination with 2ip. Similarly, **Ibrahim et al. (2011)** observed that ABA facilitated the development of numerous embryos and stimulated the maturation of embryos from the date palm cultivar Sakkoty. Various protocols have been established concerning the use of ABA to improve embryo maturation (**Jayanthi et al., 2015**). **Zouine et al. (2005)** reported that ABA at a concentration of 10–5 μM increased the accumulation of sugars and total proteins in somatic embryos of the date palm varieties Bousthami Noir and Jihel.

As noted by **Bhatia and Bera (2015)**, somatic embryos provide numerous benefits, including the capacity to produce plants in large quantities and the simultaneous generation of both roots and shoots, thereby eliminating the necessity for a separate root induction phase. This is particularly beneficial for large-scale vegetative propagation of monocots.



3.3- Germination medium

The germination of somatic embryo, could mainly affected by the type and the concentration of auxin and cytokinin. The results of **Table (4) and Fig. (8)** indicated that after 4 months of culture duration, $\frac{3}{4}$ MS medium supplemented with 0.1 mg/l NAA, 0.25 mg/l BA with added 0.2 mg/l ABA was recorded the highest percentage values of embryos germination (54.0%) as well as the highest significant number of embryos (26 embryo/culture) compared with the other studied treatments. Increasing the concentration of combination between NAA and BA to 0.5 mg/l for both of them resulted reduced the germination percentage and number of somatic embryos to (32.4% and 18 embryo/culture). On the other hand, using of BA (1.0 mg/l) in combination with NAA (1.0 mg/l) significantly reduced somatic embryo germination % and number of embryos as compared with the other studied treatments.

After 4 months of culture, the biggest number of leaves (20 leaf/culture) with the length (2.5 cm) were obtained on the $\frac{3}{4}$ MS medium supplemented with 0.1 mg/l NAA, 0.25 mg/l BA with added 0.2 mg/l ABA, as shown in Table (4). Increasing the concentration of combinations to 0.5 mg/l NAA, 0.5 mg/l BA with added 0.2 mg/l ABA, reduced the number of leaves to 10.0 leaf/culture with length 1.5 cm. While, the lowest number of leaves (3 leaf/culture) with the shortest leaf length (1.0 cm) were recoded with $\frac{3}{4}$ MS medium supplemented with 1.0 mg/l NAA, 1.0 mg/l BA with added 0.2 mg/l ABA.

Table 4: Effect of different concentrations of NAA, BA with added 0.2 mg/l ABA on germinated embryos of date palm cultured *in vitro* for 4 months.

Treatments (mg/l)		Germination percentage (%)	Number of somatic embryos	Number of leaves	Length of leaves (cm)
NAA	BA				
0.05	0.1	18.5 c	10 c	6 c	4.0 a
0.1	0.25	54.0 a	26 a	20 a	2.5 b
0.5	0.5	32.4 b	18 b	10 b	1.5 c
1.0	1.0	13.1 cd	5 d	3 cd	1.0 c

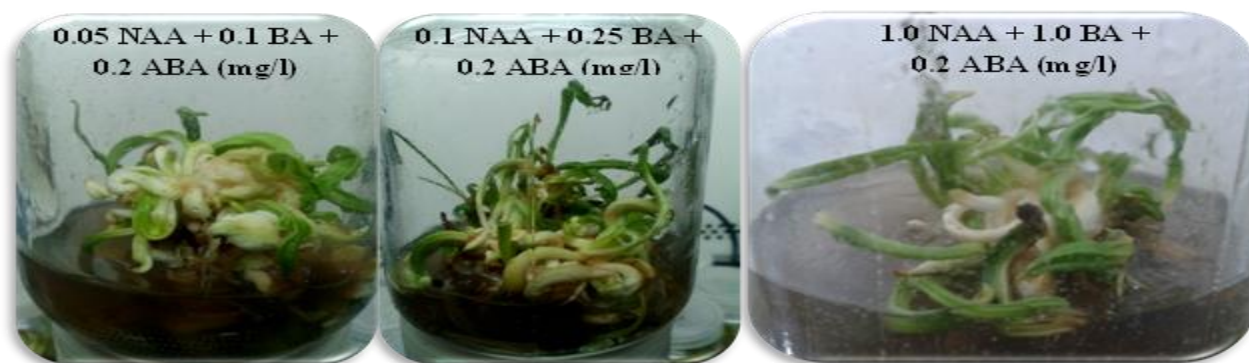


Fig. 8. Impact the various concentrations of NAA, BA with added 0.2 mg/l ABA on germinated embryos from floral buds of date palm after four subcultures.

The results obtained from the current research align with the finding of **Othmani *et al.* (2009)** who reported that in date palm, somatic embryogenesis is primarily favored due to its enhanced potential for mass propagation. The improvement of date palm cultivation depends not alone on *in vitro* propagation techniques but also on the utilization of chemical and physical mutagens (**Ghareeb *et al.*, 2022**). Growth regulators play a crucial role in somatic embryogenesis, with auxins and cytokinins being the key agents that determine cell fate (**Méndez-Hernández *et al.*, 2019**). Cytokinins, in particular, are well-known for their capacity to promote rapid cell division (**Shen *et al.*, 2018**). The inclusion of auxin and cytokinin in the nutritional medium facilitates the division, growth, and development of meristematic cells into embryonic callus and somatic embryogenesis (**Shareef *et al.*, 2016**).

Cultivating somatic embryogenesis in the germination medium exhibited varying germination percentages, with the combination of (0.1 mg/l NAA and 0.05 mg/l BA) achieving the highest germination rate of 40%. This may be attributed to the optimal concentrations provided for tissue growth by this combination (**Zayed *et al.* 2020**). These findings may be linked to the role of auxin in promoting cell division and its synergistic effect with cytokinins that enhance vegetative growth (**Asmaa *et al.*, 2019**).

The conversion of date palm somatic embryos into plantlets has been extensively researched by various studies (**Taha and Hassan, 2014; Mazri *et al.*, 2017**). The maturation and germination stages present significant challenges in the regeneration process. ABA has been commonly incorporated into culture media to encourage the maturation of somatic embryos (**Rai *et al.*, 2011**).



3.4- Multiplication medium

At this stage, the shoot clusters were cultivated on $\frac{3}{4}$ MS medium supplemented with varying concentrations of TDZ and BA, along with an addition of 0.5 mg/l NAA, which significantly influenced shoot regeneration. The combination of 3.0 mg/l TDZ and 1.0 mg/l BA, together with 0.5 mg/l NAA, was crucial in enhancing and regulating shoots regeneration; this combination yielded the highest number of shoots (20.4 shoot/culture), the greatest number of leaves (43.0 leaf/culture), and a leaf length of 3.5 cm, as illustrated in **Table (5) and Fig. (9)**. Conversely, when TDZ and BA were applied at lower concentrations, the results indicated the lowest number of shoots (3.0 shoot/culture), the fewest leaves (8.0 leaf/culture), and the longest leaf length (6.0 cm).

Table 5: Effect of different concentrations of TDZ, BA and NAA at 0.5 (mg/l) on shoots multiplication after 8 weeks in culture.

Treatments (mg/l)		Number of shoots	Number of leaves	Length of leaves (cm)
TDZ	BA			
0.5	0.05	3.0 c	8.0 d	6.0 a
1.0	0.1	5.5 c	15.0 c	5.2 ab
2.0	0.5	14.2 b	28.0 b	4.0 c
3.0	1.0	20.4 a	43.0 a	3.5 cd

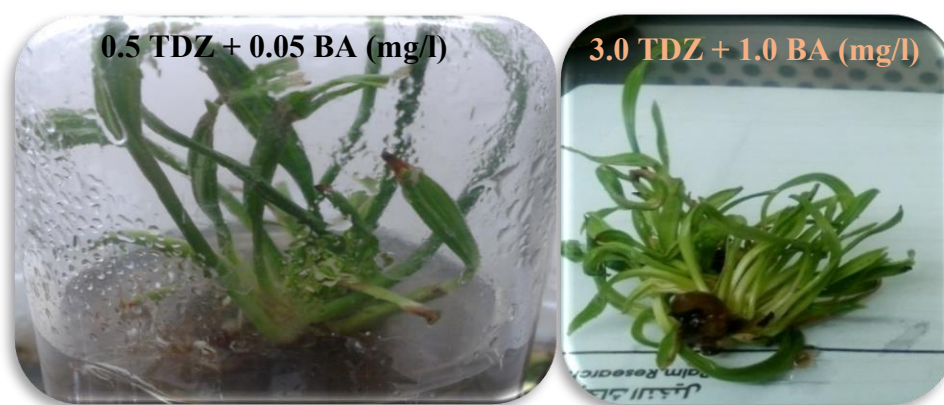


Fig. 9. Effect of TDZ and BA with added NAA at 0.5 (mg/l) on shoots multiplication of date palm after two subcultures.



The multiplication of shoots is affected by several factors, including the composition of the medium, the genotype, and plant hormones. Many researchers have sought to comprehend the action of TDZ in plants. **Dey *et al.* (2012)** observed that TDZ seems to encourage cells in the apical meristem to divide and multiply, leading to bud differentiation. Conversely, TDZ is essential for the *in vitro* propagation of date palms. The incorporation of TDZ into culture media has been shown to improve growth characteristics. This improvement is probably due to differences in the internal concentrations of growth regulators, with TDZ being essential for enhancing the biosynthesis of endogenous cytokinins or amending cytokinin metabolism. A study examining the different combinations of media and plant growth regulators utilized in the tissue culture of date palm plants reveals that shoots regeneration requires the inclusion of both auxins and cytokinins (**Ibrahim *et al.*, 2013**). This is consistent with research on the use of TDZ in shoots regeneration and its synergistic interaction with auxins in promoting the number of shoots **Yildirim and Turker (2009)**.

Mundhara and Rashid (2002) highlight that the function of TDZ in morphogenesis is associated with the metabolism of endogenous growth regulators. Additionally, it has been demonstrated that TDZ treatment enhances the levels of endogenous auxin, ethylene and ABA (**Hutchinson *et al.*, 1996**). Importantly, the application of 0.5 mg/l TDZ in combination with 1.0 mg/l BA resulted in an increase in peroxidase activity during the multiplication of date palm cv. Hillawi, where this peroxidase activity was linked to a greater number of shoot formations (**Al-Mayahi, 2014**). The findings suggest that the combination of BA with TDZ and other growth regulators in the media stimulated proliferation, as the increase in new shoots was accelerated during the multiplication phase. The noted enhancement in shoots proliferation may be ascribed to the physiological role of BA, which is acknowledged as the most usually employed cytokinin in the micro-propagation field due to its effectiveness and cost-efficiency (**Bairu *et al.*, 2007**). It promotes cell division and the differentiation of adventitious buds. Histological analyses revealed that additional shoot bud primordia were generated, facilitated by the presence of BA, within the explants just below the enlarged cells where their growth is restricted (**Fatima and Anis, 2012**). Furthermore, **Aslam and Khan (2009)** observed the positive effects of BA at 7.84 μM on shoots formation. Auxins play a vital role in the differentiation of cells, which is essential for regeneration. In addition, auxin is a crucial plant hormone necessary for the growth and development of *in vitro* organs (**Al-Mayahi, 2022**).



3.5- Elongation of shoots

The regenerated shoots were separated and moved to the elongation medium, which consists of $\frac{3}{4}$ MS medium supplemented with 1.0 mg/l BA, 0.5 mg/l 2ip, and 1.0 mg/l GA₃. The shoots exhibited successful growth with rapid elongation after 8 weeks of culture.

Auxin affects plant elongation and cell division by facilitating nuclear division (Catherine, 2010). Gibberellins promote elongation by stimulating the proliferation and elongation of sub-apical meristem cells, as well as aiding in the breakdown of polysaccharides into simple sugars that are beneficial for plant tissue (Zaid and DeWet, 2005). Following the multiplication phase and GSH treatments as described by Khierallah *et al.* (2017), the clusters of developing shoots were cultured for elongation to produce healthy shoots of suitable length for the rooting phase. The elongation culture medium consisted of MS supplemented with 0.5 mg/l BA, 0.5 mg/l 2ip and 1.0 mg/l GA₃. Al-Najm *et al.* (2018) indicated that shoots obtained from callus regeneration were transferred to elongation media with the same composition, although with different concentrations of GA₃ (0.1, 0.2, 0.5 and 1.0 mg/l). Besides exhibiting good elongation and vigorous growth, the average shoot length of the cultivar reached 7.64 cm under 0.5 mg/l GA₃, which was significantly better than other treatments.

3.6- Rooting stage

The rooting process was conducted to cultivate healthy, acclimatized plantlets. The experiment involved transferring long shoots to six rooting media to promote roots growth. The results showed clear differences among the six media in terms of root length, number of roots and the number of root hairs per root.

According to the data analysis, when using the different concentrations of IBA in rooting medium, the data show that the medium consist of $\frac{1}{2}$ MS medium supplemented with IBA at 1.0 and BA at 0.5 mg/l was resulted in the highest number of roots, with an average of 8.0 root/plantlet with length 6.3 cm, **Table (6) and Fig. (10)**. When increase the concentration of IBA to 2.0 mg/l resulted in reduce the number of roots to 6.0 root/plantlet with the highest length 7.0 cm. The low concentration of IBA at 0.5 mg/l was not good to rooting which resulted the lowest number of roots 3.0 root/plantlet with length 2.0 cm.



Table 6: Effect of different concentrations of IBA or NAA on rooting of date palm shoots after three subcultures.

IBA (mg/l)	Length of roots (cm)	Number of roots	Number of root hairs/root
0.0	1.0 d	1.0 d	0.0 d
0.5 + BA 0.5	2.5 c	3.0 c	2.0 c
1.0 + BA 0.5	6.3 ab	8.0 a	7.0 a
2.0 + BA 0.5	7.0 a	6.0 ab	3.0 b
NAA (mg/l)	Length of roots (cm)	Number of roots	Number of root hairs/root
0.0	1.0 c	1.0 d	0.0 d
0.5 + BA 0.5	1.4 c	2.0 c	1.0 bc
1.0 + BA 0.5	2.9 b	4.5 a	4.0 a
2.0 + BA 0.5	3.5 a	3.0 b	2.0 b

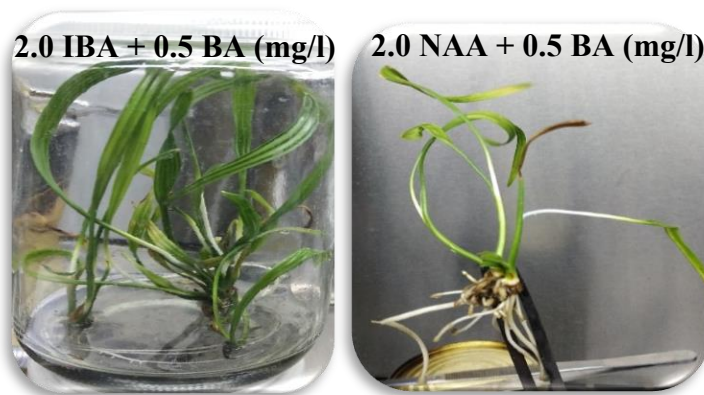


Fig. 10. Impact of various concentration of IBA or NAA on shoots rooting of date palm after 18 weeks in culture

When using the different concentrations of NAA in rooting medium, the data show that the largest number of roots was observed in the medium consist of ½ MS medium supplemented with NAA at 1.0 and BA at 0.5 mg/l which had 4.5 root with length 2.9 cm. The high concentration of NAA at 2.0 mg/l gave the lowest number of root 3.0 root/plantlet with length 3.5 cm. The treatment IBA at 0.5 and BA at 0.5 mg/l formed the lowest roots number and length.

On the other hand, the medium consist of ½ MS medium supplemented with IBA at 1.0 and BA at 0.5 mg/l was achieved the best results in number of root hairs which had 7.0 root



hairs/root more than others treatments. However, when used the treatment $\frac{1}{2}$ MS medium supplemented with NAAA at 1.0 and BA at 0.5 mg/l was gave 4.0 root hairs/root.

The rooting of plants is regarded as a crucial phase in the micro-propagation of date palms and the following acclimatization of the plants. Our findings indicated that adding IBA to the rooting medium at various concentrations improved both the number of roots and their length in comparison to the control medium.

This finding confirms that IBA is considered a superior hormone for root development. These results align with numerous other studies. Several studies have discussed root formation and development, the protocol for successful plant micro-propagation requires effective rooting (**AL-Qurainy et al., 2015**). Utilizing the root trimming technique, each plantlet produced healthy adventitious roots, which contributed to maximizing plantlet survival in the greenhouse. **Solangi et al. (2022)** achieved optimal survival and robust growth of plantlets with multiple adventive roots in a greenhouse setting through the application of the root trimming technique.

The rooting phase in date palm micro-propagation is vital for the success of date palm tissue culture (**Al-Khalifah et al., 2012**). *In vitro* rooting of date palm is influenced by the types of nutrient media, carbohydrate sources, activated charcoal, and the exogenous application of plant growth regulators, particularly auxins (**Hassan et al., 2005**). **Mazri (2012)** highlighted the beneficial impact of IBA on the rooting of date palm cv. Najda. **Fki et al. (2011)** a notable positive correlation was observed between the enhancement of rooting and the concentrations of IBA in the date palm cultivar Barhee. IBA, an auxin precursor, may regulate IAA levels through oxidation. By managing the growth of adventitious roots, oxidation can sometimes irreversibly deactivate this conjugation mechanism (**Aryal et al., 2019**).

IBA leads to alterations in the metabolic processes of carbohydrates, enzymes, DNA, RNA and proteins within the rooting zone upon external interaction with the cell, which can either inhibit or facilitate roots development, particularly during the phases of cell division and differentiation. Due to its non-toxic nature at various concentrations, IBA and NAA are the most frequently used root-promoting substances in the nursery industry (**Kentelky et al., 2021**). Nevertheless, **Al-Qurainy et al. (2015)** found that the induction of rooting was enhanced when shoots of *Tamarix nilotica* were grown on a medium enriched with 100 μ M IBA for short periods of 5, 10 and 15 days. Additionally, after transferring the shoots to a medium devoid of plant growth regulators for a month, the rooting percentages ranged from 50% to 86%.



3.7- Acclimatization

The plantlets were effectively acclimatized in a combination of peat moss and perlite (2:1), resulting in a 75% acclimatization rate following their transfer to plastic pots for a duration of 4 months (Fig. 11). All micro-propagated plants displayed no visible defects.



Fig. 11. Acclimatization the plantlets of date palm in a combination of peat moss and perlite (2:1).

Gabr and Abd-Alla (2010) emphasized that *in vitro* hardening represents a critical stage in the *in vitro* propagation process. The acclimatization of plantlets possessing a fully developed roots system to greenhouse conditions is especially essential for the successful establishment of field plants (**Blando et al., 2021**). The differing percentages of acclimatized plantlets (86.67% for the Amri variety, 82.33% for the Magdoul variety and 77.56% for the Barhy variety) in the peat and perlite mixture (2:1 v:v) can be linked to fungal infections, which were exacerbated by increased moisture levels (**Abdelghaffar et al., 2023**). Acclimatization constitutes the final phase in the micro-propagation process. **Mazri and Meziani (2013)** reported a high survival frequency in cv. Najda when a peat-gravel substrate was employed. **Meziani et al. (2015)** noted an 88% survival rate in cv. Mejhoul plantlets after 3 months in the greenhouse. The cv. Boufeggous shoots derived from semisolid medium achieved the highest survival rate of 92.5%, whereas those from stationary liquid media culture recorded a survival rate of 50% after 3 months in the greenhouse (**Mazri, 2015**). Plantlets obtained from somatic embryos of date palms have been successfully acclimatized across various cultivars. **Othmani et al. (2009)** noted survival rates of 60% and 80% for the date palm cultivars Boufeggous and Deglet Nour, respectively. **Al-Khayri (2010)** reported a survival rate of 72-84% for the Khasab and Nabout Saif cultivars after their *ex vitro* transfer.



The plantlets with developed roots were gently removed from the tubes, then thoroughly washed under running water and treated with a solution of Rezolex fungicide (100 g) for 10 minutes. Afterward, these plantlets were carefully positioned into pots filled with a peat and perlite mixture in a 2:1 v/v ratio (Rohim *et al.*, 2021).

3.8- RAPD-based genetic relationships

In the current research, we successfully regenerated shoots from callus tissues derived from floral buds, influenced by various plant growth regulators. Given that morphological assessment is not a reliable method for characterizing variability between *in vitro* shoots and the mother plant, it is essential to evaluate the genetic stability of these regenerated shoots. The eight microsatellite RAPD primers used all yielded good amplification with discernible DNA bands. Furthermore, the findings indicated that the regenerated shoots exhibited no polymorphism when compared to the mother plant's spicklets (Fig. 12). The DNA bands generated through RAPD marker amplification were monomorphic in both the mother plant and the *in vitro* leaf shoots. The genetic profiles obtained from the RAPD analysis indicate that there are no genetic alterations between the *in vitro* shoots and the mother date palm. This implies that employing this technique to generate shoots from inflorescences of a new strain under *in vitro* conditions ensures genetic stability, thereby confirming the true-to-type and genetically stable characteristics of these shoots.

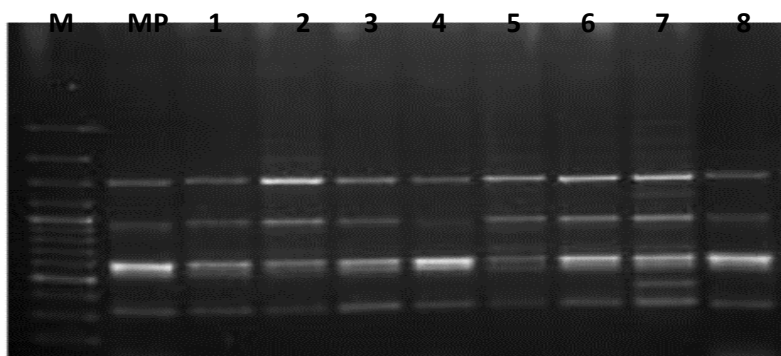


Fig. 12. Gel image shows band regenerated against genomic DNA of date palm using RAPD primer.

The date palm is a very biodiverse plant that can be propagated through tissue culture, offshoots, or seeds. In a true breeding line, off shoot methods and tissue culture are frequently employed to preserve homogeneous yield (Naqvi *et al.*, 2023). In date palms, sex has been determined using Random Amplified Polymorphic DNA (RAPD) (Moghaieb *et al.*, 2010).

Variations in regeneration capabilities across different treatments may have resulted from the varying endogenous levels of growth hormones present in these tissues. Numerous researchers



have thoroughly documented the advantages of molecular analysis for *in vitro* regenerated plantlets (Piateza *et al.*, 2015; Bhalang *et al.*, 2018). Somaclonal variation is frequently induced by the culture media and the cycles of subculture (Bidabadi *et al.*, 2010). Consequently, it is essential to assess the genetic stability of *in vitro* raised plants for the production of date palm plantlets. The use of RAPD technology has garnered significant interest among researchers. The primary reason for the effectiveness of RAPD analysis lies in its ability to generate numerous genetic markers that necessitate only small quantities of DNA, without the need for cloning, sequencing or any other form of molecular genome characterization.

An effective *in vitro* propagation method should yield true-to-type plantlets, free from genetic mutations or morphological changes (Khatab and Youssef, 2018). Likewise, no genetic variation has been reported in many instances of *in vitro* propagation of *Terminalia arjuna* using RAPD (Gupta *et al.*, 2014), and the medicinal herb *Coleus aromaticus* Benth (Govindaraju and Arulsevi, 2018), as well as date palm cv. Ashgar (Al-Mayahi, 2022). Conversely, cytokinins are crucial in DNA synthesis, cell division and plants regeneration and they regulate the protein synthesis necessary for the formation of the mitotic spindle (Karunadasa *et al.*, 2020). The RAPD technique demonstrated genetic conformity in micro-propagated plants of *H. procumbens*, whether pretreated or not with TDZ (Grabkowska *et al.*, 2014).

4. Conclusion

This research presents a simply and effective *in vitro* protocol for the large-scale cultivation of a new date palm strain (Nag Hamadi 2) derived from mature inflorescences, utilizing a simple sterilization process, followed by callus and somatic embryo induction.

The inclusion of TDZ and BA as essential cytokinins in the induction and multiplication media for date palm is recommended. We suggest using spathe sizes ranging from 37 to 50 cm and culturing on MS medium supplemented with 10.0 mg/l picloram and 3.0 mg/l TDZ for optimal callus induction. For the formation of somatic embryos, a combination of 0.1 mg/l ABA with 1.0 mg/l picloram and 2.0 mg/l TDZ proved to be critical, while the germination of embryos required 1.0 mg/l BA, 1.0 mg/l NAA, and 0.2 mg/l ABA. Furthermore, the enhancement of shoots multiplication was significantly supported by the application of 3.0 mg/l TDZ, 1.0 mg/l BA, and 0.5 mg/l NAA. The use of GA₃ at a concentration of 1.0 mg/l, with 1.0 mg/l BA and 0.5 mg/l 2ip, was essential for promoting shoot elongation. Additionally, the application of IBA at 2.0 mg/l to MS medium resulted in the highest root number and length when compared to NAA at varying concentrations, leading to the development of healthy plantlets that successfully acclimatized in the greenhouse.



The DNA bands generated through RAPD marker amplification were monomorphic in both the mother plant and the in vitro leaf shoots. The genetic profiles obtained from the RAPD analysis indicate that there are no genetic alterations between the in vitro shoots and the mother date palm. This implies that employing this technique to generate shoots from inflorescences of a new strain under in vitro conditions ensures genetic stability, thereby confirming the true-to-type and genetically stable characteristics of these shoots.

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