

DEVELOPMENT OF A SYSTEM FOR COMMERCIAL PRODUCTION OF DATE PALM (*PHOENIX DACTYLIFERA* L.) CV. MEDJOOOL

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Date palm somatic embryogenesis has become an ideal technique for large scale production of distinguish and rare cultivars. Date palm cultivar, culture medium components and culture conditions have been identified as detrimental factors affecting somatic embryo formation process. This research investigated the potential impact of ammonium nitrate (NH_4NO_3), calcium nitrate $\text{Ca}(\text{NO}_3)_2$ strengths and polyethylene glycol (PEG) concentrations on mature, germinated and vitrified embryos during maturation stage of cv. Medjool callus cultures. Also, the effect of calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) levels that could improve somatic embryo germination (shoot formation) and secondary embryo numbers during germination stage was examined. Rooting was also determined as affected by phloroglucinol (PG) levels. The ideal amount of potassium sulphate (K_2SO_4) or aluminosilicate ($\text{Al}_4\text{Si}_4\text{O}_{10}(\text{OH})_8$) (kaolin) as anti-transpirants that influenced the survival and growth of cv. Medjool plants was also investigated in relation to the *ex vitro* acclimatization conditions. Modification of Woody Plant Medium (WPM) maturation medium by using NH_4NO_3 at double strength and $\text{Ca}(\text{NO}_3)_2$ at fourth strength encouraged the highest significant value of mature embryos formation. NH_4NO_3 , $\text{Ca}(\text{NO}_3)_2$ and PEG had a clear impact on mature, germinated and vitrified embryos during maturation stage of cv. Medjool callus cultures. Also, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ levels improved somatic embryo germination (shoot formation) and secondary embryo numbers during germination stage. PG in the rooting medium had a beneficial role in root system. Using $\text{Al}_4\text{Si}_4\text{O}_{10}(\text{OH})_8$ at 0.1 mg/l as an anti-transpirant material enhanced survival percentage of plant growth during *ex vitro* acclimatization stage.

Keywords: date palm, somatic embryogenesis, rooting, acclimatization, anti-transpirant

INTRODUCTION

Because it can withstand high temperatures, drought, and salinity better than many other fruit crop plant species, date palm (*Phoenix dactylifera* L.) is regarded as a symbol of life in the desert. It is one of the first trees that man has used, and it has been cultivated for centuries (Zohary and Hopf, 2000). In many nations, date palm is a significant contributor to food security and job creation because it is a good source of food with high nutritional value (Arias et al., 2016). Offshoots are the conventional method for date palm vegetative propagation. The slow rate of propagation during the juvenile phase of a tree's life, which can range from 1 to 20 offshoots, depending on the cultivar and cultivation techniques used, is one of the challenges this type of propagation encounters. Expanding palm cultivation is severely limited by a low propagation rate. Additionally, this method of multiplication can result in the transmission of insects and pathogens that cause disease (Gueye et al., 2009 and Jain, 2012).

Tissue culture technique can be a very effective tool for date palm culture development (Jasim et al., 2009). Additionally, micropropagation is a necessary method to produce large quantities of homogeneous plants that are disease-free, pest-free, genetically identical, and high-quality planting material (Al-Mayahi et al., 2010; Al-Mayahi, 2019 and 2020). Due to the varying nutritional needs of different plant species and the numerous interactions between nutrients, optimizing growth media based on mineral nutrition for micropropagation is highly challenging (Niedz and Evens, 2006).

Techniques for *in vitro* propagation have proven to be efficient for quick and widespread multiplication using either somatic embryogenesis (Al-Khayri, 2003 and Quiroz-Figueroa et al., 2006) or organogenesis regimens (Al Kaabi et al., 2001 and Al-Khateeb, 2006). The capacity of regenerated plantlets to transfer from *in vitro* controlled circumstances and become firmly established in the recently hardened *ex vitro* environments, however, is a key factor in the economic success of these methods. Plant tissue growth and morphogenesis are significantly influenced by the growth medium composition. The most often used media in plant tissue culture is Murashige and Skoog (Murashige and Skoog, 1962). The Woody Plant medium (WPM) of Lloyd and McCown (1981) is used for culturing woody plants. When selecting a growth medium, both the type of plant and the tissue culture medium are considered (Gamborg and Phillips, 1995).

The adaptation of micropropagated plants to greenhouses involves a process called acclimatization. Typically, *in vitro* plantlets are grown in low-light environments with high relative humidity (Pospíšilová et al., 1999) and sugar is added to the culture medium as an additional source of carbon and energy.

According to these circumstances, the plantlets' underdeveloped cuticles, epicuticular waxes, and functional stomatal apparatus resulting in high transpiration rates, which lead to transplantation shock during the first stage of acclimatization (Hazarika, 2006; Chandra et al., 2010 and Kumar and Rao, 2012). The adaptation of regenerating plantlets to their environment is one of the most significant constraints on commercial date palm *in vitro* replication which depends on the rooting stage and subsequent acclimatization (Hassan et al., 2008). Additionally, plants from tissue culture are acclimated by progressively lowering relative humidity, increasing irradiance, CO₂ concentration, or adding osmotic agents to decrease water availability of the culture medium (Hazarika, 2006; Pospíšilová et al., 2007; Asayesh et al., 2017a and Vahdati et al., 2017).

The low survival and slow development rates of plants when they are transferred from *in vitro* to *ex vitro* settings during acclimatization are the two main issues that date palm tissue culture technology faces (Awad, 2008). For a few date palm varieties, Awad et al. (2006) reported survival rates of between 40 and 50 percent. The lack of structural complexity of the epicuticular, which distinguished many varieties of plants developed *in vitro* and their counterparts cultivated *ex vitro*, may be the cause of the low survival and slow development rates (Pospíšilová et al., 1998). Additionally, when plantlets were transferred from culture vessels to greenhouse conditions, high stomatal and cuticular transpiration rates of leaves exacerbated water loss and desiccation (Pospíšilová et al., 2009; Asayesh et al., 2017a, b and Vahdati et al., 2017). The rate of transpiration can be reduced, and plant water stress can be reduced by increasing the resistance of the leaves to water vapor diffusion, using specific chemicals for a variety of biological processes. Anti-transpirants are categorized into several groups based on their modes of action. These include film-forming types, which cover the surface of the leaves with a thin layer that makes them impervious to water vapor, reflecting materials, which reflect some of the radiation that falls on the upper surface of the leaves backward, and stomatal closing types, which affect the metabolic processes in the tissues of the leaves (Conde et al., 2016). By increasing leaf reflectivity, kaolin (Al₄Si₄O₁₀ (OH)₈) spray lowers leaf temperature, which reduces transpiration rate more than photosynthesis in plants cultivated in high solar radiation environments (Nakano and Uehara, 1996). According to studies of Cantore et al. (2009), the foliar spraying of Al₄Si₄O₁₀ (OH)₈ solution on tomato and potato plants reduces plant stress, which is crucial for optimal plant growth, production, and quality. To live under varied stresses, plants developed a variety of strategies. The use of minerals contributes significantly to plant tolerance to abiotic stresses.

It was discovered that film-forming and reflecting anti-transpirants are non-toxic and last longer than metabolic kinds. Additionally, unlike the majority

of film-forming anti-transpirants, which are impervious to CO₂ exchange and may slow down photosynthesis (Al Humaid and Moftah, 2005), these anti-transpirants are permeable to CO₂ exchange. In many plant species maintained at high solar radiation levels, also it was discovered that a reflective Al₄Si₄O₁₀ (OH)₈ spray reduced transpiration rate more than photosynthesis and decreased leaf temperature by increasing leaf reflecting (Nakano and Uehara, 1996). Early research showed that reflecting Al₄Si₄O₁₀ (OH)₈ enhanced tomato plants under water stress in terms of water status and yield while having no negative effects on carbon absorption (Glenn et al., 2003). It is evident that the plant transpires a significant amount of the water used for irrigation. The amount of required irrigation water might be reduced by reducing transpiration, which would significantly lessen the plant's stress from water shortages. Utilizing anti-transpirants on plants, such as Al₄Si₄O₁₀ (OH)₈ and whitewash (CaCO₃), may increase leaf resistance to water vapor diffusion, hence reducing transpiration (Abou El-Khair, 2004 and Anwar, 2005).

To the best of our knowledge, the application of calcium nitrate (Ca(NO₃)₂), potassium sulfate (K₂SO₄) or aluminosilicate (Al₄Si₄O₁₀ (OH)₈) (kaolin) as anti-transpirants was not previously published on date palm. Therefore, the main objective of this investigation was to develop an effective approach to date palm cv. Medjool regeneration through somatic embryogenesis. The potential impact of NH₄NO₃, Ca(NO₃)₂ strength and PEG concentrations was studied on mature, germinated and vitrified embryos during maturation stage of cv. Medjool callus cultures. Also, the effects of calcium chloride (CaCl₂.2H₂O) levels on secondary embryo number and germinated embryo (shoot formation) numbers were examined during germination stage. The influence of phloroglucinol (PG) levels on rooting was investigated. The study also mentions the role of the ideal amounts of K₂SO₄ or Al₄Si₄O₁₀ (OH)₈ as anti-transpirants on the survival and growth of date palm plantlets of cv. Medjool in relation to the *ex vitro* acclimatization conditions.

MATERIALS AND METHODS

The present study was conducted in Date Palm Research and Production Lab., Tissue Culture Unit, Department of Genetic Resources, Desert Research Center, Egypt during the period from 2018 to 2023.

1. Explant Selection and Disinfection

Three to five years old healthy offshoots were separated carefully from date palm cv. Medjool trees and transferred to the laboratory. Removing outer leaves, fibrous and roots was the first step of disinfection process. Shoot tips were washed under running tap water and soap for 30 min. After that, the shoot tips

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were kept in an antioxidant solution consisted of citric and ascorbic acid both at 150 mg/l for one hour at 4-5°C to minimize browning of tissue. Disinfection was performed by soaking shoot tips in 3% sodium hypochlorite solution with 2-3 drop of tween 20 for 25 min with continuous shaking, followed by three rinses with sterilized distilled water. After that, shoot tips were sliced longitudinally into several pieces and cultured on callus initiation medium (Abd Elaziem, 2017).

2. Callus Initiation Medium and Incubation Conditions

In the present work, Llyod and McCown (1981) WPM was used as shown in Table (1), supplemented with 10 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 3 mg/l 2-isopentenyladenine (2iP), 100 mg/l glutamine, 1.5 g/l activated charcoal and 30 g/l sucrose gelled with phytigel (2 g/l) as the callus initiation medium. The medium pH was adjusted to 5.7 prior the addition of phytigel, dispensed into small jars (250 ml) at rate of 40 ml/jar and sterilized at 121°C and 15 lb/ins for 20 min. Sliced explants were cultured in the initiation medium and incubated in the controlled growth room, where the temperature was 27±2°C and kept in total darkness. Explants continued in the initiation medium for six subcultures with regular transfer to fresh medium of the same supplements every 1.5 month. Within this period, shoot tip explants began to swell and started to form compact callus after five subcultures. After that, the concentration of 2,4-D was reduced to 5 mg/l in the culture medium to form friable callus. Friable callus was used as explant materials on the following experiments during maturation stage.

3. Maturation Stage

3.1. Influence of ammonium nitrate (NH₄NO₃) modified strength on the rate of somatic embryos and vitrification

Friable callus pieces (1 g) were cultured on maturation medium supplemented with 0.1 mg/l naphthalene acetic acid (NAA) and 0.05 mg/l benzyl adenine (BA), along with NH₄NO₃ at 100, 200, 400, 800 and 1600 mg/l to form somatic embryos, Callus cultures were incubated at 27°C in total darkness. After inoculating the callus in different media, the experiment's results were monitored over the course of three subcultures (four weeks intervals). After 12 weeks, the number of mature somatic embryos, number of germinated embryos, and number of vitrified embryos per jar were recorded.

3.2. Effect of calcium nitrate (Ca(NO₃)₂) on somatic embryos induction and vitrification

To form somatic embryos, friable callus (1 g) was transferred to the previous maturation medium with varying Ca(NO₃)₂ concentrations (193.16, 289.74, 386.34, 772.68 and 1545.36 mg/l) and kept at 27±2° C in darkness for three months (one month interval). Following that, the number of matured somatic embryos, germinated embryos, and vitrified embryos per jar were noted.

3.3. Effect of polyethylene glycol (PEG) on somatic embryos formation

Friable callus fragments (1 g) were cultivated on growth regulator-free WPM| with PEG-400 at 5, 10 and 20 g/l to produce somatic embryos. After 12 weeks of culture maintenance at 27°C and complete darkness, the resulting somatic embryos were counted per jar.

Table (1). Composition of basal medium of Woody Plant Medium (1981).

Constituent	Conc. (mg/l)
Macronutrients	
NH ₄ NO ₃	400
Ca(NO ₃) ₂ .4H ₂ O	386.34
CaCl ₂ .2H ₂ O	72.50
K ₂ SO ₄	990
MgSO ₄ .7H ₂ O	180.69
KH ₂ PO ₄	170
Micronutrients	
MnSO ₄ .4H ₂ O	22.30
ZnSO ₄ .4H ₂ O	8.60
H ₃ BO ₃	6.20
Na ₂ MoO ₄ .2H ₂ O	0.25
CuSO ₄ .5H ₂ O	0.025
Iron	
FeNaEDTA	36.70
Vitamins	
Nicotinic acid (free acid)	0.50
Pyridoxine-HCl	0.50
Thiamin-HCl	1.00
Myo-inositol	100.0
Amino acid	
Glycine	2.00

4. Germination Stage

The resulting embryos from previous experiments were used as explant materials for germination stage.

Effect of calcium chloride (CaCl₂.2H₂O) on germination

Clusters of matured embryos (2-3 embryo/cluster) were employed to WPM for germination supplemented with CaCl₂.2H₂O modified levels (36.25, 54.37, 72.5, 145 and 217.5 mg/l) to study its impact on somatic embryo

germination (shoot formation), secondary embryo and vitrified shoots. Culture medium of different treatments was supplemented with 0.1 mg/l NAA+ 0.2 mg/l abscisic acid (ABA), 0.25 mg/l kinetin and 1 g/l activated charcoal (Hassan et al., 2021). Explants were subcultured three times (four weeks intervals) in different treatments. Shoot number, secondary embryo number and vitrified shoots per explant were recorded after three subcultures. All cultures were kept at $27\pm 2^{\circ}\text{C}$ and a 16 h photoperiod (3000 lux).

5. Rooting Stage

Effect of phloroglycinol (PG) on root system

The primary goal was to investigate the effects of supplying culture media with PG at five levels; 20, 40, 80 and 160 mg/l, as well as control medium on root formation. Shoots from the previous stage (with two and three leaves) were transplanted to WPM supplemented with 60 g/l sucrose, 170 mg/l KH_2PO_4 , 200 mg/l glutamine, 100 mg/l myo-inositol, 0.4 mg/l thiamine-HCl, 0.1 mg/l NAA, and 7 g/l agar. Every treatment was repeated three times, each repetition was represented by a set of ten cultured tubes (2.5 in diameter and 25 cm in length) at a rate of 25 ml, each contained a single cultured shoot and capped with polyethylene closures. Throughout the three subsequent subcultures (four weeks apart) that were a part of this stage, all cultured tubes were incubated in a growth environment at a temperature of $27\pm 2^{\circ}\text{C}$ and under lighting of 4000 lux for 16 hours each day. Rooting percentage, number of developed rootlets per each cultured shoots (plantlet) and average length of root (cm), were recorded after each subculture.

6. Acclimatization Process

Rooted plantlets with 2-3 leaves (10–12 cm) were carefully taken out of test tubes and rinsed with tap water. Plantlets were submerged in a fungicide solution containing Moncut 25% at a concentration of 1% (w/v) for 20 min. The plantlets were then transferred into plastic pots (5 x 18 cm) containing peat: perlite mixtures 2:1 v/v (Hassan et al., 2021). The plantlets were kept for 4-6 weeks at 27°C with natural daylight and high relative humidity (90-95%) under a cover of white translucent polyethylene sheets (Hassan et al., 2014). The plastic sheets were gradually taken off to give the plants time to adjust to the greenhouse. Acclimatized plants pioneered numerous roots after 12 weeks of *ex vitro* conditions. After that, plants with three to four leaves were foliar-sprayed with K_2SO_4 at different concentrations (100, 200 and 400 mg/l) or $\text{Al}_4\text{Si}_4\text{O}_{10}(\text{OH})_8$ as anti-transparents at 0.1, 0.2 and 0.4 mg/l and watered twice a week for 24 weeks. After this period, survival percentage (%), number of leaves and plant height (cm) were assessed and documented.

7. Experimental Design and Statistical Analysis

Treatments were set up in a completely randomized design; each treatment reproduced three replicates, each replicate containing at least ten jars with one explant. Analysis of variance and the Duncan's multiple range test ($P \leq 0.05$) were used to analyze the evidence.

RESULTS

The present work resulted in a successful large scale *in vitro* propagation protocol of date palm cv. Medjool using shoot tip explants. Most explants responded well to the WPM as the initiation medium. Compact callus was noticed within 24 weeks through four subcultures. Friable callus was observed within 12 weeks through two subcultures on WPM with reduced 2,4-D and other supplements (Fig. 1 a-d). After that, friable callus weighing 1 g was shifted to maturation medium to form somatic embryos.

1. Maturation Stage

1.1. Influence of ammonium nitrate (NH_4NO_3) modified strength on the rate of somatic embryos and vitrification

The amount of NH_4NO_3 in WPM significantly impacted on the *in vitro* growth responses of date palm cv. Medjool. Callus cultures produced higher number of somatic embryos when NH_4NO_3 at 1600 mg/l was used. Lowering the level of NH_4NO_3 from 1600 to 100 mg/l significantly decreased the average somatic embryos from 15.26 to 5 somatic embryos/jar (Table 2). The same trend was also noticed with the number of germinated embryos, where the greatest number was achieved at 1600 mg/l (20 germinated embryos/jar) and the lowest one was recorded with 100 mg/l (8 germinated embryos/ jar) (Fig. 1e). Data in Table (2) show that vitrification was very significant and peaked on medium containing 1600 mg/l NH_4NO_3 (1.5 vitrified embryos/jar).

1.2. Effect of calcium nitrate $\text{Ca}(\text{NO}_3)_2$ on the number of matured, germinated and vitrified somatic embryo

Modifications were made to the WPM's nitrogen supply; $\text{Ca}(\text{NO}_3)_2$, to enhance the induction of somatic embryos. After 12 weeks of culture, the changed $\text{Ca}(\text{NO}_3)_2$ strength in WPM had a statistically significant impact on the quantity and quality of somatic embryos (Table 3). Maximum mature embryos/jar (25) was produced using $\text{Ca}(\text{NO}_3)_2$ at doubled strength (1545.36 mg/l) (Fig.1f). Noticeable differences between germinated embryo number/jar was recorded with various strengths of $\text{Ca}(\text{NO}_3)_2$; the highest number was obtained with doubled strength $\text{Ca}(\text{NO}_3)_2$ (30), which was statistically inferior to the 18.66 with the full $\text{Ca}(\text{NO}_3)_2$ strength (Table 3). Doubling the strength of $\text{Ca}(\text{NO}_3)_2$ in maturation medium

increased the number of vitrified embryos which was reduced to the lowest values (1) with half and quarter strengths (Fig. 1i).



Fig. (1). Stages of date palm cv. Medjool micropropagation. **a, b and c.** Callus formation sequences after nine months, **d.** mature embryos, **e.** mature embryos in 1600 mg/l NH_4NO_3 , **f.** mature embryos in 1545.36 mg/l $\text{Ca}(\text{NO}_3)_2$, **g.** mature embryos in 5 g/l PEG, **h.** secondary embryos in 288 mg/l CaCl_2 , **i.** moderate vitrified shoots from cultures containing $\text{Ca}(\text{NO}_3)_2$ at 1545.36 mg/l, **j.** root numbers during rooting stage and **k and l.** acclimatized plants after foliar sprayed and watered with 0.1 mg/l $\text{Al}_4\text{Si}_4\text{O}_{10}(\text{OH})_8$, after six and nine months, respectively.

Table (2). Influence of ammonium nitrate (NH_4NO_3) modified strength on the rate of somatic embryos and vitrification of date palm cv. Medjool.

NH_4NO_3 strength (mg/l)	No. of mature somatic embryos/jar	No. of germinated embryos/ jar	No. of vitrified embryos/jar
Quarter (100)	5.00 ^e	8 ^e	1.0 ^e
Half (200)	8.20 ^d	12 ^d	1.0 ^e
Full (400)	11.00 ^c	14 ^c	1.0 ^e
Double (800)	13.06 ^b	18 ^b	1.3 ^b
four (1600)	15.26 ^a	20 ^a	1.5 ^a

Table (3). Influence of calcium nitrate ($\text{Ca}(\text{NO}_3)_2$) different strengths on mature, germinated and hyperhydric somatic embryos formation of date palm cv. Medjool.

$\text{Ca}(\text{NO}_3)_2$ strength (mg/l)	No. of mature somatic embryos/jar	No. of germinated embryo/jar	No. of vitrified embryos/jar
Quarter (193.17)	4.10 ^e	9.13 ^e	1.0 ^d
Half (289.74)	6.06 ^d	10.00 ^d	1.0 ^d
Three-quarter (386.34)	9.00 ^c	15.33 ^c	1.3 ^c
Full (772.68)	13.03 ^b	18.66 ^b	1.5 ^b
Double (1545.36)	25.00 ^a	30.00 ^a	2.0 ^a

1.3. Effect of polyethylene glycol (PEG) on somatic embryos formation

The findings in Table (4) demonstrate how PEG directly affected the ability of cv. Medjool somatic embryos to mature. This therapy aided in the development of somatic embryos into plantlets. The maximum percentage (70%) and number of somatic embryos (7.4 somatic embryos/callus) were generated with 5 g/l PEG treatment (Fig. 1 g). Low rates of somatic embryos maturation and development were seen in medium contained 10 g/l. On the other hand, medium contained 20 g/l PEG failed completely to form somatic embryos (Table 4).

Table (4). Effect of polyethylene glycol (PEG) concentrations on somatic embryos production date palm cv. Medjool.

PEG conc. (g/l)	Percentage of explants forming somatic embryos (%)	No. of differentiated somatic embryos/jar
0	50	5.60 ^b
5	70	7.40 ^a
10	35	4.00 ^c
20	0	0.00 ^d

2. Germination Stage

Effect of calcium chloride (CaCl₂.2H₂O) on germination

Cluster of somatic embryos (2-3 embryos) cultivated in the highest concentration of CaCl₂.2H₂O (217.5 mg/l) modified WPM maximized both average number of mature embryos (13.06) and germination percentage (25%) after three months of culture (Table 5 and Fig. 1h). While CaCl₂.2H₂O concentrations below 217.5 mg/l reduced both parameters. Contrarily, explants' vitrification is decreased significantly by decreasing the concentration of CaCl₂.2H₂O.

Table (5). Effect of calcium chloride (CaCl₂.2H₂O) concentrations on somatic embryos production of date palm cv. Medjool.

CaCl ₂ .2H ₂ O conc. (mg/l)	No. of mature somatic embryos/jar	Percentage of germinated embryos (%)	No. of vitrified embryos/jar
Half (36.25)	5.00 ^e	10 ^e	1.20 ^d
Three- quarter (54.37)	7.00 ^d	13 ^d	1.20 ^d
Full (72.5)	8.80 ^c	15 ^c	1.40 ^c
Double (145)	10.10 ^b	18 ^b	1.66 ^b
Third (217.5)	13.06 ^a	25 ^a	1.90 ^a

3. Rooting Stage

Effect of phloroglycinol (PG) on the root system

Table (6) demonstrates that rooting percentage increased significantly over control at all PG concentrations (20, 40, 80, and 160 mg/l), and 40 mg/l PG was the best. On the other hand, for any of the tested PG treatments, rooting percentage rose with progress in subculture numbers (Fig. 1 j). According to data

in Table (6), the maximum number of rootlets were produced with 40 mg/l PG, which was statistically better (6.44) after three subcultures. On the other hand, control medium and medium contained 160 mg/l significantly lowered the number of rootlets per plantlet at all tested subcultures. Table (6) shows that during the first, second, and third subcultures, the tallest rootlets, measuring 3.1, 2.6, and 2.2 cm, were produced after three subcultures in the ½ WPM for rooting supplemented with 40, 20 and 0 mg/l PG, respectively with significant differences among them.

Table (6). Effect of phloroglucinol (PG) on root formation of date palm cv. Medjool plantlets through three subcultures.

PG conc. (mg/l)	Rooting percentage (%)			No. of rootlets/plantlet			Rootlets length (cm)		
	Subculture number			Subculture number			Subculture number		
	I	II	III	I	II	III	I	II	III
0	50.00 ^m	53.0 ^l	58.00 ^k	1.0 ^k	1.23 ^j	1.36 ^h	1.0 ^m	1.50 ^k	2.2 ^c
20	78.00 ^g	80.2 ^f	83.20 ^e	1.6 ^f	1.70 ^e	2.00 ^c	1.8 ^h	1.83 ^g	2.6 ^b
40	90.10 ^c	95.0 ^b	97.00 ^a	2.0 ^c	4.30 ^b	6.30 ^a	2.0 ^e	2.10 ^d	3.1 ^a
80	76.16 ^h	80.0 ^f	84.00 ^d	1.4 ^h	1.50 ^g	1.80 ^d	1.6 ^j	1.70 ⁱ	1.9 ^f
160	71.13 ^j	75.0 ⁱ	76.33 ^h	1.2 ^j	1.30 ⁱ	1.40 ^h	1.4 ^l	1.50 ^k	1.6 ^j

4. Acclimatization Stage

To create healthy plantlets for acclimatization, two steps of the rooting stage were completed. Elongated shoots were rooted in the rooting solid media prior to being moved to the pre-acclimatization liquid medium to promote root growth (Fig. 1k and l). Rooted plantlets with healthy shoots and roots acclimated to the greenhouse environment successfully. After that, plants were foliar sprayed and watered twice a week with K₂SO₄ or Al₄Si₄O₁₀(OH)₈ for 24 weeks. Table (7) and Fig. (1k and l) show that there were significant differences among treatments, foliar sprayed and watered plants with 0.1 mg/l Al₄Si₄O₁₀(OH)₈ recorded the highest significant values of survival percentage, leaf number, and plant height (96%, 7.03 and 76 cm, respectively), followed statistically by using 200 mg/l K₂SO₄. While control treatment having the lowest values of the three parameters (79.33%, 5, and 35.13 cm, respectively).

Table (7). Effect of potassium sulfate (K_2SO_4) and alumino silicate ($Al_4Si_4O_{10}(OH)_8$) (kaolin) on growth measurements of date palm cv. Medjool plants after 24 weeks in greenhouse conditions during acclimatization stage

Treatment (mg/l)		Survival percentage (%)	No. of leaves/plant	Plant height (cm)
K_2SO_4	$Al_4Si_4O_{10}(OH)_8$			
0	0.0	79.33 ^e	5.00 ^d	35.13 ^g
100	0.0	83.00 ^d	5.60 ^{bcd}	61.10 ^d
200	0.0	92.33 ^b	6.76 ^a	71.66 ^b
400	0.0	89.00 ^c	6.00 ^b	40.00 ^f
0	0.1	96.00 ^a	7.03 ^a	76.00 ^a
0	0.2	81.00 ^e	5.76 ^{bc}	66.00 ^c
0	0.4	83.00 ^d	5.30 ^{cd}	50.33 ^e

DISCUSSION

Previous studies described many factors that have been associated with somatic embryo formation process; some through modification of culture media supplements (Al-Khayri, 2010; Hassan et al., 2012 and 2021) or technical methods (Ibrahim et al., 2012). Exposing to salt stress could be another aspect to improve somatic embryogenesis of date palm (Taha and Hassan, 2014). The limited success and slow progress in date palm micropagation technology development may be attributed to the serious deficiency in basic information on tissue and cell response to various media formulation and additives. The formation, devolvement and germination of somatic embryos are controlled by basal salt composition among other known tissue culture factors. Different culture media contain defined amounts of minerals, in the form of inorganic salts, essential for *in vitro* growth and development. Nevertheless, studies related to mineral nutrients have been limited and often focused on growth responses thus overlooking their role as morphogenic elicitors (Ramage and Williams, 2002). A wide range of culture medium formulas have been arbitrarily selected as a basal nutrient medium in various plant species. The impact of different culture medium formulations on *in vitro* culture stages of date palm are scarce (Hassan et al., 2012).

In the present investigation, WPM had stimulatory effects on date palm somatic embryogenesis process. Moreover, the strength of WPM is a crucial factor in the obtained results. Date palm numbers of mature and germinated somatic embryos could be improved when NH_4NO_3 and $CaNO_3$ (1545.36 mg/l) were modified and increased in WPM. Capuana et al. (2007) found that WPM

appeared to be more conducive to faster embryo maturation for ash (*Fraxinus excelsior* L.) compared with Nitsch and Nitsch (NN) (Nitsch and Nitsch, 1969). and MS media. In date palm cv. Khalas, WPM formulation improved callus growth and embryo proliferation, secondary and germinated embryo numbers to maximum values during somatic embryo formation stages (Hassan et al., 2012). White's Medium and WPM ranked first, Schenk and Hildebrandt (SH) and MS media ranked second and NN medium ranked third in the case of callus growth in date palm cv. Khusab, while the highest regeneration percentage in cv. Berny occurred on WPM followed by MS and NN media (Al-Khayri, 2011).

The most popular nitrogen sources employed for *in vitro* growth are nitrate and ammonium ions (Murashige and Skoog, 1962 and Niedz, 1994). The concentration of nitrogen and the ratio of its forms may have an impact on the cell division, differentiation, growth, and development of tissue cultures. Nitrogen is one of the major essential nutrients present in culture medium. The various forms of nitrogen affect the endogenous levels of cell metabolites, proteins, organic acids, and plant hormones (Sotiropoulos et al., 2005). Anthocyanin production, electron transport rate, photosynthetic rate, chlorophyll content, rubisco activity, fresh mass, soluble protein concentration, and osmotic pressure of the cell sap are all influenced by nitrogen availability in addition to each other (Manoli et al., 2014). Moreover, Asemotam et al. (2007) found that the addition of 50 mM nitrogen (40 mM NO_3^- and 10 mM NH_4) enhanced callus formation and growth of leaf explants of date palm.

In the present study, increasing NH_4NO_3 and CaNO_3 in the culture medium enhanced vitrification which is considered as a big physiological disorder associated with date palm organogenesis and embryogenesis techniques. Abahmane (2011) stated that an excess of ammonium ion in culture medium of date palm micropropagation via organogenesis increased vitrified shoot numbers. Mazri et al. (2016) found that the medium containing 1650 mg/l NH_4NO_3 increased vitrification frequency of date palm cultures. Moreover, Ivanova and Staden (2009) mentioned that tissue cultured *Aloe polyphylla* exhibited higher vitrification percentage when NH_4NO_3 was used as the source of nitrogen, and when NO_3^- was used, the percentage was significantly reduced to lowest value. PEG does not damage tissue, which may do so in reaction to water stress, which could lead to plasmolysis in the tissue (Calic-Dragosavac et al., 2010).

The somatic embryo's quality is a crucial factor in somatic embryogenesis and a strong determinant of how well *in vitro* somatic embryos form. PEG at varying concentrations is added to modified growth regulator-free WPM to speed and create somatic embryos. The highest yield (70%) and somatic embryo yield (7.4 somatic embryos/callus) were produced with 5 g/l PEG treatment. When

Langhansova et al. (2004) evaluated the impact of PEG and ABA on somatic embryogenesis and plantlet regeneration, they found that the best way to improve the quality of somatic embryos was to introduce a maturation phase of culture between the multiplication (maintenance) and regeneration phases. Additionally, they stated that cultures treated with PEG 4000 and ABA showed improved growth. Both PEG concentrations examined yielded similar effects. According to Mazri et al. (2019), the medium enriched with 40 g/l PEG produced the most advanced somatic embryos (71.4 per 100 mg callus), which corresponded to the highest rate of somatic embryo maturation.

Stress that promotes embryonic development is therefore necessary for the maturation or conversion of somatic embryos. Stress also appears to be a key factor in somatic embryo maturation, similar to induction. According to Smulders and de Klerk (2011), this stress alters the DNA methylation pattern and triggers the expression of proteins, both of which are necessary for the development of somatic embryos and their proper maturation.

Somatic embryo induction and development are influenced by several parameters, including the culture medium's protein content, the explant source, the genotype, the type and concentration of plant hormones, and stress factors (Rose et al., 2010). Among these nutrients, calcium is regarded as a second messenger in many hormone-regulated activities, playing a significant part in many cellular and physiological processes in plants (Das and Pandey, 2010). It can encourage the development of plant somatic embryos, contributing to polarized growth, cell elongation and division, and cytoskeletal organization. Calcium not only encourages somatic embryo formation, but it can also maintain the embryogenetic callus friable and boost callus regeneration capacity (Arruda et al., 2000 and Takeda et al., 2003). $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ treatments significantly increased the number of mature and germinated embryos in cv. Medjool date palm callus cultures. After three months of culture, the amount of modified WPM (217.5 mg/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) provided the highest average number of mature embryos (13.06) and germination percentage (25%). Similar to this, Mazri et al. (2016) showed that the concentrations of 825 mg/l NH_4NO_3 , 1900 mg/l KNO_3 , 220 mg/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 370 mg/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 170 mg/l KH_2PO_4 are the most suitable for date palm cv. Mejhoul shoot bud multiplication.

The quality of the roots is a significant indicator of the quality of *in vitro* plantlets and a key element in adventitious rooting and transplantation. On WPM with 0.1 mg/l NAA and various amounts of PG, shoots developed roots. Higher rooting percentage, root numbers and lengths were obtained on 40 mg/l PG. However, as PG concentrations increased, root number and root length steadily reduced. This result is corroborated by several researchers. However, the majority of these studies were conducted on other fruit species, such as apricot, apple in

addition to date palm. Schmildt et al. (2000) studied *Citrus sinensis*, Erbenová et al. (2001) studied sweet cherries, and Hassan et al. (2005) studied date palm. Additionally, PG positive impact on two rooting parameters (the number and length of produced rootlets) is broadly consistent with Madgi et al. (1999) findings on apple.

A natural precursor to the formation of lignin, PG has complicated effects on plant tissue culture (Teixeira da Silva et al., 2013). According to several investigations, PG is capable of simulating auxin or cytokinin activity, and it is also effective at reducing stress damage and eliminating vitrification brought on by inadequate lignification (Tallon et al., 2012). In vitro plant mortality after acclimation, according to Ricardo et al. (2015), is significantly influenced by the genotype and PG concentration applied. Although more research is undoubtedly required, their findings showed that moderate PG concentrations during shoot multiplication (0.4 mM), reduced to 0.2 mM, six weeks before root induction, significantly improved walnut *in vitro* performance without significantly affecting rooting efficiency and plant survival. An excessively high concentration of PG, like those of most other phenolic compounds and growth regulators, would have an inhibitory effect; it is also true that PG and its precursors (such as phloridzin) or products of metabolism (such as phloretic acid) have the potential to influence a wide range of plant growth processes and development (Jaime et al., 2013).

Plantlets are subjected to different conditions, such as high relative humidity, low light intensity, and constrained CO₂ inflow, when they are being cultured in a dish. These unique circumstances result in the development of plantlets that exhibit morphological and anatomical defects that negatively affect water relations, photosynthetic effectiveness, and other physiological processes (Hazarika, 2006). After being moved to the greenhouse, these plantlets could readily deteriorate in the event of abrupt changes in the environment. Plantlets must therefore acclimatize for a period to get rid of these anomalies, maintain water balance, and ultimately boost survival rates (Chandra et al., 2010).

According to the data about date palm acclimatization, foliar applications of various concentrations of Al₄Si₄O₁₀(OH)₈ as an anti-transpiration chemical, resulted in appreciable increases in growth criteria compared with control treatment and it is being the best one. Mohamed et al. (2011) found that, spraying date palm plantlets of Sakkoty and Bartmuda cultivars during acclimatization stage with 0.5, 1.0 and 2 ml/l anti-transpiration agent; stress relief 35 (active ingredients acrylic latex polymers) enhanced survival percentages compared with control treatment. Moreover 0.5 ml/l significantly increased plantlets length and leaf number.

Recently, it was discovered that $\text{Al}_4\text{Si}_4\text{O}_{10}(\text{OH})_8$ treatment in a grapevine plant enhanced photosynthetic pigments and photochemical reflectance (Dinis et al., 2018). The activities of some compatible solutes (total soluble sugar, free amino acids, and proline), as well as some antioxidant enzymes, were significantly increased by water stress, according to the research of Abdallah et al. (2019). Growth parameters, yield components, photosynthetic pigments, and carbohydrate contents all increased when wheat plants were given foliar treatments with K_2SO_4 or $\text{Al}_4\text{Si}_4\text{O}_{10}(\text{OH})_8$ (Abdallah et al., 2019).

Potassium is necessary for the growth of plants; it also plays a crucial role in the activities of enzymes, the opening and closing of stomata, and photosynthesis (Golldack et al., 2003). It was shown by Abdelaziz and Abdeldaym (2018) that cucumber growth was significantly boosted by foliar potassium rates. Potassium is essential for increasing plant growth, photosynthetic capability, water usage effectiveness, and participation in the manufacture of metabolic chemicals. Additionally, Segura-Monroy et al. (2015) found that the use of K_2SO_4 increased the plant height and dry weight of seedlings of *Physalis peruviana* L. According to Khalil (2006), compared to the control treatment, all anti-transpirants (film-forming, stomata, and reflecting) significantly boosted all growth parameters for sesame (*Sesamum indicum*) plants. The effectiveness of foliar application of K_2SO_4 in reducing the negative impacts of water stress, enhancing the physiology and production of plants, and reducing the negative effects of water stress on photosynthesis in almond or walnut trees is assessed (Rosati et al., 2006). According to Denaxa et al. (2012), K_2SO_4 increased the amount of photosynthesis occurring in olive plants, which increased the rate at which plants could absorb water. Additionally, after K_2SO_4 treatment, Lombardini et al. (2005) noted a favorable impact on the chlorophyll index. This increase in chlorophyll concentration may be because leaves not treated with K_2SO_4 may reflect light less well, indicating that the pigments used for photosynthetic energy are degrading more quickly (Dinis et al., 2016).

CONCLUSIONS

The success of date palm micropropagation is thought to be greatly influenced by the various balanced combinations of tissue culture medium. When cv. Medjool callus cultures were in the maturity stage, the effects of NH_4NO_3 , $\text{Ca}(\text{NO}_3)_2$, and PEG were clearly seen on developed, germinated, and vitrified embryos. Additionally, the somatic embryo germination (shoot production) and number of secondary embryos were enhanced by $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ levels throughout the germination stage. The root system benefited from the addition of PG to the rooting media. The ability of regenerated plantlets to overcome the water

limitation in the greenhouse determines their vigour to transplant successfully *ex vitro*. To better comprehend how the plantlets handle such issues, it is crucial to have a greater grasp of the physiological and anatomical events that take place throughout the *in vitro* hardening process. In this study, K_2SO_4 and kaolin or $Al_4Si_4O_{10}(OH)_8$ were used to reduce the negative effects of water stress and increase date palm development. It is interesting that the current study suggested that $Al_4Si_4O_{10}(OH)_8$ (kaolin) might improve plant physiology, which would increase acclimation rates and make the material safe for the environment. Potassium is an essential nutrient that has the power to regulate several biochemical and physiological processes in plants. In the realm of micropropagation and biotechnology, cv. Medjool has proven to be a promising cultivar. Its shoots have grown into robust plantlets that have adapted to greenhouse conditions.

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تطوير نظام للإنتاج التجاري في نخيل البلح صنف المجدول

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أصبح استخدام تقنية الأجنة الجسدية لنخيل التمر تقنية مثالية لإنتاج الأصناف النادرة والمميزة على نطاق واسع. أصناف نخيل التمر ومكونات وسط الزراعة وكذلك ظروف التحضين كلها عوامل مؤثرة على عملية تكوين الأجنة الجسدية. خلال هذا البحث تم دراسة التأثير المحتمل لقوة نترات الأمونيوم، نترات الكالسيوم وتركيزات البولي إيثيلين جليكول على الأجنة الناضجة والأجنة المنبثة وأيضاً الأجنة المزججة أثناء مرحلة نضج الأجنة لصنف نخيل التمر المجدول. أيضاً، تم دراسة تأثير مستويات كلوريد الكالسيوم والتي يمكن أن تحسن من عملية إنبات الجنين الجسدي (تكوين الأفرع الخضريّة) وأعداد الأجنة الثانوية أثناء مرحلة الإنبات. كما تم تحديد نسبة التجذير وعدد الجذور/نبات ومتوسط طول الجذور (سم) على أنها تتأثر بمستويات الفلوروجلويسينول أثناء مرحلة التجذير. كذلك تم استخدام التركيز المثالي من كبريتات البوتاسيوم أو سيليكات الألومينو (الكاولين) كمضادات للنتح والذي يؤثر على بقاء ونمو نباتات نخيل التمر صنف المجهول فيما يتعلق بمرحلة التأقلم خارج المعمل. أظهرت النتائج أن استجابة النباتات للتشكل أثناء عملية النضج يتم التحكم فيها بواسطة كلاً من نترات الأمونيوم وقوة نترات الكالسيوم وكذلك تركيزات البولي إيثيلين جليكول. شجع تعديل وسط نمو WPM باستخدام نترات الأمونيوم بضعف تركيزها المستخدم ونترات الكالسيوم عند استخدامها بمقدار أربعة أمثال التركيز المستخدم على تحقيق أعلى قيمة معنوية لتكوين الجنين الناضج وأثناء استخدام كلا المركبين السابقين بقوة كاملة أدى إلى الحصول على العدد المعنوي المعتدل للأجنة الناضجة. أدى استخدام المستوى المرتفع (ثلاثة أمثال القوة المستخدمة) لكلوريد الكالسيوم إلى زيادة عدد الأجنة الثانوية وكذلك زيادة عدد النموات الخضريّة المزججة أثناء مرحلة الإنبات. بالنسبة لتكوين الجذور، فإن إضافة الفلوروجلويسينول بتركيز ٤٠ مجم / لتر إلى وسط التجذير حقق أعلى معدل لطول النبات وأعلى نسبة تجذير وأيضاً حقق أعلى متوسط طول للجذور (سم / نبات). يمكن أن يؤدي استخدام سيليكات الألومينو (الكاولين) لكاولين إلي تحسين فسيولوجيا النبات وبالتالي يؤدي إلى زيادة نسبة الأقلمة وذلك عند استخدامه بتركيز ٠,١ مجم/لتر.