International Journal of Environment, Agriculture and Biotechnology
Vol-8, Issue-5; Sep-Oct, 2023
Peer-Reviewed International Journal
Journal Home Page Available: https://ijeab.com/
Journal DOI: 10.22161/ijeab

The genetic stability of date palm shoots regenerated from leaves explant

Rasha N. Arafa[1], Sayed A.A. Elsayh[2], Marwa M. Abdalgaleel[2], Salwa El-Habashy[2], Emadeldin A.H. Ahmed[2], Eman H. Afifi[2], Alaa N. Draz[3], Rabab W. El Aramany[1], Abdallah Sharkh[3], Mohamed T. Abd El-Kader[3], Ahmed A.M. Barakat[2], Dosoky Hoda A.A.[4]

[1]Central Lab of Date Palm Researches and Development, Agricultural Research Center, Giza, Egypt
[2]Horticulture Research Institute, Breeding Research Department, Agricultural Research Center, Giza, Egypt.
[3]Agriculture Botany-Department, Genetics, Faculty of Agriculture, Al Azhar University Cairo, Egypt.
[4]Horticulture Department, Faculty of Agriculture, Benha University.
Corresponding author: Rasha N. Arafa; rashanagyarafa2022@gmail.com

Received: 25 Aug 2023; Received in revised form: 29 Sep 2023; Accepted: 07 Oct 2023; Available online: 13 Oct 2023

Abstract— *The purpose of this research was to develop a micro-propagation method for the date palm Zaghlol cv. using juvenile leaves. To produce the necessary results, different plant growth regulator combinations were used. The leaves were grown on MS medium supplemented with PVP to prevent the explants from browning. The results showed that adding PVP at a concentration of 1.0 g/l considerably reduced browning. On the induction medium, callus formation occurred during the fourth week of culture; however, callus formation (87.5%) was more prevalent on the ¾ MS medium containing with 10.0 mg/l NAA, 1.0 mg/l BA and 2.0 mg/l 2ip. The greatest development of embryogenic callus (94.50%) occurred on a ¾ MS medium supplemented with 5.0 mg/l NAA and 2.0 mg/l BA. The largest fresh callus weight (3.53 g) was produced by this treatment after four months in culture. On MS medium supplemented with 2.0 TDZ, 1.5 BA and 0.5 NAA, which was regarded as the optimum medium for increasing the number of embryos to 32.10 embryos/culture, the best results (65.67%) were obtained. Further investigation into the stimulation and development of somatic embryogenesis involved using MS basal medium supplemented with BA at 0.5 mg/l, kin at 0.1 mg/l and NAA at 0.05 mg/l; this treatment formed the most leaves (20.11 leaf/cluster). The cluster of shoots grown on MS basal medium supplemented with BA at 2.0 mg/l and NAA at 0.5 mg/l had the highest leaf number (34.25 leaf/cluster) and leaves length values after three sub-cultures (4.75 cm). The DNA-based fingerprinting technology ISSR was used to confirm the genetic stability of this protocol. The mother tree and tissue culture-derived shoots evaluated exhibited no differences in the ISSR banding patterns. The micro-propagation method could be used to produce genetically stable date palm plants.*

Keywords— ***Date palm, juvenile leaf explants, plant growth regulators, embryogenic callus induction, somatic embryos formation and maturation and Polymorphism ISSR.***

I. Abbreviation:

II. MS: Murashige and Skoog medium
III. PVP: Polyvinyl Pyrrolidone
IV. NAA: Naphthalene acetic acid
V. BA: 6-Benzyl amino purine
VI. 2ip: 6-dimethylallyl amino purine
VII. TDZ: Thidiazuron
VIII. Kin: Kinetin
IX. DNA: Deoxyribonucleic acid
X. ISSR: Inter simple sequence repeat

I. INTRODUCTION

Phoenix dactylfera L. (Family: Palmaceae) is a dioecious monocotyledonous plant. It is regarded as the most significant fruit tree in a number of Arab nations, including Saudi Arabia and Iraq **(Mirani, 2018)**. Date palm is a resistant fruit tree that grows in a variety of climates, but is most prevalent in hot, arid regions of the Middle East and North Africa. The date fruit has a large number of chemical components with nutritive and medicinal properties **(Al-Khayri and Naik, 2017)**. The yearly worldwide market value of date crops (including imports and exports) reached about 1.9 billion USD **(FAOSTAT, 2013)**, providing a solid important basis upon which a country may develop a successful economy.

Due to a shortage of suitable planting materials, expansion of date palm plantation in various locations of Egypt and replanting trees to compensate for loss due to diseases or human causes are limited. Additionally, owing to the extended generation time and heterozygosity of date palm cultivars, development of Egyptian date palm cultivars using conventional breeding programs has encountered challenges. In recent years, significant advancements in plant biotechnology have complemented traditional approaches for date palm multiplication, conservation, and genetic improvement **(Bekheet, 2013)**. The date palm may have a significant ecological impact on a variety of desert and semi–arid environments **(Badawy *et al.*, 2005)**.

Worldwide, an estimated 150 million date palm trees have been developed utilizing a variety of propagation techniques, including seed germination and offshoot transplanting as natural and traditional means of propagation, as well as small-scale tissue culture technologies **(Al-Khayri *et al.*, 2015)**. Due to the heterozygosis of the seed propagated date palm, it cannot be used for commercial production of selected cultivars **(Tisserat, 1982)**, due to the significant differences in fruit maturation, fruit quality, harvesting time, and production potential between plants propagated vegetative and seedlings **(Zaid *et al.*, 2011)**. Whereas true to type date palm propagation by offshoots is a sluggish process that is often impeded by the restricted number of offshoots that a tree produces during its life, a poor survival rate, and the potential of disease spread such as Bayoud and Red Palm Weevil infection **(Al-Khalifah and Askari, 2011)**.

The micro-propagation is a potential method for producing vast quantities of disease-free and pest-free plants for plant material exchange, as well as genetically identical and high-quality planting material. Due to the rising demand for unusual and superior quality date palm cultivars, micro-propagation is an inescapable means of propagation **(Al-Mayahi, 2020)**. The effectiveness of plant tissue culture as a technique of plant multiplication is highly dependent on the composition of the growing medium **(Al-Mayahi *et al.*, 2020)**.

The tissue culture method of propagation is the most potential tool for the efficient production of high-quality plant materials **(Sane *et al.*, 2006)**. Due of the inherent limits of traditional date palm growth by offshoots **(Al-Khayri, 2011)**. For date palm micro-propagation, somatic embryogenesis is regarded the most effective regeneration mechanism **(Fki *et al.*, 2003)**. It is considered to be a rapid and effective approach for large-scale date palm propagation and may also be very beneficial for breeding operations.

The induction of embryogenic callus in date palm is controlled by a variety of factors, including genotype, explant type, induction duration, and plant growth regulators (PGRs). In the case of either shoot tips or leaf primordial, large amounts of auxins have been employed to produce embryogenic callus. The most efficacious auxin is 2,4-dichlorphenoxyacetic acid (2,4-D), which is used to induce embryonic callus in date palm **(Behnas *et al.*, 2014)**. However, **Fki *et al.* (2011)** indicated that large dosages of 2,4-D may generate somaclonal variation. As a result, several researchers induced somatic embryogenesis using lower 2,4-D doses or different auxins.

To begin the process of *in vitro* date palm formation by indirect organogenesis or somatic embryogenesis, relatively large doses of 2,4-D or NAA must be applied. However, it is well established that these auxins are related with genetic instability in plants **(Behnas *et al.*, 2014)**. Numerous research have been conducted on date palm micro-propagation using a variety of explants, among which shoot tips of young offshoots **(Al-Khayri and Naik, 2017; Tisserat, 1984)** and juvenile spathes **(Fki *et al.*, 2003; Jatoi *et al.*, 2019)** have been proven to be sustainable and prolific. However, *in vitro* conditions may result in the development of multiple genetic and epigenetic variations in date palm plantlets generated by tissue culture owing to the stress caused by plant growth regulators and the prolonged production or multiplication cycle **(Mirani *et al.*, 2020)**.

Date palm cultivars fall into three groups: soft, semi-dry, and dry, based on the texture of the fruit when it is ripe. There are a lot of countries that produce dates. Egypt is the first country that produces the most dates. Saudi Arabia, Iran, the United Arab Emirates, Pakistan, Algeria, Sudan, Oman, Libya and Tunisia are the 10 countries that produce the most of these dates **(Kader and Hussein, 2009)**. The ISSR approach is one of the efficient methods for determining date palm genetic diversity **(Haider *et al.*,**

2012). Numerous research has examined the use of ISSR markers to measure date palm genetic variation **(Adawy *et al.*, 2011; Zehdi-Aziuzi *et al.*, 2009)**. It has been demonstrated by numerous researchers that ISSR can detect genetic variety in date palms and has the capability to amplify polymorphism fragments, which is consistent with our findings. For example, **Zehdi *et al.* (2004)** used seven ISSR primers to analyze the genetic diversity of 12 cultivars of Tunisian dates and were able to achieve a significant polymorphism rate. They found that there were enough polymorphic bands in their study to distinguish between all cultivars. **Cullis (2011)** successfully distinguished date palm cultivars in a different study utilizing RAPD, AFLP and ISSR markers. Each of the aforementioned indicators can be used effectively both individually and in combination.

II. MATERIAL AND METHODS

This investigation was conducted in the Central Laboratory of Date Palm Researches and Development - Agricultural Research Center, Egypt during 2022-2023.

2.1- Plant materials:

The date palm Zaghloul cv. offshoots had a height of 80-100 cm, a diameter of 30 cm, a weight of 25-35 kg, and was two years old. Offshoots were carefully separated from field-grown mother trees in Natron Valley and immediately transferred to the laboratory for use as mother plant material, as shown in **Fig (1)**.

Fig.1: The date palm Zaghloul cv. Offshoots

2.2- Leaf primordial explant separation and sterilization:

To remove the leaf primordial explants, the leaves and fiber sheath were cut acropetally from the offshoot using a hatchet and a sharp knife. Separation of mature leaves began at the base and continued until the shoot tip material attained a width of 18 cm and a length of 30 cm.

To prevent browning of the explants, they were placed in beakers containing antioxidant solutions (100 mg/l citric acid and 150 mg/l ascorbic acid) for 20 minutes, then thoroughly washed in running tap water for 30 minutes. The outermost leaves were then removed and the palm heart was reduced to approximately 10 cm in length (measured from the meristem base to the leaf apex of the central cylinder) using a sterile blade. It was then surface sterilized in a laminar flow chamber by flaming once. The explants (shoot tips and leaf primordial) were surface sterilized under aseptic conditions by immersion in a 50% Clorox solution (5.25% sodium hypochlorite) (NaOCl) containing two drops of tween 20 for 20 minutes with continuous stirring, followed by three sequential one-minute rinses in sterile distilled water. Following that, remove two more leaves around the shoot tip and immerse in 30% NaOCl for 30 minutes then thoroughly rinsing with sterile distilled water. Finally, the explants were soaked for 10 minutes in a 0.1% mercuric chloride ($HgCl_2$) solution and washed three times with sterile distilled water. Following surface disinfestation, shoot tips ranging in length from 3-5 cm and inner and primary leaflets generally ranging from 1-3 cm were separated and sliced into three to four pieces. The explants were split into pieces of 0.5 cm in length. On the culture medium, leaf segments were put with the abaxial surface. The trials were divided into four stages: callus induction, callus proliferation, somatic embryo differentiation and plant regeneration.

2.3- Culture Medium

The basal nutrient medium used was MS medium **(Murashige and Skoog, 1962)** supplemented with (mg/l): 0.5 nicotinic acid, 0.5 pyridoxine-HCL, 2.0 glycine, 1.0 thiamine-HCL, 100 myo-inositol, 2.0 biotin, 40 mg/l adenine sulphate, 200 glutamine, 170 mg/l $NaH_2PO_4.2H_2O$, Ca-pantothenate 0.2 g/l, 1.0 activated charcoal (AC), 35.0 g/l sucrose and solidified with 6.0 g/l agar in addition different plant growth regulators according to each growth stage. The pH of all media was adjusted to 5.7 with NaOH or HCl then the medium was distributed in 40 ml aliquots in 250 ml jars. The culture medium was autoclaved for 25 min at 121 °C and 1.4 Kg cm^{-2}.

2.4- Embryogenic callus induction

- To avoid browning of explants, all surface sterilized explants were grown for 3 weeks on MS medium devoid of growth regulators and supplemented with Polyvinyl Pyrrolidone (PVP) at doses of 0.0, 0.1, 0.5 and 1.0 g/l.
- The juvenile leaves were grown in ¾ MS medium supplemented with varied doses of Naphthalene acetic acid

(NAA) (0.0, 2.0, 5.0 and 10.0 mg/l), 1.0 mg/l 6-Benzyl amino purine (BAP), and 2.0 mg/l 6-dimethylallyl amino purine (2ip). All cultures were incubated at 26°C in the dark and re-cultured every six weeks on the same fresh medium. On the juvenile leaves, white nodular embryonic callus was noticed after three subcultures.

- After four months, juvenile leaves with initiation callus were grown on ¾ MS medium supplemented with NAA at a concentration of 5.0 mg/l and BA at 0.5, 1.0 and 2.0 mg/l. All cultures were incubated at 26°C in the dark and re-cultured every six weeks in the same fresh medium for three subcultures.

2.5- Somatic embryos proliferation

After the induction period (8 months), 0.5 g of embryogenic callus was separated from the original explants and transferred to MS medium containing 40.0 g/l sucrose, 6.0 g/l agar and 1.0 g/l activated charcoal, as well as TDZ at various concentrations (0.0, 0.5, 1.0 and 2.0 mg/l), BA at 1.5 mg/l and NAA at 0.5 mg/l (differentiation medium), in order to stimulate differentiation of embryogenic callus into somatic embryos. For three months, the cultures were kept in the dark at 27°C and sub-cultured every four weeks under the same culture conditions. After three subcultures, the proportion of somatic embryos that proliferated (%), the number of somatic embryos (embryo/culture), and the length of embryos (cm) were determined.

2.6- Somatic embryos maturation and germination

From the previous stage, the somatic embryo clusters were divided into small clusters containing around 8-13 embryos. The embryo clusters were cultivated in jars (150 ml) containing 45 ml modified MS media supplemented with BA at 0.5, N^6-furfuryladenine (Kin) at 0.1 and NAA at 0.05 (mg/l) (germination medium), as well as the control treatment. The cultures were maintained and proliferated by sub-culturing at intervals of 6 weeks for three sub-cultures, nine similar jars were utilized. The cultures were incubated at a temperature of 27±1°C and a photoperiod of 16 hours with a light intensity of 1500 lux. After three months, the germination (%), the number of shoots per jar, the number of leaves per jar and the length of the leaves (cm) were all recorded.

2.7- Regeneration and shoots multiplication

After germinating the somatic embryos, the cluster of shoots was moved to MS medium supplemented with 200 mg/l NaH_2PO_4, 40 g/l sucrose, 200 mg/l casein hydrolysate, 1.5 g/l charcoal, 2.0 mg/l BA and 0.5 mg/l NAA. The cultures were incubated in a culture room at 28±2°C with a 16-hour photoperiod provided by fluorescent lights and three subcultures were conducted at a 6-week interval.

2.8- The genetic stability among regenerated date palm shoots in comparison with their mother plant

The several regenerated shoots were studied at the molecular level using ISSR analysis in order to study the genetic similarities among the regenerated date palm shoots induced from the leaves explants of Zaghloul cultivar.

2.8-1 PCR reaction using ISSR primers

The following ingredients were used to construct the ISSR amplification reactions: 25 ng of template DNA, 0.2 µM dNTPs, 0.7 mol of primer, 1.0 µl x 10 PCR buffer, 1.5 µM of $MgCl_2$, and one unit of Taq polymerase (Cinnagen, Iran) in a final volume of 10 µl. The reactions were designed for 40 cycles of pre-denaturation at 94 °C for 4 min, followed by denaturation at 94 °C for 30 s, annealing for each primer for 45 s and extension at 72 °C for 2 min. The amplifications were carried out using a PEQStar 96 Universal Gradient 96 wells thermal cycler. After 40 cycles, there was a final 7 min extension at 72 °C. The polymorphism of DNA fragments was assessed using six ISSR primers. The DNA fragments were amplified and then separated by electrophoresis in 1.5% agarose gel. The fluoroDye dyed the DNA. Using the Uvitec Geldoc technology, DNA fragments were seen and recorded.

2.9- Statistical Analysis

The design of the experiments was completely random, with six replicates of each treatment. MSTAT, a computer program, was used to look at the best three results from each treatment. Ducan's Multiple Range Test was used to compare the means of different treatments to see if they were different.

III. RESULTS AND DISCUSSIONS

3.1- Embryogenic callus induction

- Regarding the impact of PVP on explant browning, the findings indicated that adding PVP at a concentration of 1.0 g/l greatly decreased the degree of browning when compared to the other concentrations (0.1 and 0.5 g/l), as shown in **Table (1)**.

Table 1. Effect of antioxidant treatment (PVP) on survival, growth value and degree of browning of date palm juvenile leaf explants.

Concentrations of PVP (g/l)	Survival %	Growth value	Degree of browning
0.0	35.2 d	21.4 de	5.11 a
0.1	81.0 c	28.7 c	4.90 ab
0.5	90.0 ab	67.5 b	2.80 c
1.0	100.0 a	88.0 a	1.0 d

These findings corroborated those of **Zaid (1984)**, who discovered that adding PVP to the culture medium inhibited explant browning. According to **Zaid (1987)** the browning of date palm tissue and the surrounding media is caused by phenolic compound oxidation. Browning and consequent explant mortality is a major problem in woody plant tissue culture, which is often related to phenolic chemicals **(Hesami *et al.*, 2020)**. Phenolic substances may activate the polyphenol oxidase enzyme, change cellular metabolism and ultimately oxidase to form the brown precursor Quinine. Antioxidants limit phenolic compound oxidation by altering the oxidation potential of phenolic compound explants, hence alleviating browning symptoms **(Raj *et al.*, 2020)**. Similarly, in *Curculigo latifolia*, adding PVP to the culture media at a concentration of 0.5% decreased browning and infection in shoot-tip explants **(Babaei *et al.*, 2012)**. Antioxidant substances may influence explant growth by promoting the leaf expansion process and encouraging callus formation **(Huh *et al.*, 2017)**.

Thus, the beneficial impact of PVP on lowering the degree of browning in date palm leaf primordial explants was attributed to the explants' ability to absorb phenolic substances. As a result, adding PVP to culture media at a concentration of 1.0 g/l may be reported as the treatment with the lowest infection rate and the greatest callus induction subsequently.

- Leaf segments implanted on ¾ MS medium supplemented with varying doses of NAA, 1.0 mg/l BA and 2.0 mg/l 2ip, demonstrated a variety of growth responses. Explants treated with 10.0 mg/l NAA, 1.0 mg/l BA and 2.0 mg/l 2ip had a considerably greater frequency of callus development on leaves (87.50%) than those supplied with 5.0 mg/l NAA, 1.0 mg/l BA and 2.0 mg/l 2ip (44.65%), as shown in **Table (2)** and **Fig. (2)**. The medium supplemented with 2.0 mg/l NAA, 1.0 mg/l BA and 2.0 mg/l 2ip exhibited the minimal explant response (13.20%).

According to the findings in **Table (2)**, plant growth regulators (NAA, BA and 2ip) were beneficial at initiating callus formation. On the surface of the leaf explants, the maximum percentage of callus induction (81.90%) was found in ¾ MS medium supplemented with 10.0 mg/l NAA, 1.0 mg/l BA and 2.0 mg/l 2ip; callus induction was lower in other treatments. The control treatment did not result in the formation of the callus.

Table 2. Effects of different concentrations of NAA with added BA at 1.0 mg/l and 2ip at 2.0 mg/l on callus initiation of date palm leaf primordial explants of Zaghloul cv. cultured in vitro for 4 months.

Treatments	Growth value	Callus initiation %
Control	0.00 d	0.00 d
2.0 NAA	13.20 c	24.33 c
5.0 NAA	44.65 b	50.67 b
10.0 NAA	87.50 a	81.90 a

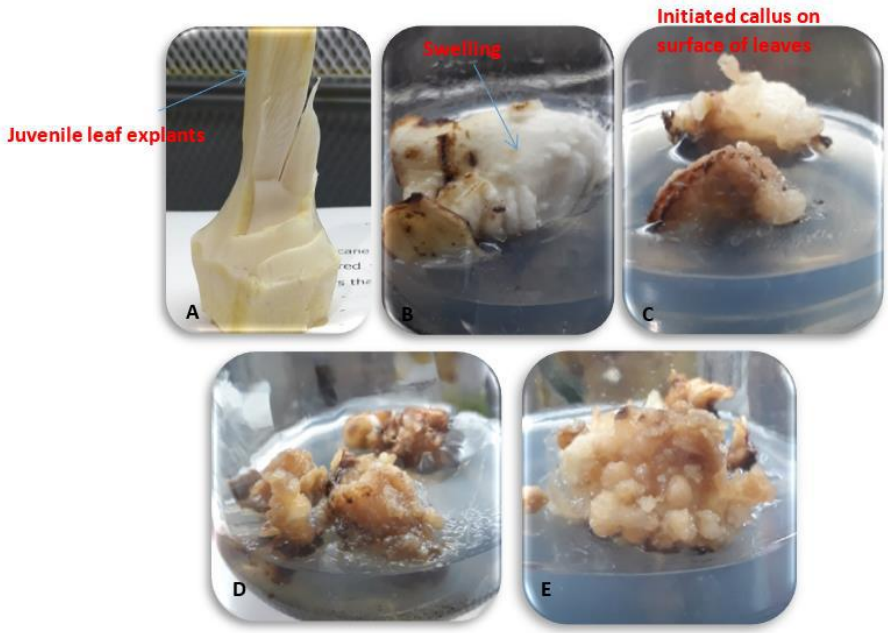

Fig.2: Induction of callus from juvenile leaf explants of date palm cv. Zaghlol; a) Juvenile leaf explants were around the shoot tip, B) the swelling of explants, C) initiated callus on the surface of leaf explants after six weeks on induction medium, D) The control treatment did not resulted in the formation of callus, E) the maximum percentage of callus induction was recorded in MS medium supplemented with 10.0 mg/l NAA, 1.0 mg/l BA and 2.0 mg/l 2ip.

The previous research has shown that plant growth regulators have an effect on the induction callus. The kind and dosage of growth regulators, as well as the type of explants, are the most critical factors in callus formation **(George *et al.*, 2008)**. Additionally, the amount of endogenous auxin and the position of explants **(Almeida *et al.*, 2012)**, particularly the kind and characteristics of tissue vascularization, are likely to affect *in vitro* responses. Numerous variables influence callus initiation, including plant species, culture conditions, explant age and explant placement **(Shin *et al.*, 2019)**. Auxin's beneficial impact on callus induction may be due to low endogenous auxin levels in the explants. Exogenous auxin may influence the amount of endogenous auxin by regulating a range of auxin-related enzymes **(Machakova *et al.*, 2008)**.

Gueye *et al.* (2009) established callus from seedling leaves of the cultivar Ahmar. A rather high auxin concentration, 15.0 mg/l NAA, either alone or in conjunction with cytokinin, significantly increased callus initiation and growth (BA or Kin). The leaf explants grown on Eeuwen's medium at various NAA concentrations (5.0–20.0 mg/l) developed callus at all NAA levels **(Asemota *et al.*, 2007)**. To date, the most frequently employed growth phyto-hormones for plant regeneration are 2,4-D, BA and NAA. In this investigation, we evaluated several combinations of NAA, 2ip and BA to see which combination produced the highest callus percentages **(Liu *et al.*, 2018)**. According to **Liu *et al.* (2021)**, an adequate concentration of auxin induced callus formation in *F. mandshurica*. The proportion of callus induction increased significantly with increasing NAA levels in the range of 0.1 to 0.15 mg/l.

- The callus began to form during the fourth week of culture on induction medium. After 4 sub-cultures, the callus generation rate remained consistent but callus weight maintained growing. The maximum development value (94.50%) of embryogenic callus was obtained on ¾ MS medium combined with 5.0 mg/l NAA and 2.0 mg/l BA. The leaf primordial explants, revealed callus development rates of 100.0%. Additionally, with this treatment resulted in the best fresh weight of callus (3.53 g), as shown in **Fig (3)**.

The Lowest concentration of BA at 0.5 mg/l with NAA at 5.0 mg/l, exhibited the least reaction as growth value of callus embryogenesis (25.33%), percentage of callus formation (43.56%) and fresh weight of callus was (1.02 g). By raise the concentration of BA to 1.0 mg/l with adding 5.0 mg/l NAA on MS medium recorded the satisfactory findings, as shown in **Table (3)**. The control treatment did not exhibited any callus development activity on the explants.

As a result, it is possible to consider that the mixture of NAA and BA acts as a critical hormone in the development of embryogenic callus.

Table 3. Effects of different concentrations of BA with added NAA at 5.0 mg/l on callus growth of date palm leaf primordial explants of Zaghloul cv. cultured in vitro for 4 months.

Treatment (mg/l)	Growth value	Callus formation%	Fresh weight (g)
Control	0.00 d	0.00 d	0.00 d
5.0 NAA + 0.5 BA	25.33 c	43.56 c	1.02 bc
5.0 NAA + 1.0 BA	38.00 b	56.77 b	1.98 b
5.0 NAA + 2.0 BA	94.50 a	100.0 a	3.53 a

Cytokinins are necessary for the formation of calluses and cell division **(Minocha, 1987)**. The addition of BA to the culture medium in combination with auxin increased callogenesis rates in *Acacia raddiana* **(Sane *et al.*, 2006)**. Callus development requires a balance of auxin and cytokinin administration **(Rout, 2004)**.

Balzon *et al.* (2013) demonstrated that lowering the auxin concentration was critical for establishing repetitive cycles of cell division and inhibiting differentiation processes, therefore enabling *E. guineensis* calli to proliferate. **Salim (2014)** cleared that among the combinations of NAA and BA evaluated for callus induction on various explants (seed, node and internode), results indicated that NAA concentrations of 0.3 and 0.4 mg/l were the most effective for callus induction. **Al-Mayahi (2015)** has observed embryos regeneration from cv. Quntar leaf explants. This innovative method has the potential to significantly improve date palm cultivar *in vitro* propagation. Nonetheless, further research is required to enhance the incidence of somatic embryogenesis from leaf explants.

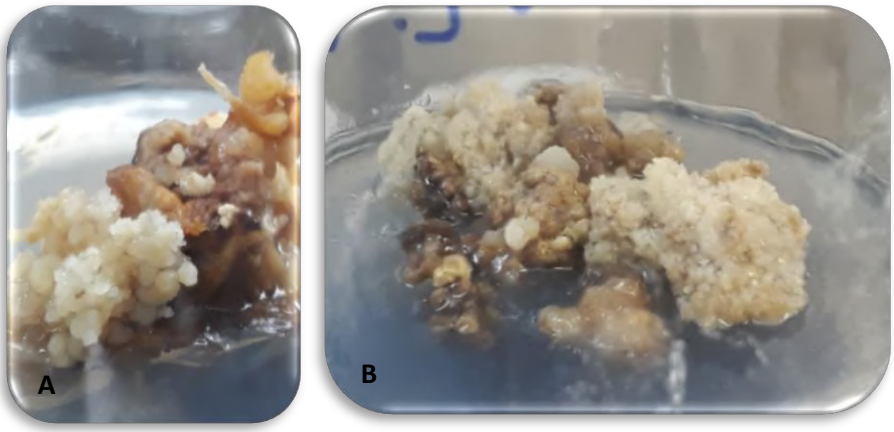

Fig.3: The embryogenic callus production from leaf explants, A) MS basal medium containing BA at 0.5 mg/l with NAA at 5.0 mg/l, exhibited the lowest result as growth value of callus embryogenesis (25.33%), B) the maximum embryogenic callus formation was obtained on ¾ MS medium combined with 5.0 mg/l NAA and 2.0 mg/l BA after 3 subcultures.

In the somatic embryogenesis of oil palm, callus formation occurred in the addition of auxin, either at full strength or ½ MS basal medium **(Rival and Parveez, 2005)**. Notably, the presence of BA to the medium seems to give additional options for the regeneration of plants. Additionally, the usage of various quantities of plant growth regulators had a substantial effect on the development of callus **(Liu *et al.*, 2018)**.

3.2- Somatic embryos proliferation

The somatic embryos were seen in this work by incubating embryogenic calluses on MS medium supplemented with various doses of TDZ, 1.5 mg/l BA and 0.5 mg/l NAA. The embryos were white in color, globular in form as they matured and appeared alone or in clusters. According to the findings in **Table (4)**, the TDZ concentration had an effect on the proliferation percentage, the number of somatic embryos and the length of somatic embryos.

Concerning the percentage of somatic embryos that proliferated, the best results 65.67% was obtained on MS medium supplemented with 2.0 TDZ, 1.5 BA and 0.5 NAA, as compared to the other TDZ doses. While raising the TDZ concentration to 3.0 mg/l lowered the proliferation rate of embryos to 40.51%, as shown in **Fig (4)**.

On the other hand, TDZ concentrations had an effect on the number of somatic embryos; MS medium contained 2.0 mg/l TDZ, 1.5 BA and 0.5 NAA was shown to be the optimal medium for increasing the number of embryos to 32.10 embryos/culture. By increasing the TDZ concentration to 3.0 mg/l, the number of embryos per culture was reduced to 23.16 embryos. Low doses of TDZ (0.5 and 1.0 mg/l) in the presence of 1.5 BA and 0.5 NAA produced the fewest somatic embryos (11.20 and 20.00 embryos/culture, respectively).

In terms of somatic embryo length, MS medium supplemented with 2.0 TDZ, 1.5 BA and 0.5 NAA (mg/l) resulted in embryos measuring 1.5 cm in length, compared to 0.5 cm in the control treatment. TDZ concentrations of 0.5 and 1.0 mg/l resulted in embryos ranging in length from 0.8 to 1.2 cm.

Table 4. Effect of different concentrations of TDZ with added 1.5 BA and 0.5 NAA (mg/l) on somatic embryos proliferation of Zaghloul date palm cultivar after 12 weeks of culturing.

Treatment (mg/l)	Somatic embryos proliferation %	Number of somatic embryos	Length of somatic embryos (cm)
Control	8.00 e	4.33 d	0.5 cd
0.5 TDZ, 1.5 BA, 0.5 NAA	22.15 d	11.20 c	0.8 c
1.0 TDZ, 1.5 BA, 0.5 NAA	30.10 c	20.00 b	1.2 b
2.0 TDZ, 1.5 BA, 0.5 NAA	65.67 a	32.10 a	1.5 a
3.0 TDZ, 1.5 BA, 0.5 NAA	40.51 b	23.16 b	1.0 bc

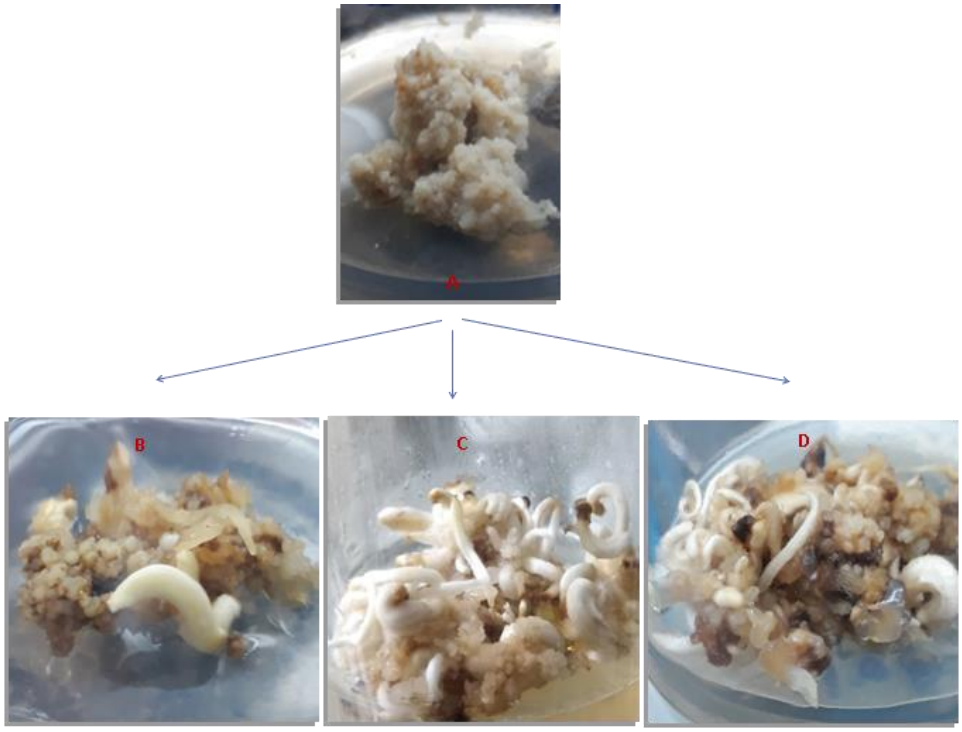

Fig. 4. The embryogenic callus germinated, A) Embryogenic callus was formed, B) The control treatment recorded the fewest number of somatic embryos, C) MS medium supplemented with 2.0 TDZ, 1.5 BA and 0.5 NAA shown to be the optimal medium for increasing the number of embryos, D) By increase the TDZ concentration to 3.0 mg/l lowered the proliferation rate of embryos.

In previous research, **Rival and Parveez (2005)** somatic embryogenesis in oil palm as a multistep process that includes induction, embryogenesis, somatic embryo development and maturation, shoot development and rooting. The explants are stimulated to develop primary callus in auxin-containing media under dark conditions during the induction stage. The callus is also transferred to auxin-containing media during the embryonic stage, however at lower concentrations and often under light conditions. This stage promotes the development of proliferating callus into embryogenic cell clusters.

Organogenesis requires a ratio of auxin to cytokinin of 10/0.4/0 or equal. In *Damask rose*, combining TDZ and BA resulted in considerably more shoots per explant than the most optimal BA treatments alone **(Mamaghani *et al.*, 2010)**. Additionally, treatment with TDZ promoted development and increased the quantity of somatic embryos. TDZ has been proposed to enhance nucleoside levels, purine cytokinin accumulation or synthesis, or to accelerate adenine to adenosine conversion **(Capelle *et al.*, 1983)**. TDZ enhanced the explants' embryogenic response throughout normal growth and development; same effect has been reported in a variety of different species. TDZ is very stable in culture medium and has a long half-life in plant tissues **(Mok and Mok, 1985)**. **Victor and colleagues (1999)** demonstrated that TDZ efficiently promoted somatic embryogenesis in peanut after a minimal exposure period. TDZ may have a double function in the induction of somatic embryogenesis: 1) cytokinin-like activity that stimulates cell division and differentiation and 2) a modest auxin-like activity that seems to be required for the development of embryogenic competence.

Wójcikowska *et al.* (2013) discovered that auxin treatment accelerated somatic embryogenesis by activating transcription factors, particularly leafy cotyledon 2, a transcription factor that regulates IAA production in explants. In oil palm, somatic embryogenesis has been described mostly as an indirect process **(Hilae and Te Chato, 2005)**. According to **Baharan *et al.* (2015)**, plant growth regulators (2,4-D, TDZ and BA) were effective in inducing callogenesis. The maximum regeneration ratio was seen in medium supplemented with 5.0 mg/l BA or 5.0 mg/l TDZ, whereas other treatments induced fewer calluses. However, when shoot tips were cultured with 2,4-D and TDZ at 10.0 mg/l, somatic embryogenesis occurred **(Sidky and Eldawyati, 2012)**.

3.3- Somatic embryo maturation and germination

MS basal medium supplemented with BA at 0.5 mg/l, kin at 0.1 mg/l and NAA at 0.05 mg/l was examined for further enhancement and maturation of somatic embryogenesis, and then these embryos formed shoots concurrently. After three weeks of culture, the mature

embryos developed a green color. Germination of somatic embryos was detected in control and MS medium treated with combinations of plant growth regulators, with germination percentages ranging from 32.4 to 87.5%. According to **Table (5)**, the most leaves number (20.11 leaf/jar) were found in MS basal medium with BA at 0.5 mg/l, kin at 0.1 mg/l and NAA at 0.05 mg/l, as shown in **Fig (5)**. In comparison, the medium devoid of plant growth regulators produced the fewest leaves (8.43 leaf/jar).

Table 5. Effect of BA at 0.5 mg/l, kin at 0.1 mg/l and NAA at 0.05 mg/l on germination of somatic embryos of Zaghloul ate palm cultivar after three subcultures.

Treatment (mg/l)	Somatic embryos germination (%)	Number of shoots	Number of leaves	Length of leaves (cm)
Control	32.4 b	2.50 b	8.43 b	1.60 b
0.5 BA, 0.1 kin, 0.05 NAA	87.5 a	8.00 a	20.11 a	3.33 a

Fig. 5: Improved the somatic embryos production and shoots regeneration of date palm, cv. Zaghloul after three sub-cultured on MS medium supplemented with BA at 0.5 mg/l, kin at 0.1 mg/l and NAA at 0.05 mg/l.

Numerous variables have been related with somatic embryo germination in prior studies. In terms of PGRs, **Othmani *et al.* (2009a)** recommended an NAA concentration of 1.0 mg/l for date palm cv. Boufeggous, whereas **Zouine and El Hadrami (2007)** enhanced somatic embryo germination in date palm cultivars Bousthami Noir and Jihel, using a mixture of NAA, IBA and BA. **Fki *et al.* (2003)** observed that when germinated date palm cv. Deglet nour embryos were transferred to a medium enriched with 1.0 mg/l NAA and BA, healthy plantlets with balanced shoot and root development were produced.

Sane *et al.* (2006) reported on the effect of NAA on the conversion of developed somatic embryos in date palm cv. Amsekchi and cv. Boufeggous cultures. When 1.0 mg/l NAA was administered, the average number of somatic embryos was 50.66 embryo/culture, the frequency of germination (83.50%) and the frequency of conversion (94.50%) were determined **(Othmani *et al.*, 2009b)**. Similar needs for cytokinins such as 2ip **(Badawy *et al.*, 2005)**, BA **(Zouine *et al.*, 2005)** and Kin **(Meziani *et al.*, 2015)** have been identified to stimulate growth, development, shoot morphogenesis and maturation into date palm.

BA is considered a possible cytokinin capable of accelerating the differentiation and development processes by causing fast cell division. **Aslam and Khan (2009)** discovered that BA was more effective than kinetin in increasing the frequency of numerous shoots in date palm, corroborating the current study's findings. The various doses of 2ip alone or in combined with Kin or IBA were extremely efficient in germination and development of somatic embryos and plantlets in both types tested **(D'Onofrio and Morini, 2005)**. We investigated somatic embryo germination and plantlet conversion using a variety of BA and Kin concentrations and discovered that BA was particularly efficient in promoting germination and plantlet conversion. Previously, it was found that the same cytokinins, alone or in association with auxin, were quite successful in somatic embryogenesis in a variety of plants **(Nasim *et al.*, 2009; Ghanti *et al.*, 2010)**.

Transfer of date palm cultivar embryos to MS medium supplemented with 1.0 mg/l NAA and 1.0 mg/l BA resulted in the formation of viable plantlets with uniform shoot and root development **(Zouine *et al.*, 2005)**. Recently, a somatic embryogenesis pathway was reported using leaf segments from *in vitro* shoots of date palm cv. Quntar **(Al-Mayahi, 2015)**; these explants would be ideal for large-scale propagation because their somatic origin, seasonal independence and availability. **Mazri *et al.* (2017)** also detected somatic embryogenesis in proximal leaf segments isolated from *in vitro*-grown shoots.

Letouze *et al.* (2000) demonstrated that when the hormonal combination during the induction stage is changed appropriately, the embryogenic callus pathway

may result in the development of somatic embryos and organogenesis. The dynamic equilibrium in the auxin and cytokinin concentrations in the media would have resulted in fast shoot and root growth, hence reducing the total time required for regeneration, which is rather unique in this research. Our findings indicated that 32.4 to 87.5 (%) of somatic embryos germinate. Other date palm cultivars have been documented to have varying rates of somatic embryos germination. **Al-Khayri and Al-Bahrany (2012)**, for example, observed germination rates of 17.5–72.5 (%) in cv. Naboul Saif. Germination frequencies of somatic embryos derived from cvs. Khusab, Berny and Barhee were between 60% and 75% **(Al-Khayri, 2011)**.

Mazri *et al.* (2018) found that MS medium supplemented with 2.5 µM NAA and 2.5 µM BA had the greatest rate of somatic embryo germination (52.0%). For the cv. Safawi, the optimal medium for promoting somatic embryos germination (%), shoots development and shoots length was 6.0 mg/l 2iP + 3.0 mg/l Kin + 0.5 mg/l IBA. While the ideal medium comprised 6.0 mg/l 2iP + 2.0 mg/l Kin + 1.0 mg/l IBA for cv. Magdoul **(Metwali *et al.*, 2020)**.

3.4- Regeneration and shoots multiplication

The results demonstrated the critical importance of using the optimal hormone combinations for successful shoots regeneration. After three sub-culturing, the cluster of shoots cultured on MS basal medium supplemented with BA at 2.0 mg/l and NAA at 0.5 mg/l possessed the best values for leaf number (34.25) and leaf length (4.75 cm), as shown in **Fig (6)**.

Fig.6: The cluster of shoots cultured on MS basal medium supplemented with BA at 2.0 mg/l and NAA at 0.5 mg/l resulted the best shoots multiplication.

Numerous parameters, most notably the combination of the culture medium and genotype, have been identified to impact shoot multiplication in date palm in earlier research. The combination of differing auxin and cytokinin concentrations may have generated a hormonal balance that enhanced shoot proliferation. Cytokinins are well recognized for their ability to decrease apical meristem dominance and encourage the development of both axillary and adventitious shoots from meristematic explants **(Madhulatha *et al.*, 2004)**. Due to its efficiency and availability, BA is now the most extensively utilized cytokinin in the micro-propagation industry **(Bairu *et al.*, 2007)**.

However, **Al-Khateeb (2006)** observed that low hormone concentrations stimulated the production of new buds, but high hormone concentrations resulted in aberrant growth devoid of budding or shoots development. Clearly, low auxin concentrations in the presence of cytokinin promoted adventitious buds proliferation **(Al-Najm *et al.*, 2018)**. For shoots multiplication, **Beauchesne *et al.* (1986)** recommended ½ MS medium supplemented with 2.0 mg/l NOA, 1.0 mg/l NAA, 1.0 mg/l IAA, 0.5 mg/l BA, 1.0 mg/l 2iP and 1.0 - 5.0 mg/l kin.

Similarly, **Taha *et al.* (2001)** developed an efficient quick technique for *in vitro* multiplication of date palm shoot buds cv. Zaghlool. They employed a medium that had a high concentration of 2ip. **Khierallah and Bader (2007)** reported that a hormone combination of 1.0 mg/l NAA, 1.0 mg/l NOA, 4.0 mg/l 2iP and 2.0 mg/l BAP increased shoots multiplication in MS medium of date palm. **Othmani *et al.* (2009a)** revealed that high rates of shoots proliferation were attained in cv. Deglet Nour on medium containing 1.0 mg/l NAA and 1.0 mg/l BA. For the cultivar Khalas, **Aslam and Khan (2009)** stated that, on medium supplemented with 7.84 µM BA, the greatest shoots regeneration response (in terms of shoot development (%), shoots number and shoots length.

According to **Zaid *et al.* (2011)**, NAA, NOA, IAA, BAP and kin may be utilized at concentrations ranging from 0.5 to 5.0 mg/l for shoots bud proliferation. **Mazri and Meziani (2013)** discovered that ½ MS medium supplemented with 0.5 mg/l NOA and 0.5 mg/l Kin resulted in the formation of 23.5 shoot buds per explant after three months of multiplication in cv. Najda. Additionally, **Bekheet (2013)** observed that adding three various kinds of cytokinins to the growth medium, BA, Kin and 2ip, at concentrations ranging from 0.5 to 5.0 mg/l, increased shoot bud development in date palm cv. Zaghlool.

The optimal medium for shoots regeneration was ½ MS medium combined with 0.5 mg/l NOA and 0.5 mg/l kin, which resulted in an average of 23.5 shoots per explant after three months of multiplication **(Mazri and Meziani, 2013)**. **Al-Mayahi (2014)** proposed growing cv. Hillawi on MS medium with 1.0 mg/l BA and 0.5 mg/l TDZ, which

resulted in an average of 18.2 buds per culture. **Mazri (2015)** suggested that MS medium with 2.5 µM IBA and 2.5 µM BA for cv. 16-bis (22.3 shoot buds/culture), whereas cv. Boufeggous (22.9 shoot buds/culture) required ½ MS media supplemented with 3 µM IBA and 3 µM BA.

After about two subcultures into medium containing 2,4-D at 0.1 mg/l, subculture of secondary shoots on MS medium supplemented with NAA and BA at (1.0 mg/l) ensures both multiplication and development of shoots capable of conversion into plantlets **(Othmani *et al.*, 2018)**. **Baghdady *et al.* (2018)** investigated for shoots multiplication, transfer of cultures to MS medium enriched with BA and Kin, either alone or in combination, after direct appearance of 2-3 shoots. The findings demonstrated that MS medium supplemented with BA at any concentration outperformed the control therapy. In this respect, introducing BA at a concentration of 1.0 mg/l to MS medium resulted in the greatest shoots number and shoots fresh weight values.

3.5- Genetic stability between mother plant and regenerated date palm shoots induced from leaves explants

In **Table (6)** ISSR analysis was done on the DNA of Zaghlol cultivar shoots produced from leaf explants in tissue culture. The ISSR profiles of tissue culture-derived shoots, particularly those induced from leaf explants, clearly showed a high similarity of 97% to the mother tree as shown in **Fig. (7)**.

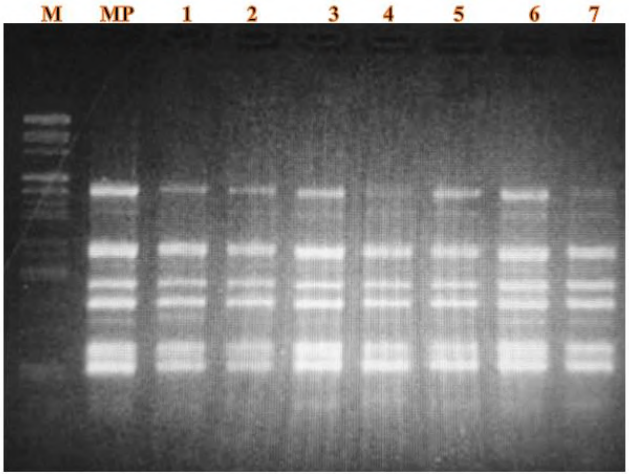

Fig. 7. ISSR profile of regenerated date palm shoots Zaghlol cv. from the leaves explants in comparison to their mother plant. M: 1kp DNA marker, MP: mother plant, Lanes 1 to 7: the regenerated date palm shoots induced from leaves explants. There were no differences in genetic variation (97%).

Table 6. Names and sequences of ISSR primers used in genetic stability among regenerated date palm shoots in comparison with their mother plant.

Primer name	Primer sequence	Annealing Temp. (°C)
DP1	(AGG)6	55
DP2	(AG)10G	60
DP3	(AG)10C	60
DP4	(AG)10T	57
DP5	(CT)10A	57
DP6	(CT)10G	60
DP7	(CT)10T	57

It is possible for regenerated somaclones to differ in chromosomal numbers and structures **(Hao and Deng, 2002; Mujib *et al.*, 2007)**. Fruit cultivars that have been micro-propagated have had their genetic variability studied using inter-simple sequence repeat (ISSR) markers.

Date palm cultivars can have polymorphisms found using the ISSR method **(Abd-Alla, 2010)**. ISSR is a practical and effective method used in many species to identify polymorphisms without knowledge of the DNA sequences. This technique is trustworthy for identifying date palm cultivars. Using ISSR, **Zehdi *et al.* (2012)** evaluated the genetic diversity of a collection of cultivars of Tunisian date palm.

Numerous authors have extensively studied the value of molecular analysis of *in vitro* regenerated plants **(Piatcza *et al.*, 2015; Bhalang *et al.*, 2018)**. Micro-propagated plants' genetic diversity has significant practical advantages and commercial implications.

A successful micro-propagation method should give true-to-type plantlets with no genetic or morphological alteration **(Prakash *et al.*, 2016; Safarpour *et al.*, 2017; Khatab and Youssef, 2018)**. The micro-propagated plants derived from shoot tips and axillary buds have been previously reported to maintain genetic stability **(Borsai *et al.*, 2020)**. Somaclonal variation is often induced by the culture media and subculture cycles **(Bidabadi *et al.*, 2010)**. Therefore, testing of genetic stability of *in vitro* raised plants is necessary to date palm plantlets production. However, in our study, no variability was detected among the plantlets by ISSR assay; Therefore, we can state that there were no somaclonal differences in the tissue culture-raised plantlets used in the current investigation.

IV. CONCLUSION

Explants of date palm Zaghloul cv. inner juvenile leaves were employed to develop an indirect somatic embryogenesis system through callus. When leaf segments were cultivated on MS medium, callus formation occurred on the surface of the leaves. MS medium supplemented with 10.0 mg/l NAA, 1.0 mg/l BA and 2.0 mg/l 2ip was used. Following that, the explants are transferred to MS medium containing 5.0 mg/l NAA and 2.0 mg/l BA to promote the development of embryogenic callus. To differentiate the embryogenic callus, it was grown on MS basal medium with 2.0 TDZ, 1.5 BA and 0.5 NAA. After that, the resulting somatic embryos were transferred to MS basal medium supplemented with BA at 0.5 mg/l, kin at 0.1 mg/l and NAA at 0.05 mg/l for maturation. The cluster of shoots cultivated on MS basal medium supplemented with BA at 2.0 mg/l and NAA at 0.5 mg/l exhibited the greatest regeneration. The date palm Zaghlol cultivar displayed the greatest genetic stability in the *in vitro* culture, according to the results of genetic variation detection using ISSR primers in date palm shoots induced from leaves explants. The detection of specific somaclonal variation in cloned plants using ISSR is another successful application.

KEY MESSAGE

In this paper, we used the juvenile leaves surrounding the shoot tips to study the capability of induction the somatic embryos or not on different media, then we did the ISSR analysis to study the genetic stability of shoots induced from leaf explants.

REFERENCES

[1] **Abd-Alla, M. M. (2010).** Genetic stability on *Phoenix dactylifera* var. karama produced *in vitro*. N. Y. Sci. J., 3: 70–75.

[2] **Adawy S. S.; Hussein, E. H. A.; El-Khishin, D.; Saker, M. M.; Mohamed, A. A. and El-Itriby, H. A. (2011).** Genotyping Egyptian date palm cultivars using RAPD, ISSR, AFLP markers and estimation of genetic stability among tissue culture derived plants. From: http://www.icarda.org/aprp/ Datepalm/Topics/ Biotech/Biotech-right.htm.

[3] **Al-Khalifah, N. and Askari, E. (2011)**. Growth abnormalities associated with micro-propagation of date palm. In: Jain S, Al-khayri J.M., Johnson D (eds) Date palm biotechnology. Springer, Dordrecht, Heidelberg, London, New York, pp 205–219.

[4] **Al Khateeb, A. A. (2006)**. Role of cytokinin and auxin on the multiplication stage of date palm (*Phoenix dactylifera* L.) cv. Sukry. Biotech., 5: 349–352.

[5] **Al-Khayri, J. M. (2011).** Somatic embryogenesis of date palm (*Phoenix dactylifera* L.) improved by coconut water. Biotechnol., 9: 477 - 484.

[6] **Al-Khayri, J. M. (2011).** Influence of yeast extract and casein hydrolysate on callus multiplication and somatic embryogenesis of date palm (*Phoenix dactylifera* L.). Sci. Hortic., 130: 531–535.

[7] **Al-Khayri, J. M. and Al-Bahrany, A. M. (2012).** Effect of abscisic acid and polyethylene glycol on the synchronization of somatic embryo development in date palm (*Phoenix dactylifera* L.). Biotechnology, 11: 318–325.

[8] **Al-Khayri, J. M.; Jain, S. M. and Johnson, D. V. (2015)**. Date palm genetic resources and utilization. Springer, Dordrecht, Heidelberg, London, New York.

[9] **Al-Khayri, J. M. and Naik, P. (2017).** Date palm micro-propagation: advances and applications. Ciência E Agrotecnologia, 41: 347–358.

[10] **Al-Mayahi, A. M. W. (2014).** Thidiazuron-induced *in vitro* bud organogenesis of the date palm (*Phoenix dactylifera* L.) CV. Hillawi. Afr. J. Biotechnol., 13: 3581-3590.

[11] **Al-Mayahi, A. M. W. (2015)**. An efficient protocol for indirect somatic embryogenesis and shoot organogenesis from leaf segments of date palm (*Phoenix dactylifera* L.) cv. Quntar Afr. J. Agric. Res., 10: 1031–1042.

[12] **Almeida, M.; Almeida, C. V.; Graner, E. M.; Brondani, G. E. and Abreu-Tarazi, M. F. (2012).** Pre-procambial cells are niches for pluripotent and totipotent stem-like cells for organogenesis and somatic embryogenesis in the peach palm: a histological study. *Plant Cell Rep.* 31: 1495–1515.

[13] **Al-Mayahi, A. M. W. (2020).** Effect of calcium and boron on growth and development of callus and shoot regeneration of date palm cv. Barhee. Can. J. Plant Sci., 100 (4): 357–364.

[14] **Al-Mayahi, A. M. W.; Jafar, O. N. and Mohsen, K. A. (2020).** Effect of glutathione (GSH) on date palm (*Phoenix dactylifera* L.) micro-propagation. Folia Oecologica., 47 (1): 64–69.

[15] **Al-Najm, A.; Brauer, S.; Trethowan, R. and Ahmad, N. (2018).** Optimisation of *in vitro* micro-propagation of several date palm cultivars. AJCS, 12 (12): 1937-1949.

[16] **Asemota, O.; Eke, C. R. and Odewale, J. O. (2007).** Date palm (*Phoenix dactylifera* L.) *in vitro* morphogenesis in response to growth regulators, sucrose and nitrogen. African Journal of Biotechnology, 6 (20): 2353-2357.

[17] Aslam, J. and Khan, S. A. (2009). *In vitro* micro-propagation of khalas date palm (*Phoenix dactylifera* L.), an important fruit plant. Journal of Fruit and Ornamental Plant Research, 17 (1): 15-27.

[18] **Babaei, N.; Abdullah, N. A. P.; Saleh, G. and Abdullah, T. L. (2013).** Control of contamination and explant browning in *Curculigo latifolia in vitro* cultures. Journal of Medicinal Plants Research, 7 (8): 448-454.

[19] **Badawy, E. M.; Habib, A. M.; El-Bana, A. and Yosry, G. M. (2005).** Propagation of date palm (*Phoenix dactylifera*) plants by using tissue culture technique. Arab. J. Biotechnol., 8: 343–354.

[20] **Baghdady, G. A.; Abdrabboh, G. A.; Hamdy. A. E. and Saad, M. M. (2018).** Micro-propagation of Date Palm

(*Phoenix dactylifera* L.) var. Zaghlol via Direct Organogenesis. Plant Biotechnology, 127-136.

[21] Baharan, E.; Mohammadi, P. P.; Shahbaziand E. S. and Hosseini, Z. (2015). 'Effects of some plant growth regulators and light on callus induction and explants browning in date palm (*Phoenix dactylifera* L.) *in vitro* leaves culture'. Iranian Journal of Plant Physiology, 5 (4): 1473-1481.

[22] **Balzon, T. A. and Luis, Z. G. and Scherwinski-Pereira, J. E. (2013).** New approaches to improve the efficiency of somatic embryogenesis in oil palm (*Elaeis guineensis* Jacq.) from mature zygotic embryos. *In vitro* Cell. Dev. Biol. Plant, 49: 41–50.

[23] **Bairu, M. W.; Stirk, W. A.; Dolezal, K. and Staden, J. V. (2007).** Optimizing the micro-propagation protocol for the endangered *Aloe polyphylla*: can metatopol in and its derivatives serve as replacement for benzyladenine and zeatin. Plant Cell Tissue and Organ Culture.

[24] **Beauchesne, G.; Zaid, A. and Rhiss, A. (1986).** Meristematic potentialities of bottom of young leaves to rapidly propagate date palm. Proceedings of the Second Symposium on Date Palm.

[25] **Behnas, A.; Reza, Z.; Hasan, H.; Mohsen. M. and Hedayat, Z. (2014).** Effect of 2,4-D and zip hormones on embryogenesis callus production and the effect of sucrose and concentrations of MS salts on somatic embryogenesis of date palm (cv. Madjool). Int. J. Farm and Alt. Sci., 3 (11): 1188-1193.

[26] **Bekheet, S. A. (2013).** Direct Organogenesis of Date Palm (*Phoenix dactylifera* L.) for Propagation of True-to-Type Plants. Scientia Agriculturae, 4 (3): 85.

[27] **Bekheet, S. A. (2013).** Date palm biotechnology in Egypt (Review article). Applied Science Reports, 3 (3): 144-152.

[28] **Bhalang, D.; Prabhuling, G.; Hipparagi, K.; Raghavendra, S.; Prakash, D. P. and Babu, A. G. (2018)**. Analysis of the genetic stability of banana tissue culture propagated plantlets cv. Ney Poovan (AB) using morphological and molecular markers. Int. J. Curr. Microbiol. App. Sci., 7 (1): 1007–1018.

[29] **Bidabadi, S. S.; Meon, S.; Wahab, Z. and Mahmood, M. (2010)**. Study of genetic and phenotypic variability among somaclones induced by BAP and TDZ in micro-propagated shoot tips of banana (*Musa spp.*) using RAPD markers. J. Agric. Sci., 2: 49–60.

[30] **Borsai, O.; Hârța, M.; Szabo, K.; Kelemen, C. D.; Andrecan, F. A.; Codrea, M. M. and Clapa, D. (2020)**. Evaluation of genetic fdelity of *in vitro* propagated blackberry plants using RAPD and SRAP molecular markers. Hortic. Sci., 47 (1): 21–27.

[31] **Capelle, S. C.; Mor, D. W. S.; Kirschnet, S. C. and Mok, M. C. (1983)**. Effect of thidiazuron on cytokinin autonomy and the metabolism of N6-(A2-isopentenyl) [18-C14] adensine in callus tissues of Phascolus L. Plant Physiol., 73: 796–802.

[32] **Cullis, C. (2011).** Molecular markers in date palm. In: S.M. Jain *et al*. (eds.), Date palm biotechnology 361-370.

[33] **D'Onofrio, C. and Morini, S. (2005).** Development of adventitious shoots from *in vitro* grown *Cydonia oblonga* leaves as influenced by different cytokinins and treatment duration. Biol. Plantarum., 49: 17–21.

[34] **Ducan, D. B. (1955).** Multiple range and multiple F test. Journal of Biometrics, 11: 1-42.

[35] Fki, L. R.; Masmoudi, R.; Drira, N. and Rival, A. (2003). An optimized protocol for plant regeneration from embryogenic suspension cultures of date palm, (*Phoenix dactylifera* L.) cv. Deglet Nour. Plant Cell Rep., 21: 517-524.

[36] **Fki, L. R.; Masmoudi, R.; Kriaa, W.; Mahjoub, A. and Sghaier, B. (2011).** Date Palm Micro-propagation via Somatic Embryogenesis. In: Date palm Biotechnology. Jain S.M., J.M. Al Khayri and D.V. Johnson (eds.). Springer, Dordrecht.

[37] **FAOSTAT (2013)**. Food and Agricultural Organization of the United Nations. Available in: www.fao.org/faostat/en/access.

[38] **George, E. F.; Hall, M. A. and De Klerk, G. J. (2008).** Plant Propagation by Tissue Culture. Volume 1. The Background, 3rd Edition, Published by Springer, Dordrecht, The Netherlands.

[39] **Ghanti, S. K.; Sujata, K. G.; Rao, S. M. and Kishor, P. B. K. (2010).** Direct somatic embryogenesis and plant regeneration from immature explants of chickpea. Biol. Plant, 54 (1): 121–125.

[40] **Gueye, B.; Saı̈d-Ahmed, H.; Morcillo, F.; Borgel, A.; Sane. D.; Hilbert, J. L.; Verdeil, J. L. and Blervacq. A. S. (2009).** Callogenesis and rhizogenesis in date palm leaf segments: are there similarities between the two auxin-induced pathways? Plant Cell Tiss. Organ Cult., 98: 47–58.

[41] **Haider, N.; Nabulsi, I. and MirAli, N. (2012)**. Phylogenetic relationships among date palm (*Phoenix dactylifera* L.) cultivars in Syria using RAPD and ISSR markers. Journal of Plant Biology Research, 1 (2): 12-24.

[42] **Hao, Y. J. and Deng, X. X. (2002).** *In vitro* Cellular Developmental Biology Plant., 38: 472-476.

[43] **Hesami, M.; Tohidfar, M.; Alizadeh, M. and Daneshvar, M. H. (2020).** Effects of sodium nitroprusside on callus browning of *Ficus religiosa*: an important medicinal plant. *Journal of Forestry Research*, 31: 789–796.

[44] **Hilae, A. and Te Chato, S. (2005).** Effect of carbon sources and strength of MS medium on germination of somatic embryos of oil palm (*Elaeis guineensis* Jacq.). Songklanarin J. Sci. Tech., (Supplement 3): pp. 629-635.

[45] **Huh, Y. S.; Lee, J. K. and Nam. S. Y. (2017).** Effect of plant growth regulators and antioxidants on *in vitro* plant regeneration and callus induction from leaf explants of purple passion fruit (*Passiflora edulis* Sims). J. Plant Biotechnol., 44 (3): 335-342.

[46] **Jatoi, M.; Abul-Soad, A.; Markhand, G. and Solangi, N. (2019)**. Micro-propagation of cv. Dhakki a high value date palm cultivar of Pakistan using offshoot and inflorescence explants. In: Zaid A, Alhadrami G. (eds) Proceedings of the sixth international date palm conference Abu-Dhabi, UAE, pp 11–16.

[47] **Kader, A. A. and Hussein, A. M. (2009).** Harvesting and post-harvest handling of dates. International Center for Agricultural Research in the Dry Areas (ICARDA).

[48] **Khatab, I. A. and Youssef, M. S. (2018)**. Micro-propagation and assessment of genetic stability of *Musa* sp. cv. Williams using RAPD and SRAP markers. Egypt J. Bot., 58 (3): 371–380.

[49] **Khierallah, H. S. M. and Bader, S. M. (2007).** Micro-propagation of date palm (*Phoenix dactylifera* L.) var. Maktoom through direct organogenesis. Acta Hortic., 736: 213–224.

[50] **Letouze, R.; Daquin, F.; Hamama, L.; Paquier, K.; Marionnet, F. and Javouhey, M. (2000).** Mass propagation of date palm (*Phoenix dactylifera* L.) through somatic embryogenesis. Histological study of embryo formation and cultivar identification by RAPD markers. In: Proceedings of the date palm International Symposium, Windhoek, Namibia, pp. 55-64.

[51] **Liu, Y.; Wei, C.; Wang, H.; Ma, X.; Shen, H. and Yang, L. (2021).** Indirect somatic embryogenesis and regeneration of *Fraxinus mandshurica* plants via callus tissue. J. For. Res., 32: 1613–1625.

[52] **Liu, J.; Liu, J.; Feng, H.; Ma, Y.; Zhang, L.; Han, H. and Huang, X. (2018).** Effects of different plant hormones on callus induction and plant regeneration of miniature roses (*Rosa hybrida* L.). Horticulture International Journal, 2 (4): 201-206.

[53] **Liu, H.; Zhang, H.; Dong; Y. X.; Hao, Y. J. and Zhang, X. S. (2018).** DNA METHYLTRANSFERASE1-mediated shoot regeneration is regulated by cytokinin-induced cell cycle in Arabidopsis. *New Phytologist*, 217: 219 - 232.

[54] **Madhulatha, P.; Anbalagan, M.; Jayachandaran, S. and Sakthivel, N. (2004).** Influence of liquid pulse treatment with growth regulators on *in vitro* propagation of banana (*Musa sp.*). Plant Cell Tissue Organ Cult., 76: 189-192.

[55] **Machakova, I.; Zazimalova, E. and George, E. F. (2008).** Plant growth regulators I introductions auxins their analogous and inhibitors. In: George EF, Hall M.A., De Klerk G.J. (eds) Plant propagation by tissue culture, 3rd edn. vol 1. Springer, Dordrecht, pp: 175–204.

[56] **Mamaghani, B. A.; Ghorbanli, M.; Assarehand, M. H. and Zare, A. G. (2010).** 'I*n vitro* propagation of three Damask Roses accessions'. Iranian Journal of Plant Physiology, 1: 85-94.

[57] Mazri, M. A. (2015). Role of cytokinins and physical state of the culture medium to improve *in vitro* shoot multiplication, rooting and acclimatization of date palm (*Phoenix dactylifera* L.) cv. Boufeggous. J. Plant Biochem. Biotechnol., 24: 268-275.

[58] **Mazri, M. A. and Meziani, R. (2013).** An improved method for micro-propagation and regeneration of date palm (*Phoenix dactylifera* L.). Journal of Plant Biochemistry and Biotechnology, 22: 176-184.

[59] **Mazri, M. A.; Belkoura, I.; Meziani, R.; Mokhless, B. and Nour, S. (2017).** Somatic embryogenesis from bud and leaf explants of date palm (*Phoenix dactylifera* L.) cv. Najda. 3 Biotech, 7: 58.

[60] **Mazri, M. A.; Meziani, R.; Belkoura, I.; Mokhless, B.; Nour, S. (2018).** A combined pathway of organogenesis and somatic embryogenesis for an efficient large-scale propagation in date palm (*Phoenix dactylifera* L.) cv. Mejhoul. 3 Biotech, 8: 215.

[61] **Meziani, R.; Jaiti, F.; Mazri, M. A.; Anjarne, M.; Ait Chitt, M.; El Fadile, J. and Alem, C. (2015).** Effects of plant growth regulators and light intensity on the micro-propagation of date palm (*Phoenix dactylifera* L.) cv, Mejhoul. J. Crop. Sci. Biotechnol., 18: 325–331.

[62] **Metwali, E. M.; Naif, R.; Kadasa, M. S.; Hemaid I.; Soliman, A.; Omar, A.; Almaghrabi and Fuller, M. P. (2020).** *In vitro* Propagation of Date Palm Cultivars Magdoul and Safwai through Somatic Embryogenesis. International Journal of Agriculture & Biology, 24: 1745–1753.

[63] **Minocha, S. C. (1987)**. Plant Growth Regulators and Morphogenesis in Cell and Tissue Culture of Forest Trees. In book, Cell and tissue culture in forestry. Library of Congress Cataloging in Publication Data.

[64] **Mirani, A. A. (2018).** Evaluating genetic stability of micro-propagated date palm (*Phoenix dactylifera* L.) varieties using inflorescence. Department of Botany, Shah Abdul Latif University, Khairpur (Ph.D dissertation).

[65] **Mirani, A. A.; Teo, C. H.; Markhand, G. S.; Abul-Soad, A. A. and Harikrishna, J. A. (2020)** Detection of somaclonal variations in tissue cultured date palm (*Phoenix dactylifera* L.) using transposable element-based markers. Plant Cell Tissue Organ Cult., 141: 119–130.

[66] **Mok, M. C. and Mok, D. W. S. (1985).** The metabolism of (C14) Tidizuron in callus tissues of *Phaseolum lunatus*. Physiol. Plant, 65: 425–432.

[67] **Mujib, A.; Banerjee, S. and Dev Ghosh, P. (2007).** Propagation of Ornamental Plants, 7: 169-174.

[68] Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant, 15: 473–479.

[69] **Nasim, S. A.; Mujib, A.; Rashmi, K.; Fatima, S.; Junaid, A.; Mahmooduzzafar (2009).** Improved Allin yield in somatic embryogenesis of *Allium sativum* L. (cv. Yamuna safed) as analyzed by HPTLC. Acta Biol. Hungarica., 60 (4): 441–454.

[70] **Othmani, A.; Bayoudh, C.; Drira, N. and Trifi, M. (2009a).** *In vitro* cloning of date palm *Phoenix dactylifera* L. cv. Deglet By using embryogenesis suspension and temporary immersion bioreactor. Biotechnol. Biotechnol. Equip., 23: 1181–1188.

[71] **Othmani, A.; Bayoudh, C.; Drira, N.; Marrakchi, M. and Trifi, M. (2009b).** Regeneration and molecular analysis of date palm (*Phoenix dactylifera* L.) plantlets using RAPD markers. Afr. J. Biotechnol., 8 (5): 813–820.

[72] **Othmani, A.; Bayoudh, C.; AL-Khayri, J. M. and Drira, N. (2018).** Cyclic somatic embryogenesis in date palm (*Phoenix dactylifera* L.). cv. Deglet bey (Mnakher). Journal of new sciences, Agriculture and Biotechnology, 51 (9): 3204-3213.

[73] **Piatcza, E.; Kuzma, L.; Sitarek, P. and Wysokinska, H. (2015)**. Shoot organogenesis, molecular analysis and secondary metabolite production of micr-opropagated *Rehmannia glutinosa* Libosch. Plant Cell Tiss. Organ Cult., 120: 539–549.

[74] **Prakash, L.; Middha, S. K.; Mohanty, S. K. and Swamy, M .K. (2016)**. Micro-propagation and validation of genetic and biochemical fidelity among regenerates of Nothapodytes nimmoniana (Graham) Mabb. Employing ISSR markers and HPLC. 3 Biotech, 6: 1–9.

[75] Raj, K. S.; Moliner, A.; Lee, E.; Nickles, E.; Sim, E.; Liu, C.; Schwarz, H. and Ibáñez, C. F. (2020). CD137 negatively affects "browning" of white adipose tissue during cold exposure. **J. Biol. Chem., 295 (7): 2034–2042.**

[76] **Rival, A. and Parveez, G. K. A. (2005).** *Elaeis guineensis* Oil Palm RE Litz (Ed.), Biotechnology of Fruit and Nut Crops, CABI Publ., USA, pp. 113-143.

[77] **Rout, G. R. (2004).** *In vitro* somatic embryogenesis in callus cultures of Azadirachta indica A. Juss.—a multipurpose tree. J. For. Res., 10: 263–267.

[78] **Safarpour, M.; Sinniah, U. R.; Subramaniam, S. and Swamy, M. K. (2017).** A novel technique for *Musa acuminata* Colla 'Grand Naine' (AAA) micro-propagation through transverse sectioning of the shoot apex. *In vitro* Cell Develop Biol-Plant, 53 (3): 226–238.

[79] **Salim, S. A. A. (2014).** Effect of explant source and growth regulators on *in vitro* callus induction and organogenesis of *Melia azedarach* L. trees. Journal of Biotechnology Research Center, 8 (3): 20-27.

[80] **Sane, D.; Aberienc-Bertossi, F.; Gassama-Dia, Y.; Sagna, M.; Trousiot, M.; Duval, Y. and Borgel, A. (2006).** Histocytological analysis of callogenesis and somatic embryogenesis from cell suspensions of date palm (*Phoenix dactylifera* L.). Annals of Botany, 98: 301- 308.

[81] **Shin, U.; Chandra, R. and Kang, H. (2019)**. *In vitro* and *ex vitro* propagations of astilboidestabularis (Hemsl.) Engl. as a rare and endangered species. J. Hort., 6 (260): 2376–354.

[82] **Sidky, R. A. and Eldawyati, M. M. (2012).** 'Proliferation of female inflorescences explants of date palm Proliferatio'. Annals of Agricultural Science, 57: 161-165.

[83] **Taha, H. S.; Bekheet, S. A. and Saker, M. M. (2001).** Factors affecting *in vitro* multiplication of date palm. Biol. Plant, 44: 431-433.

[84] **Tisserat, B. (1982).** Factors involved in the production of plantlets from date palm callus cultures. Euphytica., 31: 201–214.

[85] **Wójcikowska, B.; Jaskóła, K.; Gąsiorek, P.; Meus, M.; Nowak, K. and Gaj, M. D. (2013).** Leafy cotyledon 2 (LEC 2) promotes embryogenic induction in somatic tissues of Arabidopsis, via YUCCA-mediated auxin biosynthesis. Planta 238 (3): 425–440.

[86] Victor, J. M. R.; Murch, S. J.; Krishna, R. S. and Saxena, P. K. (1999). Somatic embryogenesis and organogenesis in peanut: The role of Thidiazuron and N.6-benzylaminopurine in the induction of plant morphogenesis. Plant Growth Reg. 28: 9–15.

[87] **Zaid, A. (1984).** *In vitro* browning of tissues and media with special emphasis to date palm cultures. Date Palm J. 3: 269-275.

[88] **Zaid, A. (1987).** '*In vitro* browning of tissues and media with special emphasis to date palm culture-a review'.Acta Horticulture, 212: 561- 566.

[89] **Zaid, A.; El-Korchi, B.; Visser, H. J. (2011).** Commercial date palm tissue culture procedures and facility establishment. In: Jain SM, Al-Khayri, J. M., Johnson DV (eds) Date palm biotechnology. Springer, Dordrecht, Heidelberg, London, New York, pp 137–180.

[90] **Zehdi-Azouzi, S.; Rhouma, S. and Ould Mohamed Salem, A. (2009).** Comparative analysis of genetic diversity in Tunisian collections of date palm cultivars based on random amplified polymorphic DNA and inter simple sequence repeats fingerprints. Acta Horticulturae, 814: 149-156.

[91] **Zehdi, S.; Trifi, M. and Billotte, N. (2004).** Genetic diversity of Tunisian date palms (*Phoenix dactylifera* L.) revealed by nuclear microsatellite polymorphism. Hereditas, 141: 278-287.

[92] **Zehdi, S.; Cherif, E. and Rhouma, S. (2012).** Molecular polymorphism and genetic relationships in date palm (*Phoenix dactylifera* L.): the utility of nuclear microsatellite markers. Sci. Hort., 148: 255–263. https://doi.org/10.1016/j.scienta.2012.10.011

[93] **Zouine, J. and El Hadrami, I. (2007).** Effect of 2,4-D, glutamine and BAP on embryogenic suspension culture of date palm (*Phoenix dactylifera* L.). Sci. Hortic., 112: 221–226.

[94] **Zouine, J.; El Bellaj, M.; Meddich, A.; Verdeil, J. L. and El Hadrami, I. (2005).** Proliferation and germination of somatic embryos from embryogenic suspension cultures in *Phoenix dactylifera*. Plant Cell Tiss. Org. Cult., 82: 83–92.