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Direct organogenesis from shoot tip of Egyptian New Valley date palm cultivars

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Abstract

Shoot tip explants of superior date palm cultivars Sewi, Tamr El wady, Hegazi and one individual unknown female from El-Kharga city (called Faleg or Meghel) were grown in the New Valley region and cultured on three different starting culture media MS+10mg/l 2,4-D+3.0mg/l 2iP (M1), MS+ 10mg/l NAA+ 3.0mg/l 2iP (M2) and MS+30 mg/l NAA+20mg/l 2iP(M3) for eight months (two months interval). All culture media were supplemented with 40g/l sucrose, 2.0 g/l PVP, and 3.0 g/l activated charcoal and solidified with gelrite at 2.0 g/l. After eight months, some morphological responses were noticed as direct shoot buds, direct embryogenesis and callus. Depending on these responses, different culture media with different combinations of auxin and cytokinin were used to differentiate and multiply these cultures. Results indicated that the highest direct shoot bud percentage was formed on M1 medium while the highest direct embryo percentage was formed on M2.Induced direct shoot buds that transferred to the half strength of 2,4D then transferred to auxin free medium showed the highest multiplied rate as clusters. Maximum shoot number was obtained when transferring these clusters to a modified medium supplemented with 3/4 MS salt + 2.0 mg/l BA+ 0.5 mg/l Kinetin+ 0.25 mg/l 2iP+ 1.0 mg/l IAA. Meanwhile, induced direct embryos transferred to a modified medium supplemented with 1/2 MS+ 0.25 mg/l ABA + 0.5 mg/l Kinetin+ 0.25 mg/l 2iP showed the highest secondary embryo number. All induced shoots were rooted successfully and transferred to the greenhouse.

Keywords: Date Palm, Direct Organogenesis, Direct Embryogenesis.

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Introduction

Date palm (*Phoenix dactylifera* L.) is a dioecious perennial monocotyledon plant that belongs to the Arecaceae family. It is the primary source of income for oases residents and a frequent staple meal throughout the Middle East, North Africa, and many other tropical and subtropical locations. (Amy *et al*., 2012).The New Valley, located in the southwestern portion of Egypt, is one of the country's largest governorates and one of the largest on the African continent.

Some of the New Valley date palm cultivars, such as Sewi, Tamr El wady, and Hegazi, have good fruit quality and are the most common genotypes in the main plantations at El-Kharga City. Fruit comes in three textures: soft (Hegazi cv.), semidry (Sewi cv.), and dry (Tamr El wady cv.). Sewi cultivar and Falege female were early in terms of fruit maturation. Tamr El wady cultivar, on the other hand, was late, while Higazi cultivar was mid. (Ibrahim *et al*., 2014).

Date palm micropropagation can be accomplished through indirect, direct somatic embryogenesis or direct organogenesis. (Letouze*et al*., 2000 and McCubbin, 2000).The direct production of adventitious buds on the explant without passing through the callus stage is known as organogenesis. After root regeneration, this could result in the growth of new plants. Organogenesis has a lesser rate of multiplication than somatic embryogenesis, but it allows for the maintenance of true-to-type plants (Mazri and Meziani, 2015, Al Khateeb, 2006). Shoot bud multiplication in date palms influenced by the culture medium's basic composition as well as the genotype (Mazriand Meziani, 2015),low concentrations of plant growth regulators (PGRs) (Abahman, 2011) and number of subcultures. Both the ability of the explant/culture to respond and the nature of the morphogenic reaction are determined by the composition and relative concentration of PGRs. In cultures of *Juglans regia* zygotic embryos, the development of somatic embryos, organs, and axillary buds depends on the PGRs composition of the medium (Fernandez *et al*., 2000).

This investigation was designed to determine different responses of three cultivars and one unknown female date palm (Falege, has a high level of quality and attractive features) which were collected from New Valley Governorate (El-Kharga and Dakhleh) as affected by 2,4-D and NAA during starting stage and subsequent growth. Different cytokinin and auxin combinations were used to multiply direct shoot bud formation

Material and Methods

Plant preparation and sterilization

Healthy offshoots of some superior date palm (Sewi, Tamr El wady and Hegazi) and one individual unknown female (Falege) grown at New Valley region were selected during the fruiting season. Selected offshoots of these different cultivars were carefully separated from the mother plant then outer leaves, roots, fibrous were removed till soft leaf appeared (shoot tips of about 10 cm). Shoot tip explants of different cultivars were cleaned with running tap water. Sterilization treatment of shoot tips under aseptic conditions was started by using sodium hypochlorite (3%) for 20 min. and then rinsed three times with sterilized distilled water. All affected tissues were removed then sterilization was repeated with 2% sodium hypochlorite for 15 min. and explants were rinsed three times with sterilized distilled water. Tween 20 at 2-3 drops was added in each sterilization steps as a detergent factor.

Culture media and incubation conditions

Sterilized shoot tips were divided longitudinally into several parts and cultured on **Murashige and Skoog (1962)** (MS) containing 40g/l sucrose, 100mg/l glutamine, 5.0 mg/l thiamine HCl, 1.0 mg/l biotin, 2.0 g/l Polyvenylpyrolledon (PVP) , 3.0 g/l activated charcoal (AC), solidified with Gelrite at 2.0 g/l and supplemented with different growth regulators as : 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), N6-Δ2 –isopentenyl adenine (2iP)

M1: MS+10mg/l 2,4-D +3.0 mg/l 2iP

M2: MS+ 10mg/l NAA +3.0 mg/l 2iP.

M3: MS+ 30mg/l NAA+ 20 mg/l 2iP

The pH of the medium was adjusted to 5.7, and the media were autoclaved for 20 minutes at 121°C and 15 Ibs. Explants from each treatment were recultured every two months for four times on the same medium composition. All of the cultures were incubated in a controlled environment in the growth room, where the temperature was maintained at 27±2°C and the cultures were kept in the dark.

After eight months, some morphological responses were noticed in some culture tubes where the direct somatic embryos, direct shoot buds would form **(Photo1 A&B,** respectively) as well as callus. All of them were estimated by percentages

Depending on different responses of growth, the following steps were done

1- Direct shoot bud formation

Tubes which differed morphologically in each starting media **(Photo1C)** were subcultured for 6 weeks on the following modified starting media (MM) which supplemented with 1.5 g/l AC + 1.0 g/l PVP as:

MM1**:**3/4 MS + 5.0 mg/l 2,4-D + 3.0 mg/l 2iP

MM2: 3/4 MS+5.0 mg/l NAA +3.0 mg/l 2iP

MM3: 3/4 MS + 15mg/l NAA+ 5.0 mg/l 2iP

After that, explants in MM1 were transferred to the modified medium containing 0.5 mg/l 2,4-D + 3.0 mg/l 2iP + 1.0 g/l PVP (MM11) ;while explants from MM2 and MM3 were transferred to modified medium containing 0.5mg/l NAA +3.0 mg/l 2iP + 1.0 g/l PVP (MM22) to multiply buds. Finally, explants from different treatments were subcultured on starting media devoid of auxins and AC that supplemented with 3.0 mg/l 2iP for two months to form shoot bud clusters **(Photo1D)**.

Multiplication of direct shoot bud

Clusters of direct shoot buds (3 in each) of four cultivars formed previously were cultured on the following multiplication media for three subcultures with regular transfer to fresh media of the same composition after (4-5 weeks) where average shoot numbers were counted.

D1: 3/4 MS salt + 1.0 mg/l 2iP+ 0.5 mg/l Thidiazuron(TDZ)

D2: 3/4 MS salt + 2.0 mg/l BA+ 0.5 mg/l Kinetin+ 0.25 mg/l 2iP+ 1.0 mg/l IAA

D3: 3/4 MS salt +2.0 mg/l 2iP +1.0 mg/l Kinetin+ 0.5 mg/l NAA

D4: 3/4 MS salt + 1.5 mg/l Kinetin + 1.0 mg/l BA+ 0.1mg/l NAA+ 0.25g/l AC

Elongation of multiplied shoot

Shoot clusters (4-5 each, 0.5 – 1.0 cm in length) were transferred to the following elongation media (E) where shoot lengths were measured (cm):

E1: 1/2 MS solid media + 1.0 mg/ l Kinetin + 2.0 mg/l IAA

E2: 1/2 MS solid media + 0.1 mg/l BA + 0.1mg/l NAA

E3: 1/2 MS solid media + 0.5 mg/l BA + 0.5 mg/l Kinetin + 0.5 mg/l NAA + 0.25g/l AC

E4: 1/2 MS liquid media + 0.1mg/l BA + 0.2mg/l NAA + 0.5g/l AC

2- Direct somatic embryos

Explants formed direct somatic embryos were transferred to control medium for six weeks and after that, clusters of somatic embryos (3 embryos each) were cultured on the following medium supplements:

S1:1/2 MS+ 0.25 mg/l ABA + 0.1mg/l NAA+ 1.0 g/l AC

S2: 1/2 MS+ 0.25 mg/l ABA + 0.1mg/l NAA +0.05mg/l BA +1.0 g/l AC

S3: 1/2 MS+ 0.25 mg/l ABA + 0.5mg/l Kinetin +0.25mg/l 2iP

All culture media were supplemented with 40g/l sucrose+ 100mg/l glutamine +5mg/l thiamine HCl and 1.0 mg/l biotin. Embryo clusters were sub-cultured three times in the fresh former medium (3 weeks interval). The number of secondary embryos and germinated embryos/embryos were counted.

Rooting stage

According to **Hassan *et al*., (2013),** healthy formed shoots (5-7 cm) were cultured on half strength MS with 1.0 mg/l IBA, 40 g/l sucrose, 2.0 mg/l glycine+ 5.0 mg/l thiamine HCl+ 1.0 mg/l biotin+ 1.0 g/l AC for 6 weeks and incubated under 4000 lux to form primary roots. After that, induced roots were trimmed to 1-1.5 cm in length, subcultured on half MS liquid medium containing 0.2mg/l NAA+ 0.1mg/l BA+0.1mg/l paclobutrazol and incubated under 6000 lux for another six weeks to form adventitious roots before transplanting to greenhouse.

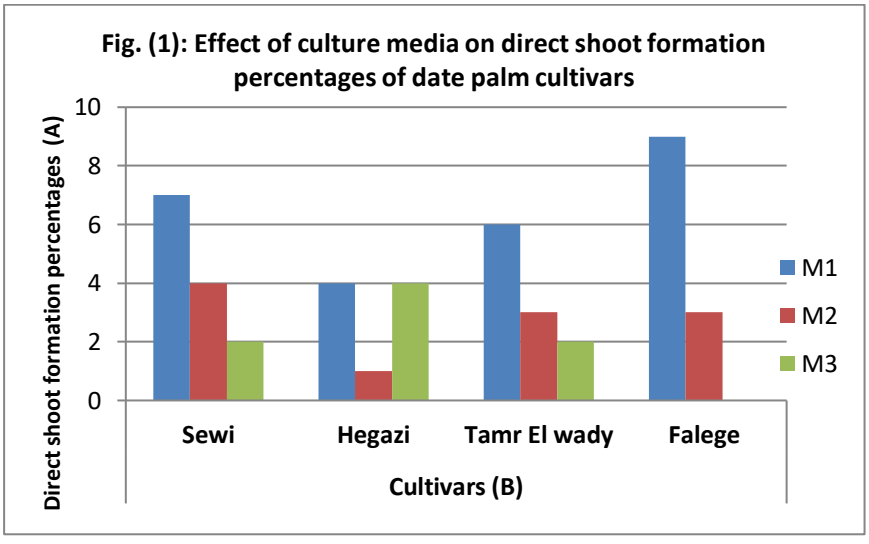
Statistical analysis:

Data obtained were subjected to the analysis of variances of randomized complete design as recommended by Snedecor and Cochran (1980). L.S.D at 5% level of significance was used to compare between means according to Steel and Torrie (1980).

Results and Discussion

Different responses were observed on shoot tip explants cultured *in vitro*. Direct shoot formation, direct embryogenesis and callus formation appeared. The type of tissue reactions were affected by growth regulators concentrations and its modifications and type of cultivars. Data presented in **Fig. (1)** indicated that explants cultured on starting medium containing 2,4-D (M1) and subsequent modifications (MM1 &MM11) then transferred to auxin and AC - free media + 3.0 mg/l 2iP statistically produced the highest percentage of direct shoot bud formation compared with other media and their modification which containing NAA .Concerning cultivars, it is clear that Sewi and Falege were statistically more responsible than Tamr El wady and Hegazicvs. Interaction revealed that Falege and Sewi in M1 produced the highest significant % (9 &7 respectively) with significant difference in between. Othmani*et al*.(2009) found that explants growing on induction medium containing 10 mg/l 2,4-D gave rise to optimal initiation of embryogenic calli which were friable and showing small (<2 mm) white

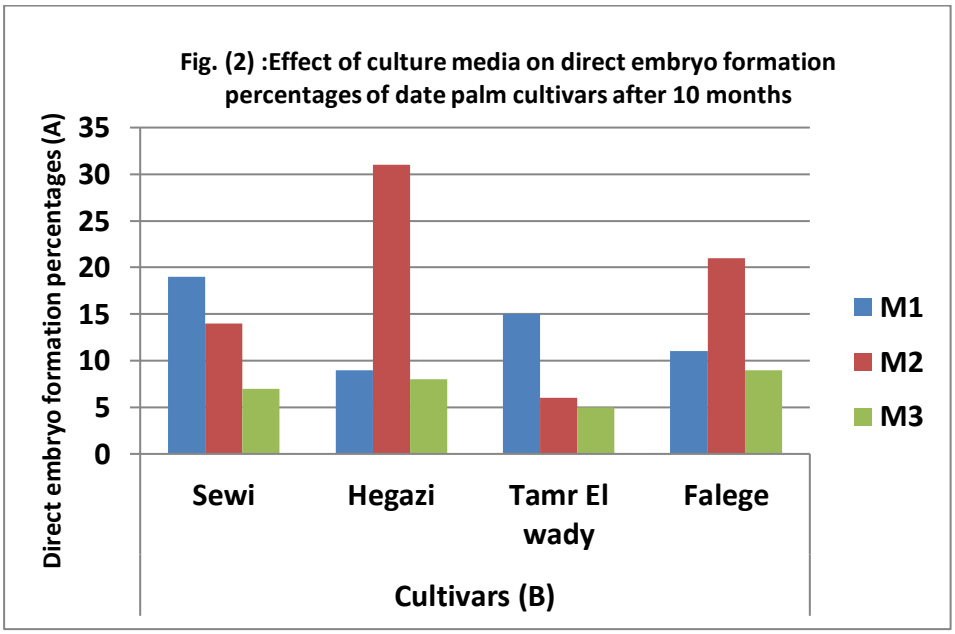
nodules as well as direct embryogenesis and direct shoot formation from the base of the leaves within 8 months of culture. This was indicative of the different modes of action of 2, 4-D. El Hadrami*et al*. (1995) recorded that *in vitro* plant regeneration of date palms occurs *via* organogensis, which is regulated by genotypes and hormonal treatment. Both the ability of the explants/culture to respond and the manner of the morphogenic reaction are affected by the composition and relative concentration of PGRs. In cultures of zygotic embryos of *Juglansregia*, somatic embryos, organs, or axillary buds developed depending on the PGR content of the culture media (Fernandez *et al.*, 2000). In addition, auxin is playing an important role for inducing direct embryogenesis (Hassan*et al*., 2021). Gaj (2004) reported that genotypes, age, developmental stage of explants, and the external environment, such as culture media composition, light, and temperatures, all influenced cell and tissue growth in vitro. The interaction of these elements enables a certain mode of cell differentiation and development to be induced. Shoot tip explants of date palm Sewi cv. were cultured on MS medium to stimulate adventitious bud formation. Media were supplemented with 1 mg/l NAA and 1 mg/lof BA, Kinetin and 2iP added individually or in combinations. The highest percentage of responsive explants (36.36%) was observed on a medium containing 1 mg/l NAA combined with 1 mg/l each of BA, Kinetin, and 2iP where 9.20 shoots per explant formed after 32 weeks of culture (Ali *et al.*, 2017).



L.S.D at 0.05 A = 0.79 B = 0.92 AB = 1.59

M2 had the largest significant average percentage of explants producing direct embryos, followed by M1 and M3, as seen in Fig. (2). The highest significant average

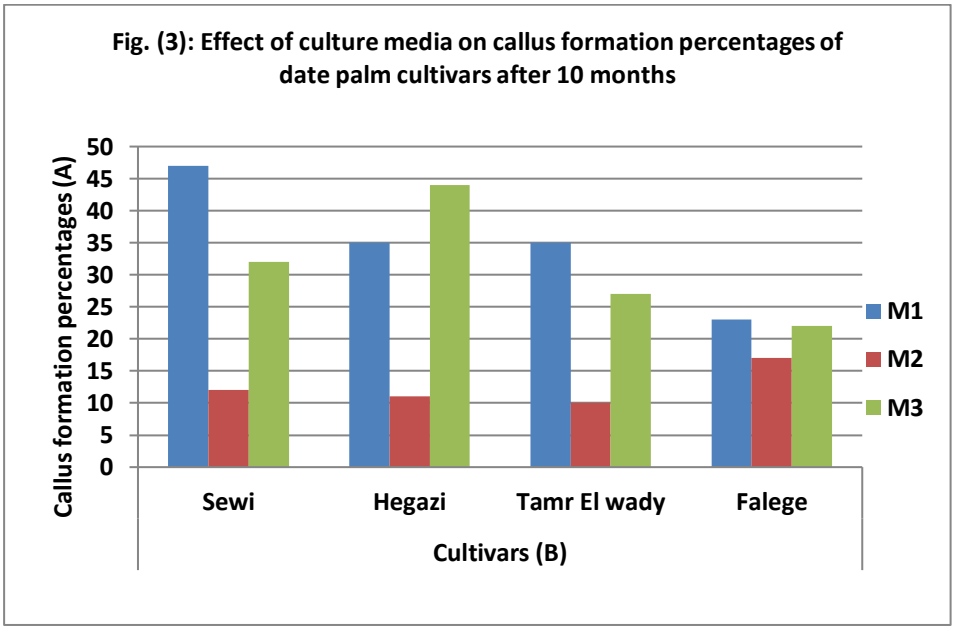
percentages of explants producing direct embryos were obtained by Sewi and Falege cvs., followed by Hegazi, while the lowest significant average was produced by Tamr El wady. In terms of the interaction, data suggested that Falege and Hegazi in M2 had the highest percentage of explants producing direct embryos, whereas Sewi and Tamr El wady in M1.Hassan (2007) succeeded to induce direct somatic embryos in dry and semi dry date palm cultivars in media with 10 mg/l 2,4-D+ 3.0 mg/l 2ip + 2.0 g/l AC in the presence of ABA for short period. The effects of date palm genotype on in vitro propagation have been documented in several studies (Al- Khayri and Al- Bahrany, 2004 and Taha*et al.*, 2021)



L.S.D at 0.05% A= 0.94 B = 1.08 AB = 1.88

Data in **Fig. (3)** revealed that M1 had the largest significant callus formation %, followed by M3 in that order. In comparison to Tamr El wady and Falege, Sewi and Hegazi gave the most significant percentages. In order to explain the interaction, M1 was found to be the most ideal for Sewi, Tamr El wady and Falege in regards to callus formation %, whilst M3 was found to be the most appropriate for Hegazi. Feher*et al.*, (2003) recorded that the auxin 2,4-D was the most widely used for callus induction among the several auxins. The remarkable efficiency of 2,4-D for inducing embryogenic response in many *in vitro* systems and plant species demonstrates that this PGR has a distinct character. This growth regulator appears to be an effective stressor as well as an exogenous auxin analogue. The fact that 2,4-D causes various changes in cell

physiology and gene expression suggests that it could play a role as a stress factor that triggers embryogenic patterns of development in developed plant cells.

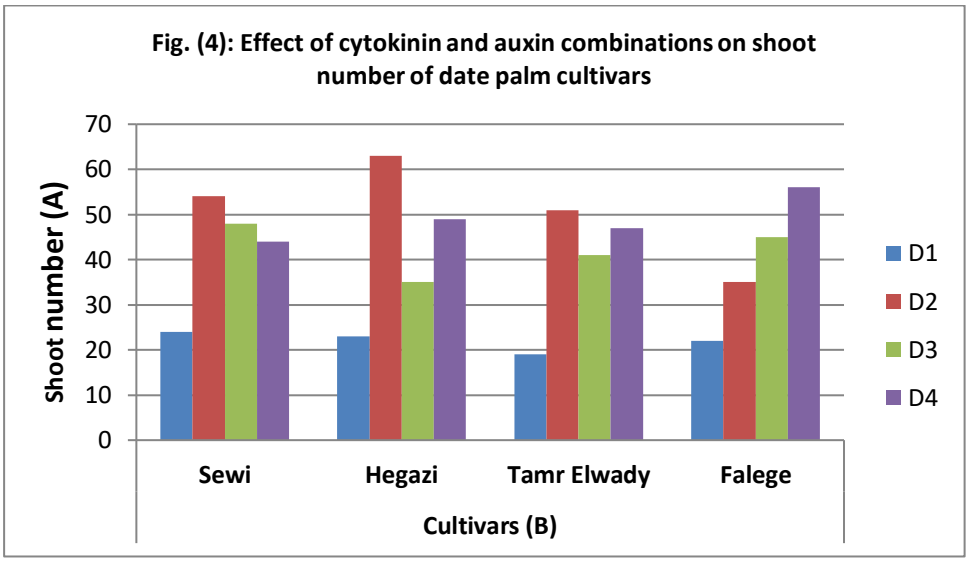


L.S.D at 0.05% A = 1.49 B = 1.49 AB = 2.589

Shoot bud multiplication

Different culture media have been tested to multiply vegetative buds obtained from the shoot tip sections. Working on derived shoots regenerated from selected cultivars, it is obvious from data in **Fig. (4)** that media containing BA was preferable for shoot bud multiplication. Multiplication medium (D2) containing combination of 2.0 mg/l BA + 0.5 mg/l Kinetin + 0.25mg/l 2iP +1.0mg/l IAA generally gave the greatest significant shoot number/cluster followed significantly by the combination of 1.5 mg/l Kinetin +1.0 mg/l BA+ 0.1 mg/l NAA + 0.25g/l AC (D4) with satisfactory shoot length (**Photo1E&F).**Working with cv, Khalas, Aslam, and Khan (2009) found that 7.84 mM of BA produced the best shoot multiplication rate. They also discovered that as the concentrations of BA and kinetin raised over 7.84 and 9.28 mM, respectively, the rate of shoot regeneration reduced. They claimed that BA was more successful during shoot multiplication than kinetin. With respect to cultivar factor, Falege gave the greatest significant mean value followed by Sewi then Tamr El wady (44, 42.5 and 42.5 shoot/ bud respectively). The interaction showed that the best result for Falege was achieved with D4 medium, while the best results of other tested cultivars were noticed with D2 medium. The culture medium of date palms is frequently modified to improve

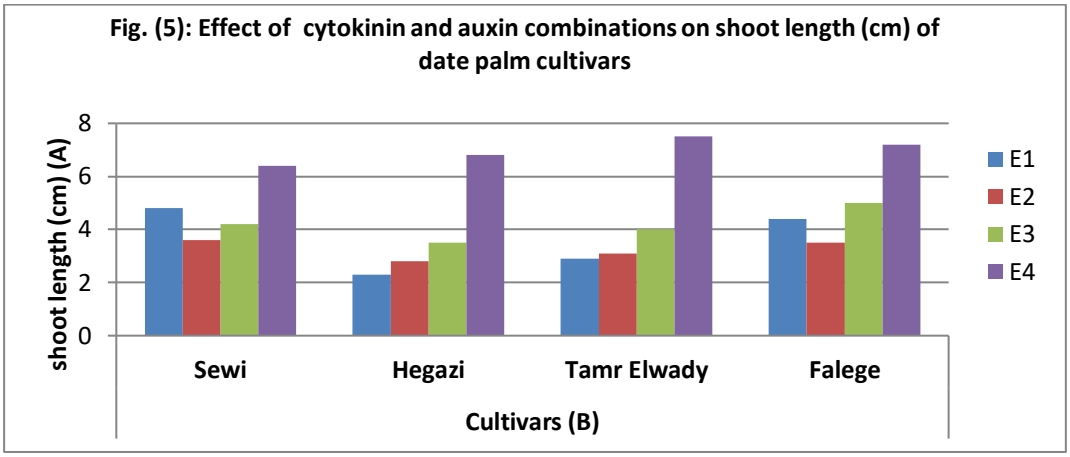
micropropagation systems for different genotypes (Al-Khayri, 2010). The lowest significant results with all cultivars appeared with culture media of adding 2iP and TDZ. On the other hand, Taha*et al.* (2021) recorded the stimulatory effect of TDZ on date palm propagated through organogensis.



L.S.D at 0.05% A = 1.17 B = 1.17 AB = 2.33

Shoot elongation:

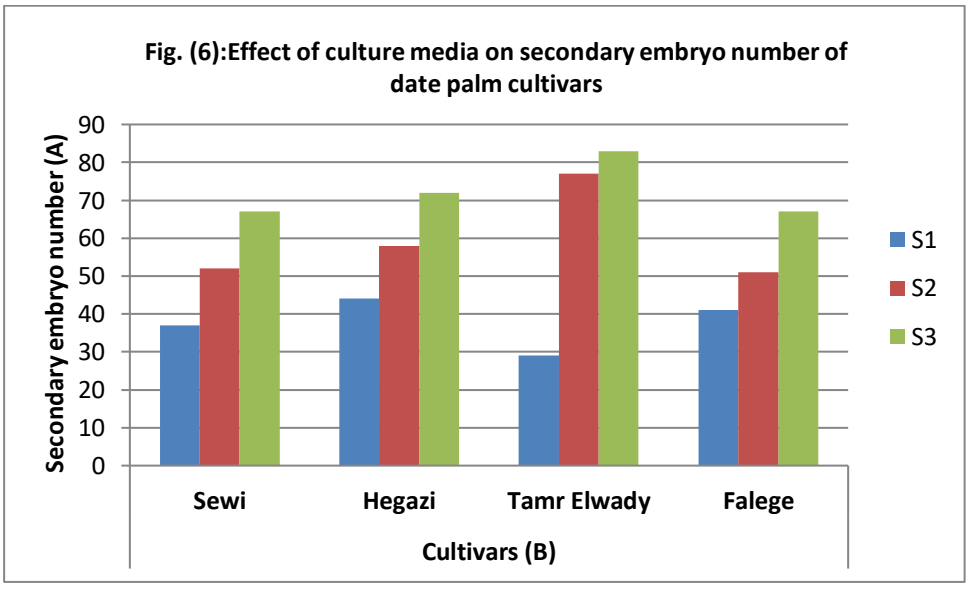
Data in **Fig.** (5) clearly showed that of all the media evaluated, 1/2 MS liquid elongation medium supplemented with 0.1mg/l BA + 0.2mg/l NAA + 0.5g/l AC (E4) exhibited pronounce significant effect on shoot length **(Photo 1G).** This medium is followed by 1/2 MS solid media + 0.5 mg/l BA + 0.5 mg/l Kinetin + 0.5 mg/l NAA + 0.25g/l AC (E3). In respect to cultivars, the results illustrated that Falege and Sewi were the most responsible cultivars than Hegazi and Tamr El wady. Interaction between cultivars and elongation media showed that the longest significant shoots (7.2 cm) were obtained with Falege cv. cultured on E4 medium. E4 medium gave the longest shoots for all cultivars under investigation. The use of a liquid medium to enhance shoot elongation in date palm has been described (Mazri, 2012). Medium containing NAA in combination with BAP and Kinetin exhibited a pronounced effect on date palm shoot elongation (Saifulland Tabassum, 2012). In addition, liquid medium enhances shoot growth and elongation of date palm shoots (Al-Khateeb and Alturki, 2014).



L.S.D at 0.05% A = 0.53 B = 0.53 AB = 1.08

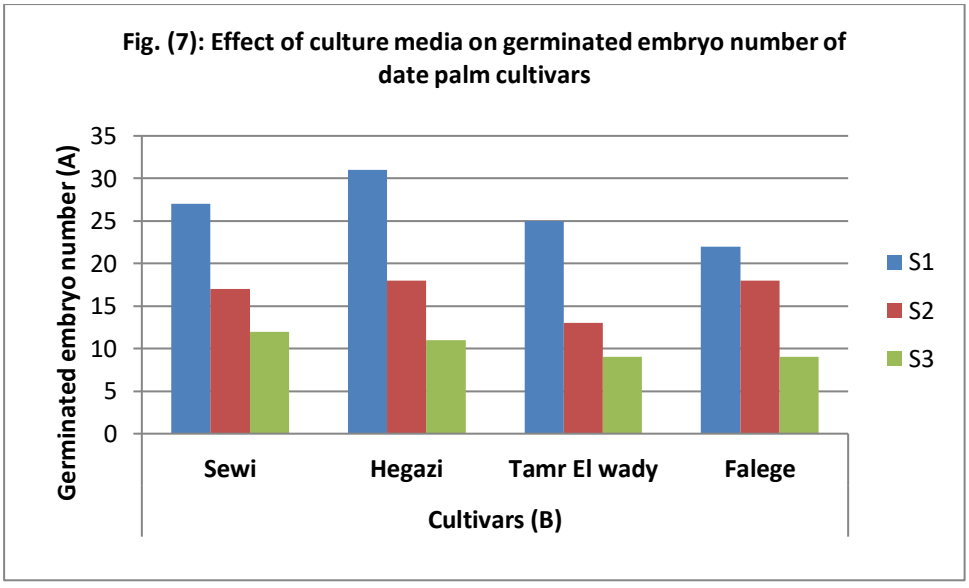
Secondary embryo multiplication

After three cultures, the influence of different culture media (S1, S2, and S3) on secondary embryo numbers was shown in Fig. (6). In comparison to the other two culture media, 1/2 MS + 0.5 mg/l kinetin + 0.25 mg/l 2iP (S3) appeared to be more promising for producing the largest significant number of secondary embryos (71.25/ cluster). In regards to cultivars, data showed that Tamr El wady cv. was the most competent among them. Interaction revealed that all cultivars cultivated on S3 medium produced the most embryo numbers/cluster (Photo 1H), followed significantly by S2. media.



LSD at 0.05 for A= 1.383 B= 1.383 AB= 3.183

Data in **Fig. (7)** showed that using culture medium free of cytokinin (S1) was preferable to promote germination of embryos followed by (S2) which containing low concentration of cytokinin compared with medium (S3) with significant differences. With respect to cultivar factor, with Hegazi cv., the greatest significant value of germinated embryo was observed (20) reduced significantly to (18.66) with Sewi cv. while, Tamr El wady cv. produced the lowest significant one (15.66). Interaction between cultivars and culture media showed that within all cultivars tested, S1 medium recorded the highest significant values while,S3 medium resulted in the lowest significant values. Gadalla (2003) found that medium containing MS salts+0.1mg/l NAA recorded superior for stimulating germinated embryos percentage of date palm cultivars while, result in the lowest significant values of secondary embryo formation. Hassan (2007) found that date palm direct somatic embryos (dry and semi-dry cultivars) treated with ABA and subcultured on 0.1 mg/l NAA + 0.05 mg/l BA+ 1.0 g/l AC exhibited good in vitro growth. Hassan *et al.* (2021) showed that when asexual embryos of date palm transferred to medium with low level of NAA (0.1 mg/l), they grow into plantlets.



LSD at 0.05 for A= 1.143 B= 1.143 AB=1.981

Healthy shoots resulted from direct shoots and embryos rooted well in double phase rooting media described by Hassan *et al.* (2013) and successfully transferred to greenhouse conditions with higher survival percentages **(Photo 1I&J).**

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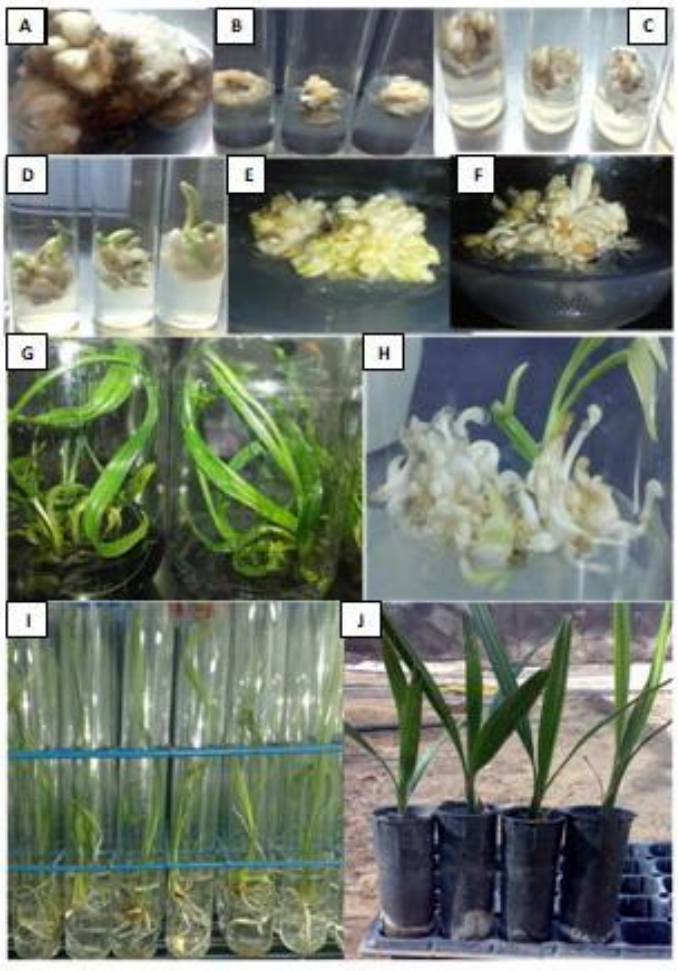

Photo 1.A: Direct somatic embryos formed after eight months, **B:** Shoot tip explants cultured on modified media MM1, MM2 and MM3, starting media for 6 weeks, **C:** Explants in modified starting media with 0.5 mg/l 2,4-D or NAA + 3mg/l 2iP, **D:** Shoot tip explants started to form buds after two months cultured on 3/4 MS+3mgl/l 2ip, **E:**Shoot bud multiplication in 2 mg/l BA + 0.5 mg/l kinetin+ 0.25mg/l 2iP +1mg/l IAA, **F:**Shoot bud multiplication in ¾ MS +1.5 mg/l kinetin +1mg/l BA+ 0.1 mg/l NAA + 0.25g/l AC, **G:** Cluster of shoots cultured on ½ MS liquid medium+0.1mg/l BA + 0.2mg/l NAA + 0.5g/l AC, **H:** Clusters of direct embryo on 1/2 MS + 0.5mg/l kinetin + 0.25mg/l 2ip, **I:** Plantlets in pre - acclimatized liquid media and **J:**Plants in greenhouse

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التكوين العضوي المباشر من القمم النامية لأصناف نخيل التمر المصرية بالوادي الجديد

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5.معمل تقنية زراعة الأنسجة ، شبكة المعامل المركزية ، المركز القومي للبحوث.

الملخص العربى

تمت زراعة القمم النامية من أصناف نخيل التمر المتميزة، سيوي، تمر الوادي، حجازي، وصنف غير معروف من مدينة الخارجة (يسمى فالج أو مجهل) المزروعة في منطقة الوادي الجديد على ثلاثة بيئات زراعة أولية مختلفة MS +10 2,4 D + 3.0 mg / l 2iP (M1), MS + 10 mg / l NAA + 3.0 mg / l 2iP (M2), MS + 30 mg / l NAA + 20 mg / l 2iP (M3) لمدة ثمانية أشهر (بفاصل شهرين). تم امداد جميع بيئات الزراعة بـ 40 جم / لتر سكروز و 2.0 جم / لتر PVP و 3.0 جم/ لتر من الفحم النشط و الجيلريت بتركيز2.0 جم/ لتر. بعد ثمانية أشهر، لوحظت بعض الاستجابات المورفولوجية مثلتكونالبراعم المباشروالتكوين الجنيني المباشر والكالس. اعتمادًا على هذه الاستجابات، تم إستخدام بيئات زراعة أخرى مختلفة مع مجموعات مختلفة من الأكسينات والسيتوكينين لتتميز هذه الزراعات وتتضاعف. وقد أشارت النتائج إلى تكون أعلى نسبة براعم مباشرة على وسط M1 بينما كانت أعلى نسبة أجنة مباشرة على M2. أظهرت البراعم المتكونة المباشرة التي نقلت إلى بيئة بها نصف تركيز (2,4-D) وتم نقلها إلى وسط خالٍ من الأكسين أعلى معدل تضاعف. وقد تم الحصول على أعلى عدد للأفرع عند نقل هذه البراعم إلى بيئة معدلة 3/4MS salt + (2.0 mg/l BA+ 0.5 mg/l Kin+ 0.25 mg/l 2iP+ 1.0 mg/l IAA). تم الحصول على أعلى عدد أجنة ثانوية في البيئة المعدلة بإضافة(1/2 MS+ 0.25 mg/l ABA + 0.5 mg/l kin + 0.25 mg/l 2iP). تم تجذير جميع البراعم المستحثة بنجاح على بيئة 1/2MS + 0.25 mg / l ABA + 0.5 mg / l kin + 0.25 mg / l 2iP ونقلها إلى صوبة الزراعة.

الكلمات الدالة: نخيل البلح، تكوين أفرع خضرية مباشرة، تكوين أجنة مباشرة.