*Plant Archives* Vol. 18 No. 2, 2018 pp. 2199-2206 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

# HISTOLOGICAL STUDY OF DIRECT SOMATIC EMBRYOGENESIS IN DATE PALM CULTURED *IN VITRO*

**Abdulminam Hussien Ali and Eman Mohammed Abdulzhara**

Department of Biology, College of Science, Basrah University, Basrah, Iraq.

## Abstract

Somatic embryogenesis is a sexual reproduction in which a bipolar structure is derived from somatic (non-zygotic) cell without vascular connection in the original tissue. This process can by achieved in vitro through using the different types of auxin and cytokinins. In date palm (*Phoenix dactylifera* L.) some research workers were able to obtained by using different compination of auxins and cytockinins. In present study we try to induced the direct somtic embryogenesis on shoot tip explants derived from in vitro microshoots. Shoot tip explants were inoculated on medium containing Cytokinins thidiazuron (TDZ) alone or in combination with auxins. It was found that MS medium enriched with 10 and 15 mg/L TDZ only was more effective to induce direct somatic embryogenesis in compared with others containing auxins, Beta naphthoxyacetic acid (NoA) or 1- napthaleen acetic acid (NAA) in combined with TDZ. While, the minimum response rate obtained when auxins NoA and NAA combined with TDZ. Transferred of somatic embryos to maturation and germination medium induced secondary embryogenesis and were developed after 4 weeks. Four weeks later somatic embryo start germination and developing to plantlet. In this medium direct organogenasis were also recognized. Histoligical examination of explants grown on medium containing 10 mg/L TDZ revealed that the somatic embryo originated from two area. First somatic embryo induced from the sub-epidermal area and second from the vascular cells. Histological analysis showed that the division pattern of the cambical cells during the somatic embryo formation tock place in one side that caused the sweeling of the explants. Subsequent cell division led to progress in somatic embryo developmental stages. The first distinct appearance of embryogenesis was marked by development of globular forms that were embedded in the explants tissue, but there were separated from the adjusent tissue by vaculated cells and became a single embryo. No vascular connection between a somatic embryos and surrounding tissue has been recognized. More development of these embryos caused raputureing of surrounding tissue and embryo emergence.

***Key words :*** Embryo formation, date palm cultured, histological examination, globular forms.

## Introduction

Micropropagation of date palm through somatic embryogenesis or organogenesis have been developed in several genotype using various meristematic explants including shoot tips, axillary buds and inflorescences, Al-Khairy (2010), Abhamane **(**2011) and (2013), Bekheet (2013), Jazinizadeh *et al*. (2015). Somatic embryogenesis is a sexual reproduction in which a bipolar structure is derived from somatic (non-zygotic) cell without vascular connection in the original tissue (Zimmerman, 1993 and Namasivayam, 2007). However, this process is considered the most efficient regeneration way for date palm micropropagation due to high number of regenerants and highly useful for breeding program, also, it give an opportunity to creation of cycling culture through the use of secondary embryogenesis and it could be used for genetic transformation (El-Hadrami *et al*., 1998; Levee *et al*., 1997). Moreover, this technology opening the way for the production of artificial seeds (Bekheet *et al*., 2002).

Two pathways in the production of somatic embryos are available. The first is an indirect method which is based on the induction of embryogenic callus. The second is direct somatic embryogenesis (somatic embryos are formed directly from cells of the explants without callus formation (El-Hadrami *et al*.,1998). The first occurrence of direct somatic embryogenesis in date palm was observed on the leaf of *in vitro* plant by Sudhersan *et al*.

(1993). Later, Othmani *et al*. (2009) found that direct somatic embryos were formed on the base of young leaf explants when cultured on MS medium enriched with 10mg/L 2,4-D. Sidky and Zaid (2011) observed direct globular somatic embryo when treating shoot tip explants by thidiazuron (TDZ) alone or in combination with 2,4-D. Whereas, Sidky and Eldawyati (2012) were able to induce direct somatic embryogenesis from female inflorescences explants of date palm on MS medium containing 2, 4-D 5 mg/L, 2ip 0.5 mg/L and abscisic acid 1.5 mg/L. Abdulminam *et al*. (2015) found that using of NAA 50mg/L in combination with 5 mg/L 2,4-D and 3mg/L 2ip in addition to 50 g/L sucrose-induced direct somatic embryos on shoot tips of date palm cultured *in vitro*.

However, direct somatic embryogenesis has rarely occurred in date palm *Phoenix dactyilfera* cultured *in vitro* ( Hegazy, 2003). Also, explants used to induce direct somatic embryo is not clear yet. Some workers used the young explants like the leaf of in vitro plant or female inflorescence, whereas the others able to induced direct somatic embryos from mature explants like shoot tip (Sidky and Zaid, 2011; Al-Musawi *et al*., 2015). In addition, there are no accurate histological studies dealing with initiation of direct somatic embryogenesis were cited in the work literatures in compared with indirect somatic embryogenesis (Tisserat and De Mason, 1980; Sané *et al*., 2006; El Dawayati *et al*., 2012).

The aim of the present investigation was to study the induction of direct somatic embryos by culturing of in vitro shoot tips of date palm as explants on MS medium supplemented with different growth regulators. Furthermore, the morphological and histological changes during the ontogeny of the direct somatic embryos from the initial cells until the establishment of the bipolar embryos and their development into plantlets were been investigated.

## Methods

### Plant materials

Present study conducted in Date Palm Research Center and Cell and Biotechnology Unit, Collage of Science, University of Basrah, during the period 2015-2016. *In vitro* shoots of date palm cv. 'Barhee' grown in the liquid medium by using bioreactor system (fig. 1). Shoot tip (2-3 mm in diameter) were excied from *in vitro* microshoots and inoculated to direct somatic embryo media.

### Induction of direct somatic embryogenesis

Entire shoot tips excied from microshoots were cultured on Murashige and Skoog 1962 (MS) basal medium enriched with different concentrations of Thiadiazuron (TDZ) and auxins (table 1). The following chemicals are supplemented routenly to the reference MS medium sodium dehydrogen phosphate dihydrate (170 mg/L), calcium nitrate (1.5 g/L), myo-inositol (100 mg/L), adenine sulphate (40 mg/L), nicotinic acid (1 mg/L), glutamine (200 mg/L), Ca-pantothenate (20 mg/L), thimanie-HCl (0.4 mg/L), pyridoxine-HCl (1 mg/L), biotin (1 mg/L), sucrose (50 g/L), polyvinyl pyrilodine (500 mg/L) and agar (7 g/L). Cultures kept at 27°C and under dark conditions.

**Fig. 1 :** Shoots of date palm *Phoenix dactylifera* L. cv. Barahee grown in liquid medium on palntform bioreactor.

### Maturation and germination of somatic embryos

For further maturation and germinations of somatic embryos. Direct somatic embryos obtained in step one were transferred to MS medium enriched with all supplements mentioned above and replace the growth regulators mentioned above with kinetien, N6-(2-Isopentenyl) adenine 2ip and benzyl adenine 0.1 mg/L each.

pH of the medium in step one and two was adjusted to 5.8±0.2 before adding the agar. Media then autoclaved at 118°C and at 1.2 Kg/cm$^2$ for 10 minutes.

### Histological study

Explants at different stages of development were collected and diped in a fixative solution formaline, acetic acid (FAA) for two days at rome temperature 25°C. Samples then transferred to 70% alcohol and stored at cold conditions in refrigerator. The fixed samples were washed with 70% ethanol and then dehydrated using graded alcohol series, then clearing with xylen series, after that explants were embedded in paraffin wax. The explants were sectioned at (10µ) thick using Rotary microtome. Slides deparaffinised was achieved by using

xylem, whereas slides rehydrahation performed by using etanol series. Sections stained with safranien and fast green, mounted with *Canda balsam*, Slides examined under the light microscope and photographed with digital camera.

## Results and Discussion

### Induction of direct somatic embryogenesis

In recent year, direct somatic embryogenesis and organogenesis have been used as a route for *in vitro* propagation of date palm (*Phoenix dactylifera* L.). But, a less histological studies of orgin and ontogeny of direct somatic embryogenesis have been reported by authors.

Study revealed that there are three types of response were recognoized. First type, the explant was slightly swelling without subsequent modifications, after few weeks the explants turn brown and died (fig. 2 a). Second type of response, include direct somatic embryo formation without callus production with no callus formed. The explants developed a numbers of smooth small globules somatic embryo after initial swelling (fig. 2b). Third type of response the explants produced callus, these calli turned brown and die when transferred to fresh medium.

Results also showed that the best response of direct somatic embryogenesis obtained when the explants inoculated on MS medium fortified with 10 and 15 mg/L of TDZ, where the rate of somatic embryo formation ranged between 50 and 80% (table 2). While, the minimum response rate obtained when auxins NoA and NAA in combined with TDZ. Transferred of somatic embryos to maturation and germination medium induced secondary embryogenesis and were developed after 4 week (fig. 2). Four weeks later somatic embryo start germination and developing to plantlet. In date palm induction of direct somatic embryogenesis is very rare, in current study, we able to induced somtic embryogenesis without callus formation by using the TDZ.

TDZ is a substituted phenyl urea has been used for rapid plant regeneration of different plant species because it has a cytokinin-like activity (Malik and Saxena, 1992). It was believed that the applying low concentrations of TDZ will substitute the using of auxin and cytokinins for inducing somatic embryogenesis in many species (Gill and Saxena, 1993; Murthy and Saxena, 1998). But, Chhabra *et al.* (2008) found that the low concentrations of TDZ induced organogenesis while the high concentrations shifted the response to somatic embryogenesis.

In the present study, we applied a high concentration

**Fig. 2 :** (a) Explant grown on somatic induction medium showed slightly swelling without subsequent modifications. (b) Explant showed direct somatic embryo formation without callus production.

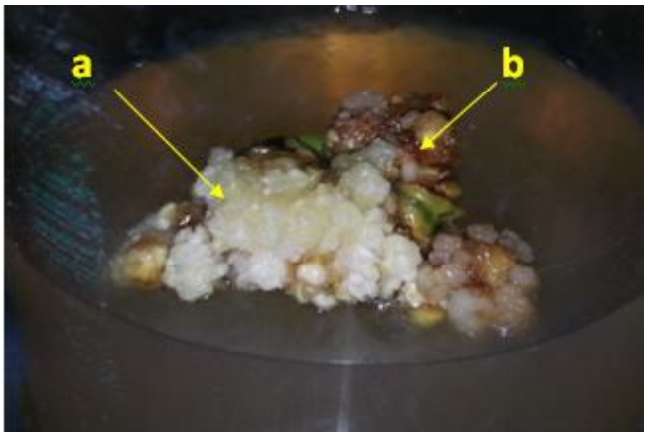


**Fig. 3 :** Secondary embryogenesis on MS medium containing with kinetien, 2ip and BAP 0.1 mg/L each. (a) secondary embryogenesis, (b) original explants.

of TDZ (15 mg/L) for induced direct somatic embryogenesis. This result is not in line with Aboshama (2011) and Khadke and Kuvalekar (2013), who reported that the high concentrations of TDZ declined the frequency and numbers of somatic embryos per explants. On the contrary, Abdulminam *et al*. (2015) observed that the low concentrations of TDZ did not increase the frequency of direct somatic embryogenesis per explants.

### Histological study

### Development of direct somatic embryo from expands

Histological study of the plants cultured *in vitro* are very important, because they explain the origin and development of organs under these conditions. Anatomical examination of explants grown on MS medium containing 15 mg/L TDZ showed a somatic embryos at different stages in combined with explants tissues expansion (fig. 4a). Histoligical examination also exposed that the somatic embryo originated from two zones, first from the sub-epidermal area (fig. 4a and b) and second from

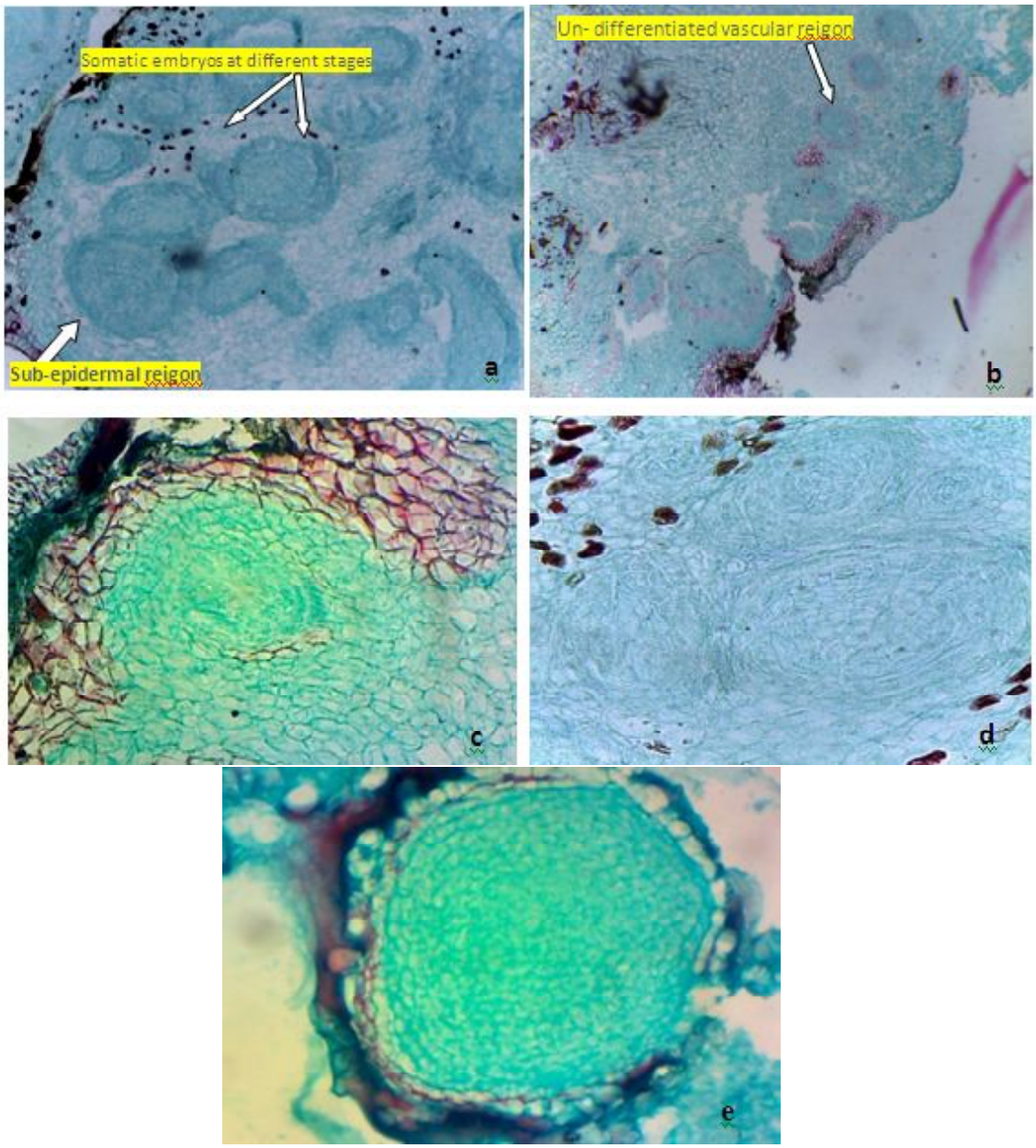


**Fig. 4 :** Direct somatic embryos at different stages of develompemt from sub-epedirmal 100X(a) and un-differentiated vascular reigon100X (b) during initiation stage. (c) Enlarged view of subepidermal reigon 400X, (d) enlaraged view of un-differentiated vascular reigon 200X. (e) Enlarged view of somatic embryo.

the un-differentiated vascular bundle (Figs. 4b and d).

The first distinct appearance of embryogenesis was marked by development of globular forms that were embedded in the explants tissue, but there were separated from the adjusent tissue by vaculated cells and became a single embryo. Subsequent cell division led to progress in somatic embryo developmental stages. However, No vascular connection between a somatic embryos and surrounding tissue has been recognized (fig. 4e).

The cells of pro-embryogenic structure characterized by a small prominent nucleus, dense cytoplasm. And no starch grains storage has been recorded in these cells.

Histological analysis of somatic embryos at un-differentiated vascular bundle showed that the further division of cells formed multicellurar ovate proembryo connected to vascular strand (figs. 5a and b). This results is line with Abd El Bar and El Dawayati (2014), who reported that in date palm the direct somatic embryo induced from young *in vitro* leaf. These embryos were originated from undifferentiated vascular cells. Intensive division of the multicellular proembryo from both direction

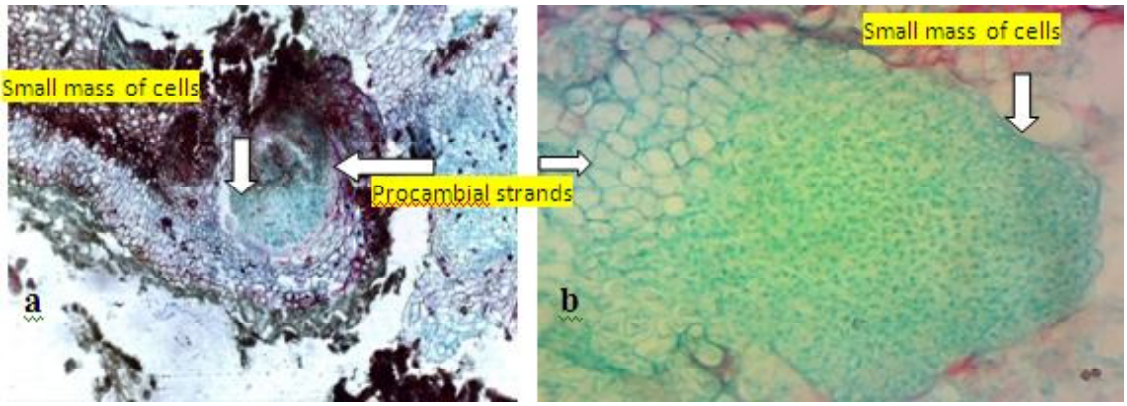


**Fig. 5 :** (a) A small mass of cells connected to procambial strands 100X.(b) Enlarged view of intensive division of ovate proembryo madding a small mass of cells connectet to procambial strands 200X.

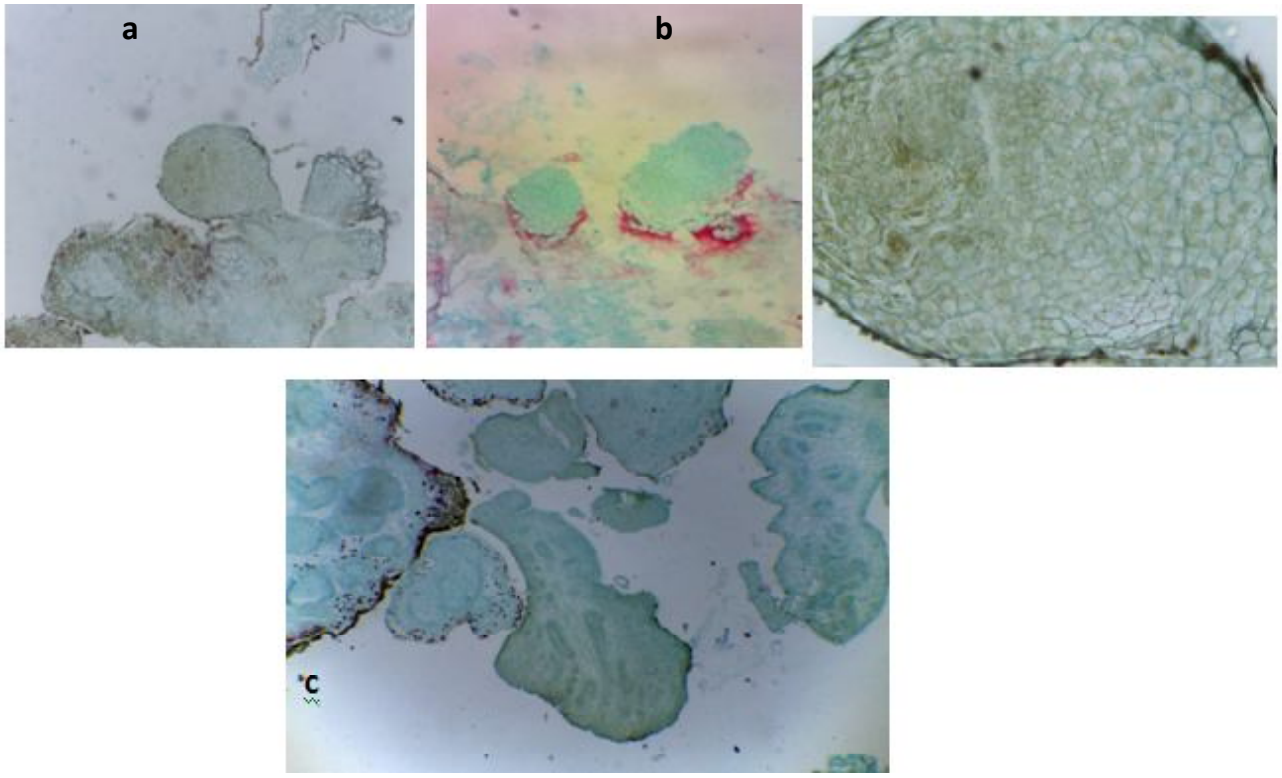


**Fig. 6 :** (a) Direct somatic embryos emerged from mother explant by made a high pressure caused a rapturaing on the surrounding tissues 100X. (b) Enlarged view of bipolar embryos showing the two marstimatic and vaculalted ends 400X. (C) longitudinal section showing the bipolar embryo resembling to the zygotic embryos.

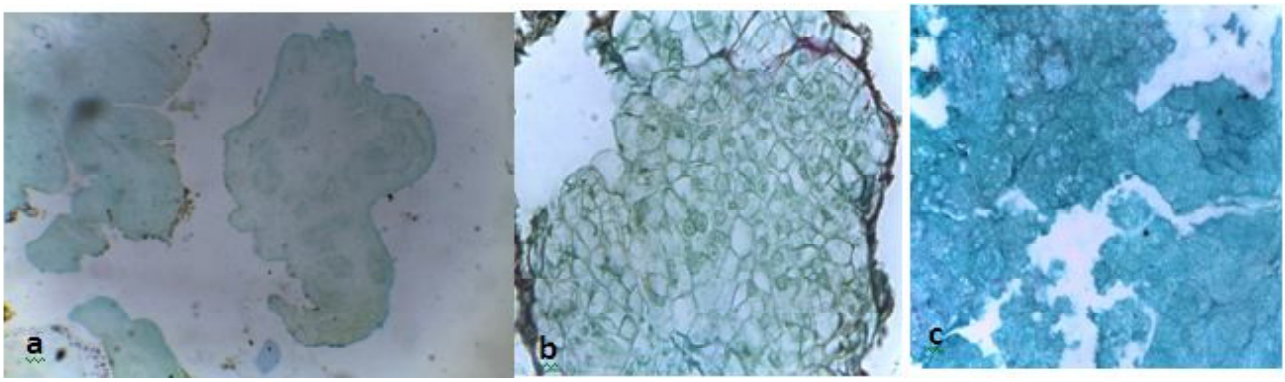


**Fig. 7 :** (a) Secondary embryogenesis formed from somatic embryo (b)Enlarged view showing the pericclinal division of pro-embryo cells produced secondary embryogenesis (400X). (c) Compact meristematic masses delimited by prododerm.

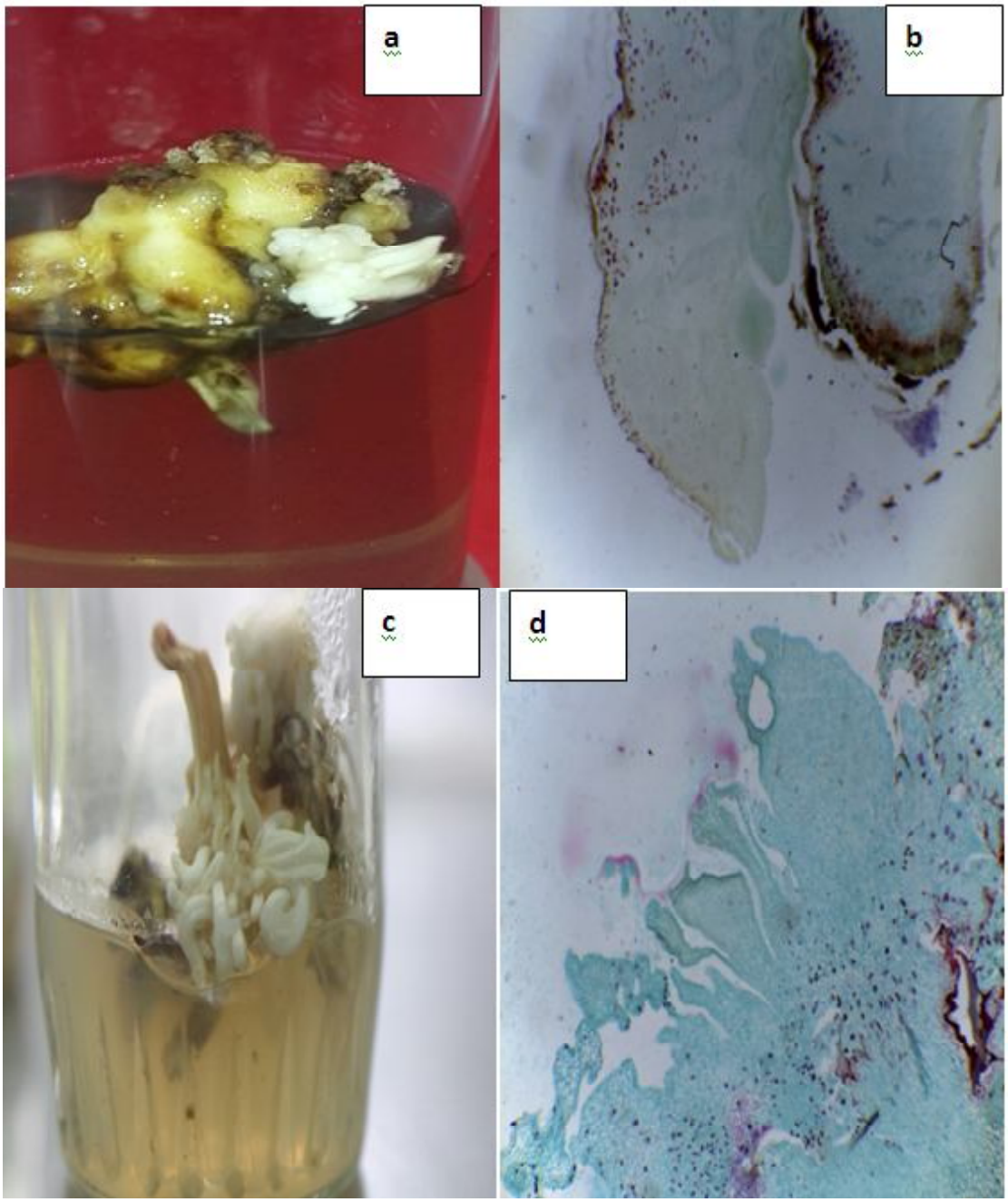


**Fig. 8 :** (a) Shoot formation by direct organogenesis from somatic embryos. (b) Meristematic centers formed from epidermal cells of bipolar somatic embryo. (c) shoot formation from germinated embryo. (d) leaves protuberance emerged from epidermal area of embryo.

**Table 1 :** Auxins and cytokinin treatments.

| Treatmets | Auxins/ $mg^{-1}$ | Cytokinins $mg^{-1}$ TDZ |
|---|---|---|
| 1 | 0 | 10 mg/L |
| 2 | 0 | 15 mg/L |
| 3 | 1 mg/L NAA | 10 mg/L |
| 4 | 1 mg/L NoA | 10 mg/L |

IBA= Indole-3-butyric acid
NAA= 1-Napthaleneacetic acid
NoA= 2-Naphthoxyacetic acid

**Table 2 :** Effect of auxin cytokinin treatments on direct somatic embryogenesis formation.

| Treatmets | Rate of direct somatic embryogenesis |
|---|---|
| 1 | 50% |
| 2 | 80% |
| 3 | 20% |
| 4 | 30% |

(anticlinal and preclinal) resulting a small mass connected to vascular strand. So, these cell masses considered the globular embryos.

Figs 6 a and b showing the somatic embryos at different stages of development. Further cell division of these embryos lead to form the bipolar structure. These structure consisted of a highly meistematic end and more differenited and highly vaculalted end procabial strands have been seen in this stage (fig. 6c). However, the somatic embryo showed no vascular connection with mother tissue. More development of these embryos caused raputureing of surrounding tissue and emergence of these embryos. All the obtained results matched with the results reported by Abd ElBar and El Dawayati (2014).

On maturation and germination medium some embryos formed secondary embryogenesis (fig. 7) because the somatic embryo failed to germinate. The budding or secondary embryogenesis seems un controlled (fig. 3a). Histological analysis of somatic embryo at this stages exhibited a peirclinaldivision of pro-embryos (figs. 7 a and b). Then, the division lost their oritention and producing compact meristematic masses and determined by a protoderm (fig. 7c). These findings corresponding to formation of secondary somatic embryogenesis was mentioned by Maheswaran and Williams (1986), Zegzouti *et al*. (2001). In date palm Ahloowalia *et al*. (2004) reported that the primary somatic embryo tends to form many embryos through secondary embryogenesis. On the other hand, because the somatic embryo grown on MS medium containing a low concentrations of cytokinins for a long time, so some embryiods tend to form direct organogenesis (fig. 8 a). Histological study of this part shows that meristemic centers formed as a result of epedirmal division of somtic embryos (fig. 8b). Further development of these centers caused the first leaves protuberance emerged from perferial area (fig. 8c and d).

## References


Abahmane, L. (2013). Recent achievements in date palm (*Phoenix dactylifera* L.) micropropagation from inflorescence tissues. *Emir. J. Food Agric*., **25 (11)** : 863-874.

Abd, El Bar1 O. H. and M. M. El Dawayati (2014). Histological changes on regeneration *in vitro* culture of date palm (*Phoenix dactylifera*) leaf explants. *AJCS*. **8(6)** : 848-855.

Abdulminam, H. A. Almusawi, Ansam S. M. Alshnawa, Aqeel A. S. Alkhalifa and Rayad F. T. Almesfer : Induction of direct somatic embryogenesis in date palm (*Phoenix dactylifera* L.) cv. Hellawi. *AAB BIOFLUX*, **7(2)** : 80-89.

Abhamane, L. (2011). Date Palm Micropropagation *via* organogenesis. Jain, S. M., J. M. Al-Khayri and D. V. Johnson (eds). *Date Palm Biotechnology*. Springer, Netherlands. pp 69-90.

Aboshama, H. M. S. (2011). Direct Somatic Embryogenesis of Pepper (*Capsicum annuum* L.). *World J. Agric. Sci*., **7(6)** : 755-762.

Al-Khairy, J. M. (2010). Somatic embryogenesis of date palm (*Phoenix dactylifera* L.) improved by coconut water. *Biotechnology*, **9** : 477-484.

Bekheet, S. (2013). Direct Organogenesis of Date Palm (*Phoenix dactylifera* L.) for propagation of true-to-type plants. *Sci. Agri*., **4(3)** : 85-92.

Bekheet, S. A., H. S. Taha and M. M. Saker (2002). A synthetic seed system of date palm through somatic embryogenesis encapsulation. *Ann. Agric. Science*, **47** : 325-337.

Chhabra, G. D., M. Chaydgart, M. Varma, M. Sainger and P. K. Jaiwal (2008). TDZ-induced direct shoot organogenesis and somatic embryogenesis on cotyledonary node explants of lentil (*Lens culinaris* Medik.). *Physiol. Mol. Biol. Plants*, **14(4)** : 347-353.

El Dawayati, M. M., O. H. Abd El Bar, Z. E. Zaid and A. F. Zein El Din (2012). *In vitro* morpho-histological studies of newly developed embryos from abnormal malformed embryos of date palm cv. Gundila under desiccation effect of polyethelyne glycol treatments. *Ann Agric Sci*., **57(2)** : 117–128.

El-Hadrami, l.; M. El-Bellaj, A. El-ldrissi, J. F.'Aitti, S. El-Jaafari and F. Daff (1998). Biotechnologie végétalers et amelioration du palmier dattier (*Phoenix dactylifera* L.) pivot de l'agriculture oasienne marocaine. *Cah. Agric*., **7** : 463-468.

Gill, R. and P. K. Saxena (1993). Somatic embryogenesis in Nicotiana tabacum L.: induction by thidiazuron of direct embryo differentiation from cultured leaf discs. *Plant Cell Rep*., **12** : 154-159.

Jazinizadeh, E., R. Zarghami, A. Majd, A. Iranbakhsh and G. Tajaddod (2015)/ *In vitro* production of date palm (*Phoenix dactylifera* L.) cv. Barhee plantlets though direct organogenesis. *Biological Forum – An International Journal*, **7(2)** : 566-572.

Khadke, S. and A. Kuvalekar (2013). Direct somatic embryogenesis and plant regeneration from leaf and stem explants of *Nothapodytes foetida* : A critically endangerd plant species. *IJPAES*, **3(1)** : 257-264.

Levee, V., M. A. Lelu, L. Jouanian, K. Comu and G. Pilate (1997). *Agrobacterium tummefaciens* mediated transformation of hybrid larch (*Larix kaempferi* o L. Deciduas) and transgenic plant regeneration. *Plant Cell Report*, **16** : 680-685.

Maheswaran, G. and E. G. Williams (1986). Primary and secondary direct somatic embryogenesis from immature zygotic embryos of *Brassica campestris*. *J. Plant Physiol*., **124** : 455–463.

Malik, K. A. and P. K. Saxena (1992). Thidiazuro induces high frequency shoot regeneration in intact seedlings of pea, chickpea and lentil. *Aust. J. Plant Physiol*., 731-740.

Murthy, B. N. S. and P. K. Saxena (1998). Somatic embryogenesis and plant regeneration of Neem (*Azadirachta indica* A. Juss). *Plant Cell Rep*., **17** : 469-475.

Namasivayam, P. (2007). Acquisition of embryogeneic competence during somatic embryogenesis. *Plant Cell Tissue Culture*, **90** : 1-8.

Ola, H. Abd El Bar1 and Maiada M. El Dawayati (2014). Histological changes on regeneration *in vitro* culture of date palm (*Phoenix dactylifera*) leaf explants. *Australian Journal of Crop Science*, **8(6)** : 848-855.

Othmani, A., C. Bayoudh, Drira and M. Trifi (2009). *In vitro* cloning of date palm *Phoenix dactylifera* L., CV. Deglet Bey by using embryogeniec suspension and temporary immersion bioreactor (TIB). *Biotechnology and Biotechnologenical Equipment*, **23(2)** : 1181-1188.

Redouane Zegzouti, Marie-France Arnould and Jean-Michel Favre (2001). Histological investigation of the multiplication step in secondary somatic embryogenesis of *Quercus robur* L. *Ann. For. Sci*., **58** : 681–690.

Sané, D., F. Aberlence- Bertossi, Y. K. Gassama-Dia, M. Sagna, M. F. Trouslot, Y. Duval and A Borgel (2006). Histocytological analysis of callogenesis and somatic embryogenesis from cell suspensions of Date Palm (*Phoenix dactylifera*). *Ann Bot.*, **98** : 301–308.

Sidky, R. A. and Z. E. Zaid (2011). Direct Production of Somatic embryos and plant regeneration using TDZ and CPPU of date palm (*Phoenix dactylifera* L.). *International Journal of Academic Research*, **3(2)** :792.

Tisserat, B. and D. A. Mason (1980). A histological study of development of adventive embryos in organ culture of *Phonenix dactylifera* L. *Ann Bot*., **46** : 465–472.

Zimmerman, J. L. (1993). Somatic embryogenesis: a model for early development in higher plants. *Plant Cell*., **5** : 1411-1423.