Australian Journal of Crop Science AJCS
AJCS 8(6):848-855 (2014) ISSN:1835-2707

Histological changes on regeneration *in vitro* culture of date palm (*Phoenix dactylifera*) leaf explants

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Abstract

A tissue culture experiment was conducted to investigate the effect of some growth regulators and two different ages of *in vitro* leaves of date palm c.v. sakoty on regeneration process. Leaves were cultured on Murashige and Skoog's medium (MS) supplemented with each or combined with the following growth regulators 4 mg l^{-1} 2,4-D, 40 mg l^{-1} NAA, 2 mg l^{-1}BA, (2 mg1 $^{-1}$BA+ 40 mg l^{-1} NAA), (2mgl^{-1} BA+ 4 mg l^{-1} 2,4-D) and (2 mg l^{-1}BA+40 mg l^{-1} NAA+4 mg l^{-1} 2,4-D). The results showed that *in vitro* young leaf explants treated with 2 mg1 $^{-1}$ BA was more efficient in inducing direct somatic embryos and subsequent plantlets growth in a short time duration ,i.e.8-10 months. However, the other treatments or the old leaves had no response to induce direct somatic embryos. Also, histological examinations were made during the initiation and development of direct somatic embryos. It was revealed that the proembryos originated from procambial cells, whereas the mesophyll parenchyma and the epidermal cells did not participate in this process. Procambial cells acquired the embryonic competence within 15 days form culturing. The embryonic structures were generated along the vascular strands, which subsequently evolved into somatic embryos. After two months, different developmental stages of somatic embryos were observed, i.e. (pre-globular, globular, early bipolar, bipolar and cotyledonary-shaped). The regenerated embryos were transferred onto both multiplication and rooting media for 8 months to obtained healthy plantlets. Therefore, this protocol provides a simple and rapid way to regenerate date palm *via* direct embryogenesis. This result obtained herewith was discussed with other results elsewhere which depend on callus formation as an intermediate phase to obtain somatic embryos indirectly.

Keywords: Date palm, *In vitro* leaf explants, Direct somatic embryogenesis, Auxins, Cytokinin, Histology, Procambial cells.
Abbreviations: BA_6-benzyl aminopurine; NAA_α-naphthalene acetic acid; 2,4-D_2, 4- Dichlorophenoxy acetic acid.

Introduction

The date palm, *Phoenix dactylifera* L., is one of the most economically important fruit trees in the desert areas of the Middle East and North-Africa (Al- Khayri, 2007). Palm groves were planted from offshoots which were produced by the date palm trees in the early part of their life. However, this traditional method is relatively slow to establish new date palm plantations since a limited number of offshoots are produced by the tree during its lifespan. In addition, seed-propagated palms do not bear true type fruits due to heterozygosis and require up to 7 years to reach the adulthood fruiting stage (Othmani et al., 2009). *In vitro* micropropagation thus became an essential and effective means to ensure the renewal and the extension of palm plantations (Smith and Aynsley, 1995). Since 1970, intensive efforts have been undertaken into large-scale micropropagation of date palm using techniques such as somatic embryogenesis and organogenesis (Tisserat, 1979; Drira, 1983; Singh and Shekhawat, 2009). Palms are considered as a recalcitrant species to be used in tissue culture. Thus, the *in vitro* regeneration of date palm using somatic embryogenesis still requires more refined and optimized protocols. Till now, the use of *in vitro* leaf explants as a source for regeneration of date palm to induce direct somatic embryos is not clearly established. Whereas, it is widely used in many other plants that produced normal plantlets through this technique (Chevreau et al., 1989; Yepes and Aldwinckle, 1994; Chen and Chang, 2002). In addition, there are no precise histological studies on the different developmental stages during the initiation of direct somatic embryogenesis were reported in the literature cited, particularly from *in vitro* leaf explants of date palm. However, the origin and ontogeny of indirect somatic embryos of date palms have been extensively presented by many authors (Tisserat and Mason, 1980; Sané et al., 2006; El Dawayati et al., 2012). The aim of the present work was to investigate the possible induction of direct somatic embryos by culturing two different ages of *in vitro* leaves 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 investigated.

Results and Discussion

Induction of direct somatic embryos

In vitro old leaves failed to regenerate under different treatments of culturing media. These leaves acquired a brownish color without any signs of further development during the culturing period. Histological examination of the *in vitro* old leaves indicated that all leaf tissues, particularly at the basal portion were fully developed and there were no undifferentiated cells (unpublished data). Therefore, it is more than likely that the fully developed cells not responsive to the different growth substances in the culturing media. On the contrary, the histological examination of the young *in vitro* leaf explants revealed the existence of undifferentiated vascular cells (procambium strands), particularly at the basal portion of the leaf.. (See histological study below) The response of *in vitro* young leaves cultured on different treatments of MS medium was greatly varied during the regeneration process (refer to Table 2). *In vitro* young leaf explants cultured on regeneration medium (M3) treatment supplemented with $2mg1^{-1}$ BA showed the highest direct somatic embryos formation followed by the (M4) treatment supplemented with 2 mgl^{-1}BA+ 40 mgl^{-1} NAA and (M6) treatment supplemented with 2 mgl^{-1}BA+ 40 mgl^{-1}NAA+4 mgl^{-1} 2,4-D respectively. In this respect, the effect of cytokinins and/or auxin in the culture media treatments on the regeneration behavior of explants was investigated by several authors. Caboni et al. (1999) found that a concentration of 8.8 µM BA induced the highest number of adventitious shoots from *in vitro* leaves culture of wild pear genotypes. Also, Chen and Chang (2002) studied the effect of auxins and cytokinins on direct somatic embryogenesis on *in vitro* leaf explants of *Oncidium* orchid. They found that direct embryo formation was reduced by exogenous auxins, but was promoted by exogenous cytokinins. On the other hand, Guohua and Qiusheng (2002) studied the induction of somatic embryos and adventitious shoots from immature *in vitro* leaves of cassava shoots by culturing on different induction media which contained 4 mg l^{-1} 2,4-D, 40 mg l^{-1} NAA , 2 mg l^{-1}BA , 2 mg l^{-1}BA+40 mg l^{-1} NAA , 2 mgl^{-1} BA + 4 mg l^{-1} 2,4-D. They revealed that 2,4-D is more effective in inducing somatic embryogenesis than NAA whereas BA had an inhibitory effect in the induction phase. Again, Caboni et al. (1999) found that the lowest NAA concentration (1.0µM) slightly enhanced the regeneration rate when used together with 8.8 µM BA in wild pear tested genotypes. A low effect of NAA concentration compared to the effect of cytokinins was also reported by Leblay et al., (1991).

The obtained results reveal that, yellow compact callus was observed on (M5) treatment supplemented with $2mgl^{-1}$ BA + 4 mgl^{-1} 2,4-D, and (M1) treatment supplemented with 4 mg l^{-1} 2,4-D (Table 2) . Callus was initiated at the end of the first 8 weeks of culturing on (M5) treatment then obviously increased at the end of the second re-culture. Cultured young leaves explants on (M4) treatment supplemented with 2 mg l^{-1}BA+ 40 mg l^{-1} NAA , and (M6) treatment supplemented with $2mgl^{-1}$ BA+ 40 mg l^{-1} NAA + 4 mg l^{-1} 2,4-D showed quite a clear appearance of brown granulated callus formation. Meanwhile, adventitious roots formation were differentiated directly at the base of young leaf explants without any signs of callus formation when cultured on (M2) treatment supplemented with 40 mg l^{-1} NAA. In contrast, no root formation was observed in other tested treatments. High significant vitrification was observed especially on the direct differentiated somatic embryos cultured on (M4) treatment and followed by (M6) treatment. Whereas low level of vitrification degree was recorded in callus induced on (M5) treatment followed by (M2) treatment. No vitrification signs were observed on direct somatic embryos developed from young leaf explants on (M3) treatment.

Histological study

Structure of the young leaf

The *in vitro* young leaf at the time of culturing was white in color, 0.5-1cm long and 1mm thick. The basal part was nearly undulated adaxially and the surface was generally smooth (Fig. 1a). Successive transverse sections in the basal part showed that both the upper and lower epidermis are uniseriate. The ground tissue consisted of parenchyma cells interspersed with vascular strands, few intercellular spaces were observed (Fig. 1b). Undifferentiated vascular cells "procambial stands" were observed. Some cell divisions were recorded in these undifferentiated cells (Fig. 1c). Some of the epidermal cells were generally isodiametric with dense cytoplasm, anticlinal divisions were observed.

Ontogeny of direct somatic embryos

After fifteen days from culturing with 2 mg l^{-1}BA, the leaf acquired a green color and expanded to give more dimensions. The earliest stage of embryo formation was recorded as few cell divisions in the undifferentiated vascular cells "procambial cells" (Figs. 2a&2b). These cells were characterized by small vacuoles and dense cytoplasm. In transections, most of these divisions took place on one side of each vascular bundle except in few cases in which several anticlinal and periclinal divisions were shown around one vascular bundle (Figs. 2c&2d). Further divisions led to form a multicellular small ovate proembryo (Fig. 3a). Intensive cell divisions of the abovementioned proembryo were observed in both periclinal and anticlinal directions, resulting in the presentation of small masses connected to the vascular strands (Fig.3b). These masses are considered the globular embryos. With further growth, the initial meristems of the embryo began to differentiate. These appear as few concentric rings encircling a mass of disarranged cells (Fig. 3c). The globular embryos ranged from 200 to 250 µm in diameter. After 30 days from culturing, all the individual globular proembryos were observed embedded in degenerating parenchyma inside the leaf tissues (Fig. 3b). Morphological examinations revealed that the basal part of the young leaf appeared to be swollen and there were few small protuberances arising from the adaxial surface, i.e. upper side (Fig. 3d).

Development of direct somatic embryos

The globular embryo slightly expanded to give an early bipolar shape, which consisted of a meristematic end and a more differentiated of highly vacuolated opposite one (Fig. 4a). After 45 days from culturing, a procambium strand in the bipolar-shaped embryo was observed with formation of the cotyledon (Fig. 4b). Further growth took place in the bipolar embryo which became visible outside the epidermis of the foliar explant.

Table 1. Components of different culture medium treatments.

Medium No.	Components of the induction medium
M1	MS + 4 mgl^{-1} 2,4-D
M2	MS + 40 mgl^{-1}NAA
M3	MS +2 mgl^{-1}BA
M4	MS +2 mgl^{-1}BA+ 40 mgl^{-1} NAA
M5	MS +2 mgl^{-1}BA+4 mgl^{-1} 2,4-D
M6	MS +2 mgl^{-1}BA+ 40mgl^{-1}NAA +4 mgl^{-1} 2,4-D

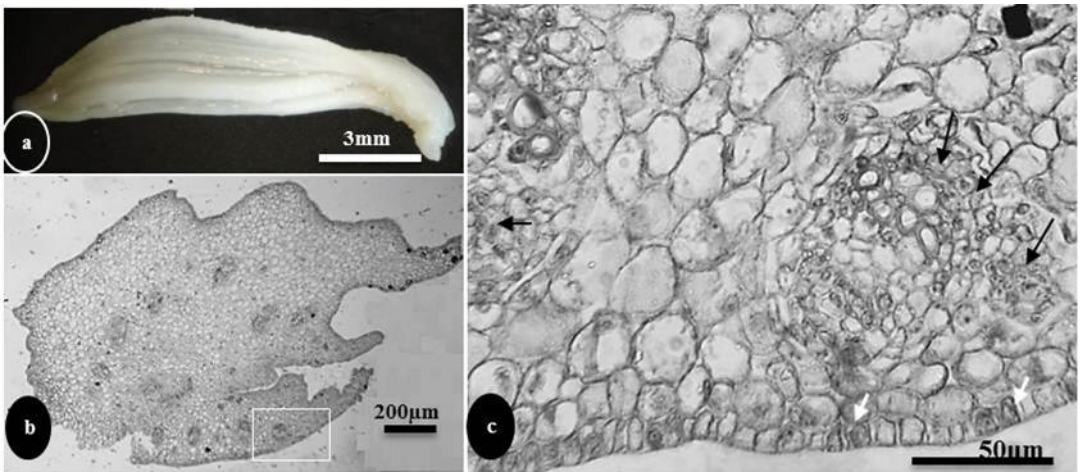

Fig 1. Morphology and histology of the small leaf of date palm c.v. Sakoty before culture. (a) Morphology of the young leaf showing that the basal part is white and smooth. (b) Cross section in the basal part of the young leaf showing uniseriate epidermis, parenchymatous ground tissue embedding the vascular bundles (c) Enlargement area of figure (1a) showing anticlinal cell divisions in the epidermal layer (white arrows), the regular compact ground tissue and procambial strands (black arrows).

The latter was broken due to the pressure of the growing inner embryos (Figs. 4a &4d). After 60 days from culturing, longitudinal sections of elongated embryos showed fully organized shoot and root apex, in addition to well-differentiated procambial strands along the embryo axis and the cotyledon were similar to that which occurred in the zygotic embryo (Fig. 4c). No vascular connections were observed between the vascular system of the somatic embryos and those of the parent tissue. The appearance of independent vascular systems in these embryos indicated that the structures possessed the ability to form normal somatic embryos and plantlets. Somatic embryogenesis was asynchronous process on the proliferation medium. Histological examinations of the present study also revealed that the procambial cells of the basal part of the *in vitro* young leaves have the ability to produce direct somatic embryos. Whereas the mesophyll parenchyma and epidermal cells did not participate in this process. This finding matched well with results of Kwaaitaal and De Vries (2007) on *Arabidopsis*. They further determined the gene SERK1 (somatic embryogenesis receptor kinase 1), which is originally identified as a marker for embryonic competent cells in plant tissue culture. This gene is expressed in procambium cells and in immature vascular cells. Furthermore, many reports stated that the totipotency is not an intrinsic property of all plant cells but can be acquired when the explant is exposed to potent synthetic auxins such as the 2,4-D (De Vries et al., 1988; Mordhorst et al., 1998). According to Nolan et al. (2003) the expression of SERK1 gene in *Medicago truncatula* can be induced by auxin and augmented by cytokinin during the process of cell division and differentiation leading to somatic embryos. The present results revealed that application of 2mg 1^{-1}BA (cytokinins) alone promoted the formation of somatic embryos in high frequency directly from the young leaf explants without passing through callus phase or needing auxin. This result agrees with Chen and Chang (2001) who reported that the direct somatic embryos in *Oncidium* orchid leaf cultures were generally promoted by cytokinins which were applied in both the synthetic form {(6-g, g-(dimethylallylamino) purine (2iP), BA, kinetin and thiadiazol} and the natural form (zeatin). Using cytokinin in the hormonal balance proved elsewhere to promote the expression of somatic embryogenesis followed by development for these embryos (Dhed'a et al., 1991). In oil palm, Aberlenc-Bertossi et al. (1999) observed that cultivating the cell suspensions for 1 month in a large quantity of liquid hormone-free medium, followed by planting the cells on BA-enriched medium, promoted the growth of proembryos (developed from the globular stage to form bipolar stage).The importance of BA during this phase of development was confirmed by experiments of Sane´ et al. (2006) with date palm. Indeed, the histological examination of date palm tissues showed that the application of cytokinin promoted the appearance of meristematic zones in the tissues. Then successive divisions led to the polarization of the proembryo within 3–4 weeks of culture. The basal portion (about 3-4 mm) of *in vitro* explant was only considered the responsive site to the tested culture media as compared to the middle or upper portion. This result was found to be agreeing with data of Welander, 1988; Pawlicki and Welander, 1994; Caboni et al., 1996. The increasing gradient in morphogenic ability from the leaf tip towards the base of the leaf has already been explained

Table 2. Effect of different growth regulators in culture medium treatments on the regeneration process of *in vitro* young leaf explants of date palm cv. sakoty for the duration of 8-10 months.

Observations Treatments	No. of direct somatic embryos formation	Callus formation degree	Callus shape &color	Percentage of adventitious root formation	Vitrification degree	Percentage of conversion plantlets
M1	0	1.33	Yellow compact callus	0%	0	0%
M2	0	0	-----	55.55%	1.33	0%
M3	18	0	-----	0%	0	66.66%
M4	10	1.33	Brown granulated callus	0%	2.66	11.11%
M5	0	2.66	Yellow compact callus	0%	2	0%
M6	7	1.33	Brown granulated callus	0%	2.33	0%
L.S.D.	0.92	0.83		8.17	0.71	14.7

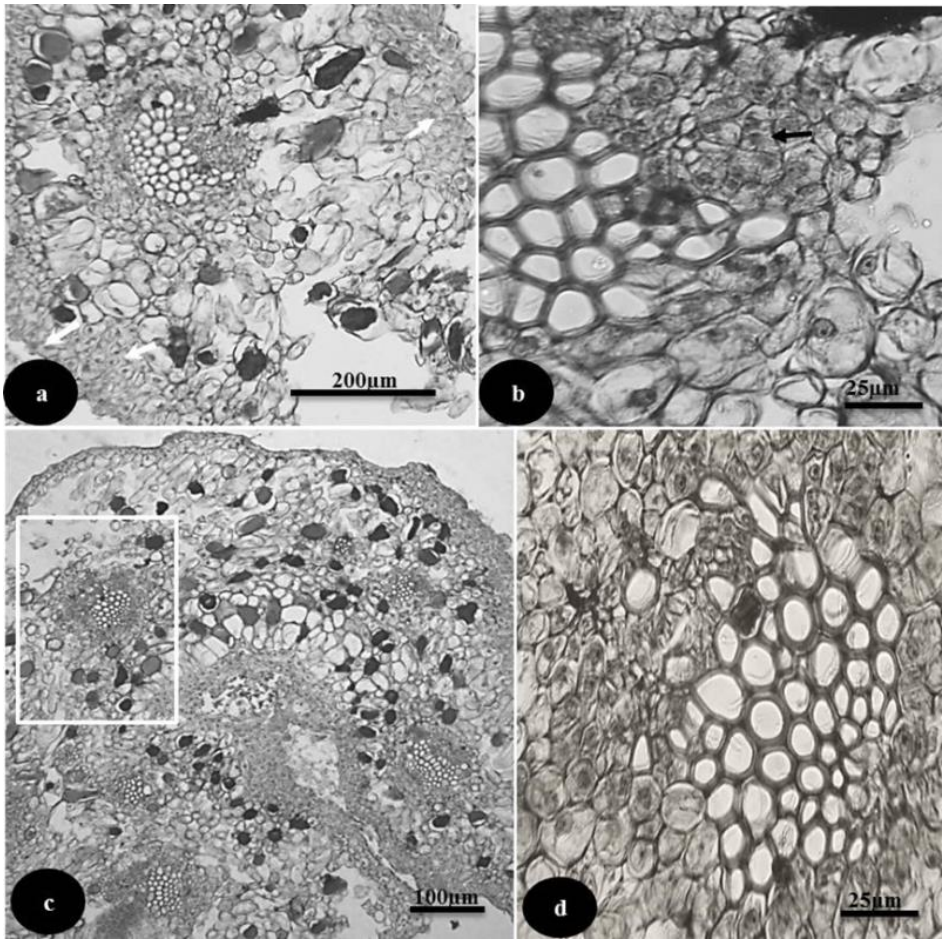

Fig 2. Ontogeny of direct somatic embryos. (a) Fifteen days after culture showing a differentiated vascular bundle. Note the expanded ground tissue compared with those in figure (1c), the arrows point to high mitotic activity occurred in the epidermal and sub epidermal layers. (b) An enlarged view of the same bundle in figure (2a) showing cell divisions occurred on one side of this vascular bundle adjacent to the xylem elements (arrow). (c) Transection in the culture young leaf, the rectangle reveals a vascular bundle which was enlarged in figure (d). Note the meristematic features in the cells surrounding the bundle.

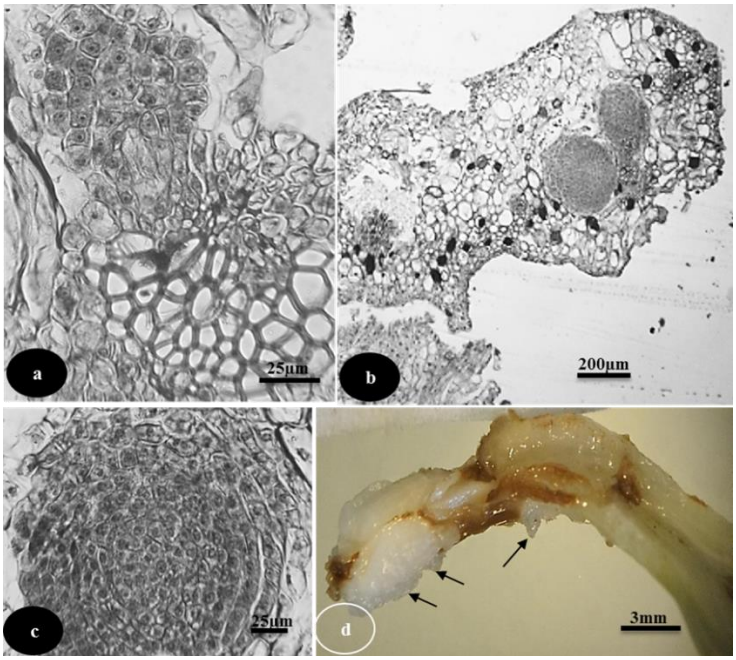

Fig 3. Development of direct somatic embryos. (a) The globular proembryo. (b) Thirty days from culturing, two globular proembryos. Note the degeneration of parenchymatous cells near the globular embryos. (c) An enlarged view of the globular embryo. Note the beginning of the differentiation of the meristematic tissues. (d) Morphology of the *in vitro* young leaf 30 days from culturing showing the swollen leaf base and few small protuberances arise from the adaxial surface (arrows).

by the fact that leaves mature first at the distal part and maturity extends basipetaly towards the proximal part (Welander, 1988). The Embryogenic cultures of date palm tissues initiated indirectly from shoot tips, young off-shoot leaves or from immature inflorescences have already been used with success for true-to-type propagation of some commercial varieties cultivated in North Africa e.g. Medjoul, and Barhe´ (Daguin and Letouze´, 1988), Boufeggous (Othmani et al., 2009) or Sakoty (Zein El Din, 2010). However, in all these cases the process needs as long as 6-8 months of culture before the primary calli are obtained. The subculture of these calli resulted in the formation of proembryos. So, it takes about 8-10 months to reach the cotyledonary embryo by the indirect somatic embryogenesis of date palm. We noted that, the culture of *in vitro* young leaf explants excised from differentiated mature somatic embryos in the presence of 2 mg l^{-1} BA resulted in embryo formation *via* direct embryogenesis without a callus phase. It takes about 2 months to reach the cotyledonary embryo. Compared with indirect somatic embryogenesis, the induction of embryos directly on leaf explants would obviously reduce somatic embryos production times. In addition, the *in vitro* young leaves are considered as valuable material for mass micropropagation of date palm. Direct somatic embryogenesis does not have the problems of phenolic accumulation in the medium which occurs with some crops, and the contamination rates are generally very low (Muniswamy and Sreenath 1996). Furthermore, the genetic variability is relatively low (Cuenca et al. 1999).

Developing of plantlets

All direct somatic embryos and callus cells were transferred to a germination medium to test their ability for resuming further development. As shown in (Table 2) M3 treatment supplemented with 2mg 1^{-1}BA recorded the highest significant plantlets which had the capacity to further multiply forming shoots and secondary embryos (Fig.5a). After three subcultures on the same germination medium, healthy plantlets obtained were transferred to a greenhouse to resume their normal life cycle (Fig. 5b). On the other hand, percentage of plantlets obtained from direct somatic embryos on (M4) treatment supplemented with 2.0 mgl^{-1}BA+ 40.0 mgl^{-1} NAA were significantly low which could not resume their development in a normal way. Furthermore, no plantlets were observed from direct somatic embryos on (M6) treatment supplemented with 2 mgl^{-1}BA+ 40 mgl^{-1}NAA +4 mgl^{-1} 2,4-D. This may due to the appearance of vitrification in the primary regeneration on both M4 and M6 treatments. It is well known that hyperhydricity (vitrification) is a serious problem during *in vitro* of date palm which directly affects the production at commercial level. Thus, *in vitro* cultured plantlets do not survive when transferred to soil due to yellowing, swelling, glassiness and leaf curling of plantlets (Wetzstein and Sommer, 1982; Donelly and Vidaver, 1984). Data in (Table 2) showed that the regenerated compact callus cells obtained from tested media did not show any ability for further differentiate into somatic embryos during the subcultures on the germination medium. Root formation was the only observation from callus cells on (M5) treatment which was supplemented with 2.0 mgl^{-1}BA+4.0 mgl^{-1} 2,4-D. It is worthwhile to mention here that granulated callus obtained from (M4 and M6) treatments gave small globular embryos like structures and it emerged on the mid-rib of leaf explants without any signs for further development during the three subcultures on the germination medium. Although there was an increase in the mass of callus induced from these treatments (unpublished results).

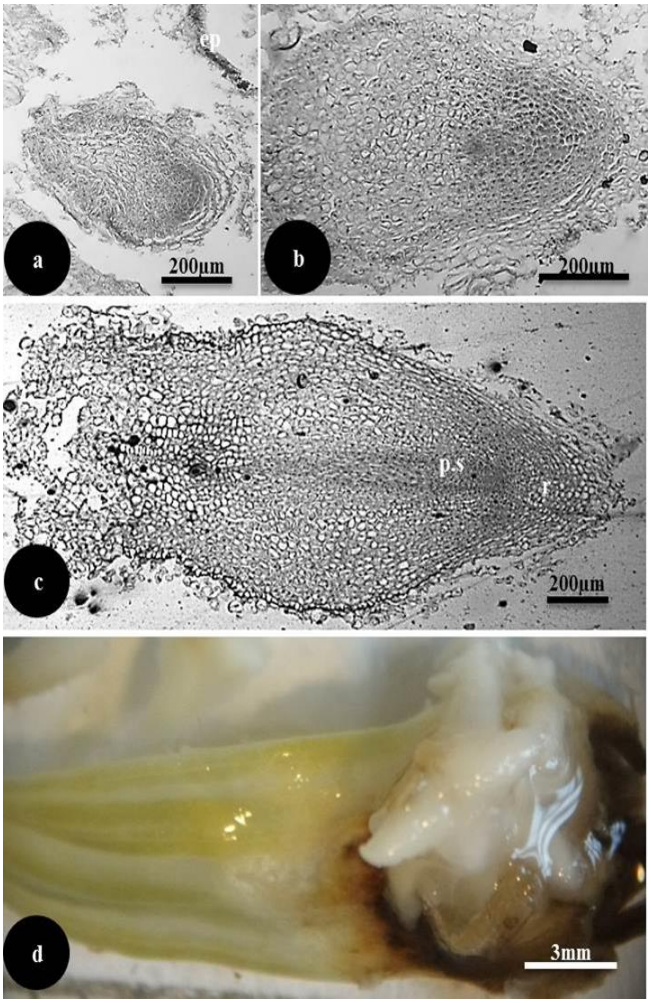

Fig 4. Development of direct somatic embryos. (a) After 45 days, the globular embryo expanded forming early stage of the bipolar embryo which is characterized with high dense meristematic region and a vacuolated one; note the degenerated parenchymatous ground tissue of the young leaf and the collapsed upper epidermis (ep). (b) The bipolar stage, note the cotyledone formation. (c) After sixty days, the complete bipolar embryo, the root meristem (r), procambial strands (p.s) and the cotyledone (c) were clearly visible, no vascular connection between somatic embryo and explant was observed. (d) Morphology of the cultured small leaf after 60 days showing a cluster of direct somatic embryos arises from the basal part of the leaf surface. Note the color transformed into green color comparing to fig. (1a), while the basal degenerative tissues became necrotic with a brownish color.

Materials and Methods

Plant material

Explants were taken from an already previous tissue culture protocol implemented for induction of indirect somatic embryos of date palm according to Tisserat and Mason, 1980. The first explants were characterized by the young leaves with white color, 0.5-1cm long and 1-2 weeks old (Fig. 1a). These leaves

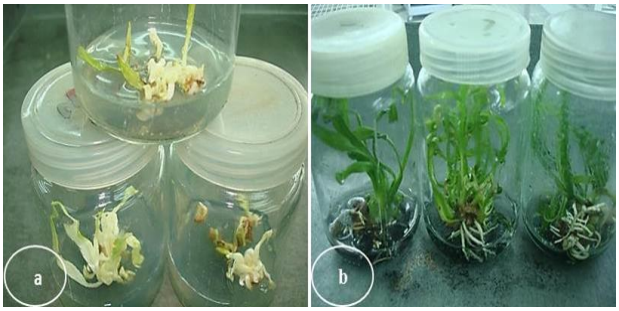

Fig 5. Different stages of plantlets. (a) Early stage of new shoots germinated from the direct somatic embryos. (b) Full-developed date palm plantlets derived from direct somatic embryos.

were excised from differentiated mature somatic embryos initiated as mentioned above. The second explants were characterized by the older leaves with green color, 3-5 cm long and 12-16 weeks old, excised from full developed plantlets of *in vitro* re-culturing from similar mature somatic embryos after being placed on a germination medium.

Induction of direct somatic embryos

Culture media were supplemented with 2,4-D, NAA and BA in different combinations and concentrations as shown in (Table 1). The effect of previous growth regulator treatments on induction of direct somatic embryos was examined. All culture media used in the present study consisted of an MS basal nutrient medium supplemented with vitamins (Murashige and Skoog, 1962), 170 $mgl^{-1}NaH_2PO_4$, 200mgl^{-1} glutamine , 40 gl^{-1}sucrose , and 5.5 gl^{-1} agar. The pH was adjusted to 5.7± 0.1 before adding the agar and medium was dispensed into small jars 150 ml (40ml/jar) before autoclaving at 120ºC and 15 Ibs/in^2 for 20 min. Leaf explants were cultured on the treated media and re-cultured every 8 weeks. Culture jars were divided into two groups according to leaves age. Each treatment consisted of ten jars. Five leaves per jar were incubated at 27± 2ºC in darkness.

Data were taken during two subcultures as follows:

-The average value of number of direct somatic formation/explant
-The average value of callus cells formation/explant
-The average value of vitrification appearance/explant
-The average percentage of adventitious roots formation/explant
-The average percentage of conversion to plantlets/explant

These data were scored visually according to Pottino (1981) as follows:

1	(-)	Negative result
2	(+)	Below average result
3	(++)	Average result
4	(+++)	Good result
5	(++++)	V. good result

Experimental design and statistical analysis

The experiment was conducted in a complete randomized design with three replicates. The obtained results were subjected to statistical analysis of variance according to the

method described by Snedecor and Cochran (1980) using LSD test at 5%.

Histological study

Samples of young leaves were collected at 0,15,30,45 and 60 days of culture from the most effective regenerative treatment (2.0 mgl^{-1} BA) to observe the morphological changes by OPTICA SZM-2 binocular. Samples were killed and fixed in FAA solution (Formalin, acetic acid and 50% ethyl alcohol, 5:5:90 by volume) for 24 h. The schedule of the paraffin method was followed as described by Johansen (1940). Serial transverse and longitudinal sections (8-10 µm thickness) were made by LEICA rotary microtome model RM 2125 RTS and fixed on slides by means of Haupt´s adhesive (Sass, 1951). The sections were stained with a Safranin–Fast green combination, and then mounted in Canada balsam (Sass, 1951). Observations and photomicrographs were obtained with LEICA light research microscope model DM 2500 supplied with a digital camera.

Conclusion

A new approach for rapid micropropagation of date palm by induction of direct somatic embryos from *in vitro* young leaves explants was established (without passing through the callus phase). The plantlets obtained from direct somatic embryos required 8-10 months comparing to 12-15 months in the indirect somatic embryogenesis. Obviously, cytokinins and explants having meristematic zones or procambial cells were necessary for induction of direct somatic embryos in *Phoenix dactylifera*. Further studies are needed to examine the selection of the right explants and cytokinins types or concentrations.

Acknowledgements

The authors wish to thank Dr. Sami, A. Habib and Dr. Said, A. Shehata for their helpful discussions on the results of the histological changes. The authors thank Dr. Abd Elaziz Hosny for critical reading of the manuscript.

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