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A comparative study between solid and liquid cultures relative to callus growth and somatic embryo formation in date palm (*Phoenix dactylifera* L.) cv. Zaghlool

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Abstract

Tissue culture techniques enable mass propagation of elite cultivars of date palm (*Phoenix dactylifera* L.). The main limitations for date palm in vitro multiplication are the low rates achieved using solidified medium and the long period needed to produce acclimatized plantlets. This research focuses on the comparison between different culture types and plant growth regulator (PGR) combinations on callus growth and somatic embryo formation of cv. Zaghlool. For callus growth, 200 mg friable embryogenic callus dispensed in Rasotherm and Phytacon flasks containing 200 ml liquid (100 rotations per minute) and in temporary immersion system, RITA® bioreactor (5 min immersion every 12 h), were compared with cultures grown on 200 ml solidified medium. For somatic embryo formation, 500 mg of friable embryogenic callus grown in Erlenmeyer flasks filled with 50 ml liquid or solid MS medium. The medium was supplemented with 0.1 mg l^{-1} Naphthaleneacetic acid (NAA), 1.5 g l^{-1} activated charcoal (AC) with or without 0.05 mg l^{-1} 6-Benzyl amino purine (BAP) compared to PGR-free medium. Results proved that cell suspension cultures produced the highest callus fresh mass as compared to the other systems tested, and the callus fresh mass reached 4 g after 16 weeks. The temporary immersion system did not significantly enhance the fresh mass of callus compared to the solidified medium. For somatic embryo induction, the number of somatic embryos increased in cell suspensions 6-16 fold compared to the solidified medium. Using liquid MS medium enriched with 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC gave rise to the highest number of somatic embryos formed from 500 mg initial callus: 160 embryos. The number of somatic embryos was also affected by the callus source. The calli induced from leaflet segments excised from converted somatic embryos resulted in a lower somatic embryo number than those of shoot tip origin with about 60 somatic embryos per 500 mg callus. The formation of somatic embryos using liquid medium required only 6 weeks, thus considerably reducing the previously reported period of 18 weeks which is required for somatic embryo formation using solidified media.

Key words: Cell suspension, Fresh mass, Growth curve, Leaflet segments, *Phoenix dactylifera*, Phytacon, RITA®, Secondary embryogenesis, Temporary immersion system

Introduction

Date palm treeshave high socioeconomic and nutritional value (ElHadrami and Al-Khayri, 2012); their seed propagation resultsin inferior quality. Offshoot use is restricted due to supply in limited numbers (Al-Khayri, 2012). Tissue culture techniques provide an alternative method to large-scale propagation of date palm (Zaid et al., 2011).

Current techniques for micropropagation require a large number of small containers, solid

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media and aseptic conditions, resulting in high cost of production (Berthouly and Etienne, 2005). The use of solid medium for commercial production is still hampered by low plantlet production rates, high labor cost and more space requirement (Soh et al., 2006). Our previous results on date palm cv. Zaghlool showed that the highest number of somatic embryos induced from initial 100 mg callus was about 7-8 embryos on a solid MS medium containing 0.1 mg l^{-1} NAA and 1.5 g l^{-1} activated charcoal (AC). Furthermore, the somatic embryos required about 18 weeks to develop (Ibraheem et al., 2010).

Liquid media have been used as an efficient method for mass propagation facilitating automation and a reduction in cost and time (Aitken-Christie, 1991; Etienne and Berthouly, 2002). Commonly, using liquid culture for plant propagation has been mainly reported either as cell

suspension cultures or in bioreactors (Preil, 2005). The advantages of liquid culture systems are: uniform culture conditions, easy media replacement without changing the container, sterilization with ultra-filtration and easier container cleaning after use. In addition, with liquid culture media, containers of different volumes can be used, whereas agar media necessitate surface culturing of tissues (Berthouly and Etienne, 2005).

Cell suspension culture is applicable for efficient mass micropropagation, and provides a versatile tool for various in vitro studies (Al-Khayri, 2005). Numerous secondary metabolites of medicinal uses were detected in cell suspension cultures of various plant species (Preil, 2005; Wilken et al., 2005). Several reports have been published on the establishment of cell suspensions of African oil palm (*Elaeis guineensis*) (de Touchet et al. 1991; Teixeira et al., 1995; Aberlenc-Bertossi et al., 1999). Under the best conditions, the initial weight of cells increased about 4-fold in 1 month (de Touchet et al., 1991). Similarly, many researchers carried out work on cell cultures of different date palm cultivars including cv. Barhi (Bhaskaran and Smith, 1992; Al-Khayri, 2012), cv. Deglet Nour (Fki et al., 2003), cvs. Bousthami noir and Jihel (Zouine et al., 2005; Zouine and El-Hadrami, 2007) and cv. Khalas (Gadalla, 2007).

Cell suspensions cultures were used either for callus growth (Al-Khayri, 2012) or for somatic embryo formation (Fki et al., 2003; Zouine et al., 2005). The productivity of embryogenic cell suspension cultures increased 20-fold (from 10 to 200 embryos per month per 100 mg fresh weightof embryogenic callus) when embryogenic suspension were used instead of agar-solidified media (Fki et al., 2003). Zouine et al. (2005) reported that cell suspension resulted in higher somatic embryo number compared to solid medium; their induction period was reduced by 2 months. Normally the plant growth regulators are reduced or abandoned in the cell suspension cultures to induce morphogenesis leading to plant regeneration. Daguin and Letouzé (1988) reported cell suspension growing on half strength MS medium devoid of plant growth regulators. Yadav et al. (2001) initiated cell suspensions by transferring embryogenic callus to MS liquid medium containing 0.1 mg l^{-1} NAA. Fki et al. (2003) used ½ MS supplemented with 1 mg l^{-1} 2,4-D. Gadallah (2007) reported using ½ MS amended with 0.5 mg l^{-1} 2,4-D. Zouine and El Hadrami (2007) tried ½ MS containing 0.1 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BAP. Understanding the behavior of date palm (*Phoenix dactylifera*) cell suspension growth and

differentiation would promote effective utilization for mass micropropagation and various in vitro investigations (Al-Khayri, 2012). In this context, Al-Khayri (2012) recently reported investigating the growth curves of cell suspension and their importance for evaluating its efficiency.

Currently, bioreactors play a crucial role in scaling up the production of somatic embryogenesis and multiplication of clusters of meristem- and bud-based plant micropropagation (Tahardi et al., 2003; Ducos et al., 2007). The temporary immersion system has positive effects on micropropagation as indicated for shoot proliferation in potato (Abdullateef et al., 2009), microtuberization (Jimenez et al., 1999) and somatic embryogenesis for coffee (Ducos et al., 2007). Furthermore, the performance of micropropagated plantlets in temporary immersion system was better during acclimatization over those cultured in solid medium (Berthouly and Etienne, 2005). Immersion time, i.e. duration or frequency, is the most decisive parameter for system efficiency (Etienne and Berthouly, 2002). To meet the increasing demand for date palms, Okere et al. (2010) recommended complementing the tissue culture techniques with temporary immersion bioreactor systems (TIBs) to enhance the commercial production of date palm plantlets. Tisserat and Vandercook (1985) developed an automated plant culture system and investigated it on some plant species and explants and they included date palm callus in their study. Their results showed better callus growth on this system compared to agar medium. The application of temporary immersion systems in date palm tissue culture was applied by Othmani et al. (2009) using the RITA® system (Vitropic-Cirad, France) for either somatic embryogenesis or for shoot proliferation of cv. Deglet Bye. They found that this system was suboptimal for callogenesis and somatic embryo formation; however, better yield of regenerated shoots from the shoot clusters was found with this liquid system compared to the agar-solidified cultures.

To the best of our knowledge there is no published report on the comparison between different liquid culture systems and its effect on date palm callus cultures. Furthermore, there is no specific literature on using liquid cultures for somatic embryo formation of cv. Zaghlool. The aim of this research was to investigate the effect of different liquid culture on the callus growth of date palm cv. Zaghlool and on the number of the induced somatic embryos.

Material and Methods

Plant material and explant preparation

Shoot tips of date palm cv. Zaghlool were separated from healthy offshoots (3-4 years old) of 5-7 kg in weight and about 50-70 cm in height, grown in the Central Laboratory of Development of Date Palm Research at Giza, Egypt.

Outer leaves were acropetally removed, exposing the hearts of the offshoots (15-20 cm length, 6-8 cm width) which were transported to Germany. To prevent browning, the hearts were immersed in a chilled antioxidant solution consisting of 100 mg l^{-1} ascorbic acid and 150 mg l^{-1} citric acid until the time of culture. The outer leaves of the offshoot hearts were removed under aseptic conditions exposing the shoot tip region (3-4 cm length, 1-1.5 cm width) with 3-4 primary leaves.

The shoot tips were disinfected by immersion in 0.3% $HgCl_2$ with 3 drops of Tween 20 for 5 min under agitation and then washed three times in sterile distilled water before dividing them to small squares (0.5-1 cm^2) which formed our initial explants.

Callus induction

The explants were cultured on a medium consisting of MS salts (Murashige and Skoog, 1962) supplemented with (per liter) 170 mg NaH_2PO_4, 100 mg myo-inositol, 200 mg glutamine, 2.5 mg thiamine-HCl, 0.2 mg biotin, 0.2 mg pyridoxine-HCl, 30 g sucrose and 7 g agar (Serva, Kobe I). The medium was enriched with 50 mg l^{-1} picloram, 3 mg l^{-1} N-(3-methyl-2-butenyl)-1Hpurin-6-amine (2iP) and 1.5 g l^{-1} activated charcoal (AC) during the first 8 months of culture, then the explants were transferred to a medium supplemented with 10 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D), 3 mg l^{-1} 2iP and 1.5 g l^{-1} AC for additional 2-3 months (Ibraheem et al., 2010). The pH was adjusted to 5.7 and distributed into 100-ml Magenta vessels (Sigma-Aldrich) containing 35 ml of the medium/vessel, caped with Magenta B-cap and autoclaved at 121°C for 15 min. Culture incubation conditions consisted of complete darkness and 24 ± 2°C. Resultant callus from the explants served as a source of callus for the following two experiments.

Experiment 1: The effect of culture system and medium composition on callus growth

Solid cultures

Portions of embryogenic friable callus (200 mg) of the same quality were chopped and cultured in 500-ml Phytacon tissue culture vessels (Figure 1a) (Sigma) containing 200 ml solid MS medium as described below.

Cell suspension establishment

Portions of embryogenic friable callus (200 mg) of the same quality were chopped and cultured in 200 ml liquid MS media dispensed in either 500 ml Phytacon^{TM} tissue culture vessels (Sigma) or 500 ml Rasotherm flasks (Figure 1b). The suspension cultures were incubated on a rotary shaker set at 100 rotations per minute (rpm) using electric shaker (Certomat® R).

Temporary immersion system

A temporary immersion system (TIS) was conducted using RITA® (Vitropic-Cirad, France) bioreactor (Figure 1c). The lower compartment of the RITA® bioreactors was filled with 200 ml liquid medium. The calli were cultured in the upper compartment at 200 mg in each bioreactor. The immersion frequency was 5 min every 12 h controlled by a digital programming timer (REV Ritter GmbH, Germany). The calli in RITA® bioreactors remained in their culture vessels and a new liquid medium was supplied aseptically after removing the old one at 5-6 week-intervals. The fresh mass of callus was evaluated at every subculture.
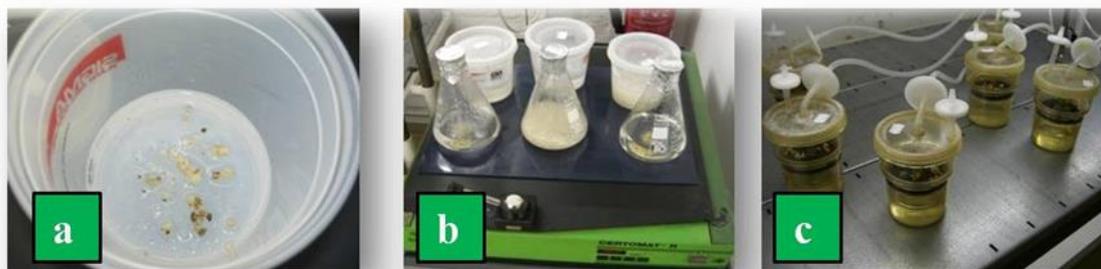

Figure 1. Date palm callus cv. Zaghlool cultured in: a) Solid medium in Phytacon vessels, b) Cell suspension culture in Rasotherm and Phytacon vessels, c) RITA bioreactor.

Culture medium

Three medium compositions were chosen according to previously reported protocols for other date palm cultivars: ½ MS with 0.5 mg l^{-1} 2,4-D (Gadallah, 2007), ½ MS with 1 mg l^{-1} 2,4-D (Fki et al., 2003) and MS with 10 mg l^{-1} Naphthaleneacetic acid (NAA)+1.5 mg l^{-1} 2iP (Al-Khayri, 2012). The pH was adjusted to 5.7 before the addition of 30 g l^{-1} sucrose and further 7 g l^{-1} agar to the solid culture medium (Serva Kobe I). The media were autoclaved at 121°C for 15 min. The cultures were incubated under 16-h photoperiods of cool-white florescent light (35 µmol m^{-2} s^{-1}) at 24 ± 2°C.

Cell growth curve

The fresh mass of the cell suspension cultures was determined from the cell mass collected on 800-µm stainless steel sieve at each transfer. Cells were then transferred to a sterile petri dish and weighed aseptically, following the procedures used by Teixeira et al. (1995). For the calli grown in RITA bioreactors and on solid media, the cells were weighed aseptically at every subculture before transferring to the fresh medium. The growth curve was obtained using Excel for Windows 2007 depending on the fresh mass of the cells (g) evaluated at every transfer.

Starch identification

A few samples were taken from each treatment of the cell suspensions and treated with a tincture of iodine/ potassium iodide to identify starch. The samples were examined under a microscope (Axiovert 100 Carl Zeiss. Germany) and photographed by digital Camera (Olympus c3 040-ADU. Japan).

Somatic embryo induction

At the end of the 16th week the calli were transferred to a somatic embryo induction medium with 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC for 18 weeks. The calli were subcultured every 6 weeks and the number of formed somatic embryos was determined after 18 weeks on this medium.

Experimental design and statistical analysis

The experiment was set up as a 4x3 factorial in Completely Randomized Design (CRD) comprising two main factors: culture system as 4 levels and medium composition as 3 levels. Each treatment consisted of 2 replications and the experiment was repeated once. The data present an average of the repetitions. Data were subjected to analysis of variance (ANOVA) and the means were separated using Tukey test.

Experiment 2: The effect of culture system and medium composition on somatic embryo formation

Callus source

Callus was induced from either shoot tips or from leaf segments excised from converted somatic embryos. Embryogenic friable callus induced from shoot tips of cv. Zaghlool as described above.

The somatic embryos were induced from shoot tip-callus on MS medium with 0.1 mg l^{-1} NAA (Ibraheem et al., 2010) and converted on MS medium with 1 mg l^{-1} NAA under darkness. The white leaflet segments were cut out from the basal part of the first leaf appeared from these converted embryos. Embryogenic callus was induced from these leaf segments using MS medium enriched with 10 mg l^{-1} picloram and 1.5 g l^{-1} AC for 2 months with 1 month subculture.

Cell suspension establishment

For both callus sources, portions of embryogenic friable callus (500 mg) were finely chopped and cultured in 50 ml liquid MS media dispensed in 125 ml Erlenmeyer flasks. The suspension cultures were incubated on a rotary shaker set at 100 rpm using an electric shaker (Certomat® R). The cultures were transferred to new medium once after 3 weeks of initial culture.

Culture medium

The media supplemented with 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC with or without 0.05 mg l^{-1} BAP compared to PGR-free medium with 1.5 g l^{-1} AC as control. Sucrose was added at 30 g l^{-1} and agar for the solid media at 7 g l^{-1}.

Experimental design and statistical analysis

The experiment was set up as a 2x3 factorial in Completely Randomized Design (CRD) comprising two main factors: culture system as 2 levels and medium composition as 3 levels. Each treatment had 5 replicates and the experiment was repeated twice. The fresh mass and the number of somatic embryos were evaluated after 6 weeks of initiation of culture. The data present an average of the repetitions. Data were subjected to analysis of variance (ANOVA) and the means were separated using Tukey test by using the program SPSS 10 for Windows.

Results and Discussion

Experiment 1: The effect of culture system and medium composition on callus growth

The embryogenic friable callus continued to grow on all of the studied media and culture systems (Table 1). Data demonstrated differences in

callus fresh mass depending on the treatments. The callus reached between 4-fold and up to 20-fold mass after 16 weeks. Regardless of medium composition, the fresh mass of callus increased significantly in cell suspensions grown in Rasotherm flasks or Phytacon vessels. However, the calli grown in Rasotherm flasks showed a slight increase over those grown in Phytacon. The calli subjected to temporary immersion system showed less growth, but the lowest increase in mass was recorded on solid medium, however the difference was not significant compared to RITA (Table 1, Figure 2a, 2c). Regarding the difference between RITA® and suspension cultures, the fresh mass of calli grown in Rasotherm flasks was significantly higher than those of RITA® (Figure 3a, b). However, no significant difference was noted between the calli grown in Phytacon vessels and RITA® system (Table 1, Figure 2a, 2d).

Concerning the effect of the medium PGR contents, adding 0.5 mg l-1 2,4-D was slightly superior to 1 mg l-1 2,4-D, but without significant difference in all culture systems (Table 1). Both concentrations of 2,4-D showed higher callus mass than 10 mg l-1 NAA combined with 1.5 mg l-1 2iP. This increase was significant for two of the tested culture systems (solid and liquid in Phytacon vessels), but not significant for the RITA® and liquid culture in Rasotherm flasks (Table 1).

Similar to the current observation the positive influence of liquid medium on fresh mass development was reported for African oil palm (Teixeira et al., 1995; Kanchanapoom and Chourykaew, 1998) and date palm (Tisserat and Vandercook, 1985; Al-Khayri, 2012). A higher mass of African oil palm callus as compared to solidified medium was also reported using TIS (Sumaryono et al., 2008). The use of Phytacon vessels for cell suspension, to our knowledge, has not been reported for date palm cell suspensions. The use of Phytacon vessels were reported to maintain lower air exchange, and thus a higher CO_2 concentration inside these vessels as compared to other tissue culture vessels (Tisserat et al., 1997) and offer reduced cost and labor (Muralidharan, 1998). The lower callus mass in the Phytacon

vessels compared to Rasotherm flasks may be due to better fluidity efficiency which may lead to better gaseous exchange of the Rasotherm flasks, nonetheless the difference was not significant.

The biomass was higher in suspension cultures than temporary immersion system (Table 1). This superior effect of suspension cultures was reported recently for African oil palm cultures (Sumaryono et al., 2008) who noted that the callus mass of African oil palm was lower in RITA® bioreactor than suspension cultures. It is worth mentioning that they immersed their calli for 3 min every 6 h. In contrast to our results, temporary immersion culture systems have proved to be more successful in achieving embryogenic tissue proliferation than conventional systems using an agar medium or suspension cultures for *Coffea arabica* (Berthouly et al., 1995; Berthouly and Etienne, 2005). The lower callus mass in a RITA® bioreactor (Table 1) might be due to a suboptimal immersion duration and/or frequency (5 min every 12 h). To our knowledge there is no optimal protocol for using RITA® system for date palm tissue cultures. Tisserat and Vandercook (1985) reported developing an automated plant culture system (APCS) for date palm callus with 5-10 min immersion every 2 h. They found superior growth of callus which reached up to a 4-fold increase as compared to those grown on agar medium. In our experiment callus increased only 1.5-2 fold compared to agar-medium (Table 1). The immersion system we used involved 5 min every 12 h. This duration was arbitrarily selected based on reports with other perennial crops which varied from 1 to 15 min immersion every 2 to 12 h (Etienne and Berthouly, 2002). Similar to our results, Othmani et al. (2009) recently reported that the embryogenic calli of date palm cv. Deglet Bey turned brown and died using a RITA® bioreactor with immersion frequency of 5 min every 8 h. They found that temporary immersion system was better than the solid medium only for shoot proliferation. The immersion frequency tested so far appeared to be suboptimal for date palm callus growth and could be optimized by testing different durations.

Table 1. The effect of different tissue culture systems and medium compositions on callus fresh mass of date palm cv. Zaghlool after 16 weeks of initial culture starting with 200 mg callus per culture The different letters indicate significant differences according to Tukey (P<0.05).

Callus growing medium	½ MS with 0.5 mg l^{-1} 2,4-D	½ MS with 1 mg l^{-1} 2,4-D	MS with 10 mg l^{-1} NAA, 1.5 mg l^{-1} 2iP
Solid	1.99 cde	1.57 ef	0.76 f
RITA®	2.86 bcd	2.05 cde	1.80 def
Suspension in Rasotherm	4.09 a	3.79 ab	3.02 abcd
Suspension in Phytacon	3.08 abc	3.48 ab	1.76 def

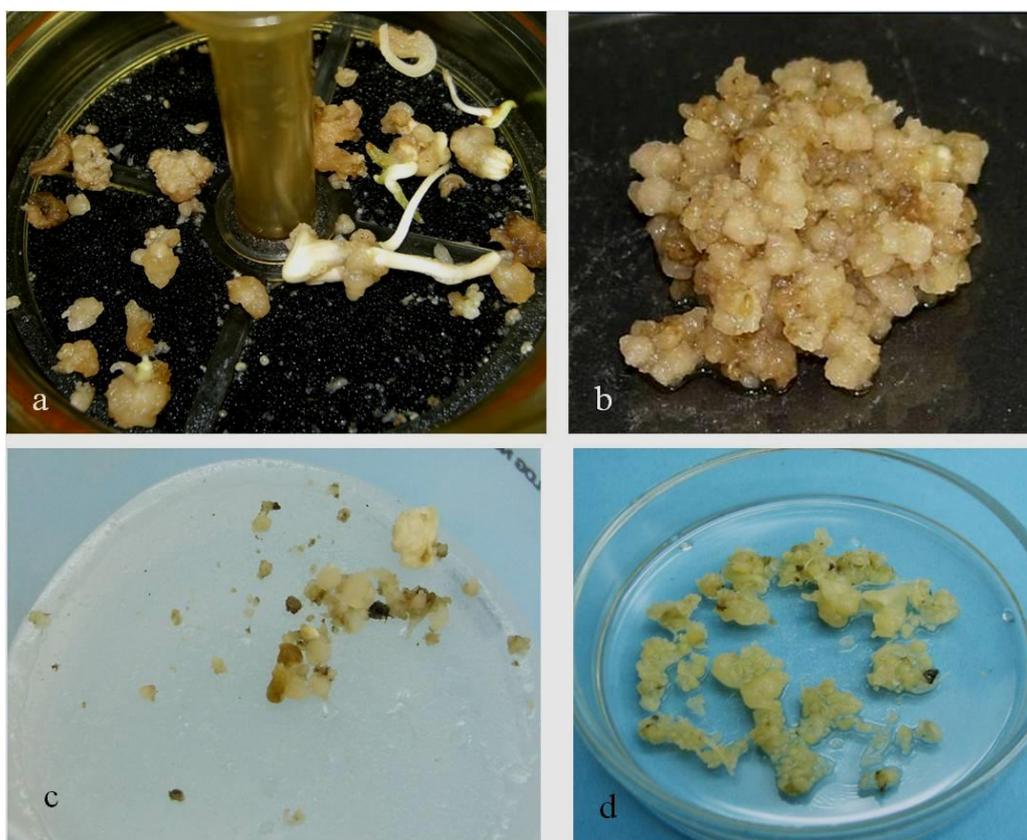Figure 2. Callus of cv. Zaghlool after 16 weeks in ½ MS liquid medium enriched with 0.5 mg l-1 2,4-D a) In a RITA® bioreactor, b) In Rasotherm flasks, c) In Phytacon vessels with solidified medium, d) In Phytacon vessels with liquid medium.

Table 2. The growth of 0.2 g friable calli of date palm cv. Zaghlool in solid and liquid culture systems after 5, 10 and 16 weeks of incubation in ½ MS with 0.5 mg l^{-1} 2,4-D.

Callus growing medium	5 weeks	10 weeks	16 weeks
Solid	0.55	1.08	2.00 cde
RITA®	0.62	1.59	2.86 bcd
Suspension in Rasotherm	0.63	1.71	4.10 a
Suspension in Phytacon	0.69	1.55	3.08 abc

Callus growth curve

The trend of the callus growth curve was the same in all medium compositions tested. For instance the callus growth curve on ½ MS supplemented with 0.5 mg l^{-1} 2,4-D is demonstrated in Table 2. The fresh mass of callus or cell suspension showed no difference between all culture systems at the first subculture. This stage is called the lag phase in which the cells prepare themselves for the division (Al-Khayri, 2012). In the second subculture the growth curve differentiated between the liquid culture systems and the solid medium. However, no difference was found in the biomass at this point between the three studied liquid culture systems. The differencebetween liquid culture systems appeared only at the third subculture (between 10-16 weeks after inoculation). The cell suspension grown in Rasotherm flasks showed the best growth followed by those grown in the Phytacon vessels. Using PhytaconTM tissue culture vessels for liquid cultures was reported for the first time in date palm tissue culture. The calli grown in RITA® bioreactor showed lower mass but it remained higher than those developed on solid medium (Table 2). Generally, the callus fresh mass increased about 9 times in solid medium and between 13-20 times in the liquid cultures, clearly illustrating the advantage of liquid medium for plant micropropagation.

Various methods are employed to measure in vitro cell growth, including cell or colony counting, dry and fresh weight and packed cell volume (Dixon, 1985). The growth curve shape depends upon the measurement method used to evaluate cell growth (Yamamoto and Yamada, 1986). Studies on growth curves of date palm suspension cultures are scant. Recently, Al-Khayri (2012) studied the growth curve of date palm cell suspensions using a packed cell volume method which demonstrated five growth phases: lag, exponential, linear, declaration and stationary. Sumaryono et al. (2008) reported a similar growth curve trend in African oil palm cell suspension using the fresh mass measurement method. Growth curves are essential to assess culture performance and metabolic activities at various growth phases (Al-Khayri, 2012). The method we used showed no stationary and/or declaration phase as we reported for the cell suspension growth curves used the packed cell volume method (Abbade et al., 2010; Al-Khayri, 2012). That may be due to the long intervals between the readings we took (5-6) weeks. It is recommended for cell suspension culture studies that the evaluation of the mass must be evaluated many times during the same subculture to obtain more precise growth curves that may help in defining the best time for subculture to fresh new medium. Otherwise, using other growth curve methods such as packed cell volume may give a better understanding of the growth nature of the cell suspensions.

Somatic embryo formation

The calli grown on the aforementioned culture systems and media were cultured on somatic formation solid medium amended with 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC (Ibraheem et al., 2010). Somatic embryo number was evaluated after 18 weeks (Table 3). The cells obtained from Rasotherm flasks and Phytacon vessels containing 10 mg l^{-1} NAA and 1.5 mg l^{-1} 2iP produced the highest number of somatic embryos (Table 3, Figure 3a,b). The somatic embryo number was rather low from the calli grown in all culture types enriched with 0.5 and 1 mg l^{-1} 2,4-D and also from the calli grown on solid and in a RITA® bioreactor on medium enriched with 10 mg l^{-1} NAA and 1.5 mg l^{-1} 2iP (Table 3).

Table 3. The effect of callus growing medium on the number of induced somatic embryos/callus cv. Zaghlool after 18 weeks of transfer to MS medium supplemented with 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC

Callus growing medium	½ MS with 0.5 mg l^{-1} 2,4-D	½ MS with 1 mg l^{-1} 2,4-D	MS with 10 mg l^{-1} NAA, 1.5 mg l^{-1} 2iP
Solid	3.00 c	3.00 c	3.00 c
RITA®	5.00 c	3.00 c	4.00 c
Suspension in Rasotherm	2.00 c	2.00 c	35.00 a
Suspension in Phytacon	3.00 c	2.00 c	22.00 b

The different letters indicate significant differences according to Tukey (P<0.05).

Figure 3. a) Embryogenic nodular callus cv. Zaghlool grown in liquid MS with 10 mg l⁻¹ NAA, 1.5 mg l⁻¹ 2iP after transferring to somatic embryo formation medium with 0.1 mg l⁻¹ NAA and 1.5 g l⁻¹ AC for 9 weeks, b) Somatic embryo formation from callus grown in liquid MS with 10 mg l⁻¹ NAA, 1.5 mg l⁻¹ 2iP after transfer to MS with 0.1 mg l⁻¹ NAA and 1.5 g l⁻¹ AC for 18 weeks.

The induced number of somatic embryos was very low compared to our observations on callus of cv. Zaghlool by using solid medium. From about 3 g callus grown in liquid MS with 10 mg l⁻¹ NAA and 1.5 mg l⁻¹ 2iP only 35 somatic embryos were formed (Table 3) while we could obtain in previous findings 75 somatic embryos from only 1 g callus in solid MS enriched with 0.1 mg l⁻¹ NAA and 1.5 g l⁻¹ AC (Ibraheem et al., 2010). This difference in somatic embryo number may be due to lack of desiccation treatment of the calli grown in liquid media. Othmani et al. (2011) reported that a desiccation procedure before transferring the calli to agar medium enhanced the number of induced somatic embryos. Othmani et al. (2011) suggested that a complex interaction exists between the water content of embryogeniccalli and development of somatic embryos. They found that a desiccation treatment of 12 hours induced significantly more somatic embryos than 6, 24 or 48 hoursdesiccation. For Indica rice, Rance et al. (1994) assumed that the desiccation treatment might trigger rapid biochemical changes in the calli and under water stress specific enzymes or polypeptides probably appear in callus culture.

Starch content

To understand the difference in resulting somatic embryos between the callus grown in MS medium contained 10 mg l⁻¹ and 1.5 mg l⁻¹ 2iP and those grown in ½ MS enriched with 2,4-D at 0.5 and 1 mg l⁻¹ (Table 3) samples of the cell suspensions were studied under the microscope (Figure 4). The histological observation for the cell suspensions indicated that the cell suspension contained densely cytoplasmic cells (Figure 4b, d). These cells were either individual or grouped in cell aggregates. Furthermore, they varied in shape from spherical to oblong. The diameter of these cells was between 40-60 μm and the length varied between 100-250 μm. The main difference between the cells grown in 10 mg l⁻¹ NAA medium (Figure 4c, d) and those grown in 2,4-D media (Figure 4a, b) was the appearance of starch grains (S). These starch grains appeared clearly in the cells grown in MS medium enriched with10 mg l⁻¹ NAA (Figure 4c, d) while no or very few starch grains existed in the cells grown in ½ MS enriched with 0.5-1 mg l⁻¹ 2,4-D enriched media (Figure 3a,b). This difference possibly explains the variation noted in the number of induced somatic embryos from these media after transferring to somatic embryo formation medium (Table 3). In further experiments it should be clarified whether the auxin type or the macronutrient content was responsible for starch accumulation and the somatic embryo formation. Our results support those published by Sharma et al. (1986) who reported that the samples of cell suspensions of date palm contained a mixed population of actively dividing, cytoplasmically rich, globular cells, elongated cells and cell aggregates. In line with our results, Sané et al. (2006) observed a significant accumulation of starch and proteins in date palm liquid cultures. In African oil palm cell suspensions, de Touchet et al. (1991) reported that the proliferating embryogenic aggregates composed of meristematic cells in active division.

Experiment 2: The effect of culture system and medium composition on somatic embryo formation

Callus induced from shoot tips

The fresh mass was significantly higher on liquid media compared to solid media (Figure 5a); it mass increased in 6 weeks 2-3 fold: 1.5-2 g in solid media versus 4.5-5 g in liquid media. There was no significant difference among the PGR treatments for each culture system individually. However, a slight increase in the fresh mass was found on the medium containing only 0.1 mg l^{-1} NAA.

The somatic embryos appeared at the 3rd week after transferring the calli to the liquid medium and the number increased obviously in the next 3 weeks. The number of somatic embryos varied according to the culture system and the medium composition (Figure 5b). The culture system obviously had a crucial effect on the number of somatic embryo formation. For all media composition the number of somatic embryos increased dramatically and significantly on liquid medium (Figures 5b, 6). This number increased 8-16 fold: 5-10 somatic embryos on solid media versus 40-160 somatic embryos in liquid media (Figure 5b). There was also a significant difference among the media compositions used in liquid cultures. The medium enriched with 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC showed the highest number of somatic embryos (Figure 5b). The number of somatic embryos was significantly lower on medium enriched with 0.1 mg l^{-1} NAA, 0.05 BAP and 1.5 g l^{-1} AC. Nevertheless, this medium was significantly superior to the PGR-free liquid medium (Figure 5b).

The number of somatic embryos (Figure 5b) showed either better or worse results in comparison to other date palm genotypes cultivated in liquid cultures. On the same medium, Saker et al. (2007) induced 120 somatic embryos from 500 mg of friable callus of cv. Sewi. This difference may be due to the genotypic effect (Pinker et al., 2009). Gadallah (2007) and Al-Khayri (2012) initiated 69 somatic embryos from 500 mg callus of cvs. Khalas and Barhi, respectively. Badawy et al. (2009) induced 48 embryos from 200 mg callus, equivalent to 120 embryos from 500 mg, on cv. Sakkoty. However better results were reported by Fki et al. (2003) with 200 embryos per 100 mg initial callus weight from cv. Deglet Nour. Furthermore, Othmani et al. (2009) produced a yield of 501 embryos beginning from 500 mg callus on suspension cultures of cv. Deglet Bey.

The medium enriched with only NAA was better than that enriched with both NAA and BAP (Figure 5b). This ensured our previous results for solid cultures where addition of BAP to the somatic embryo formation medium was also unfavorable (Ibraheem et al., 2010). Adding BAP to the cell suspension cultures seemed to be controversial. Using liquid medium with NAA and without cytokinins as we found, was reported to be optimal in some date palm liquid cultures (Tisserat and Vandercook, 1985; Sharma et al., 1986; Saker et al., 2007). In contrast to our results, Gadallah (2007) reported higher fresh mass and somatic embryo number in date palm cv. Khalas suspension cultures after adding BAP at 1 mg l^{-1} to the medium enriched with 2,4-D. Adding BAP to date palm suspension cultures was also reported by Zouine et al. (2005) and Zouine and El Hadrami (2007). For other species, Yamamoto and Yamada (1986) reported that the hormonal combination of NAA and BA was the most suitable for cell suspension culture for snakeroot (Rauwolfia serpentine). Furthermore, Stafford (1996) stated that plant cell cultures are normally established and maintained on media containing an auxin and a cytokinin. Removal of either hormone from the medium would normally result in culture death.
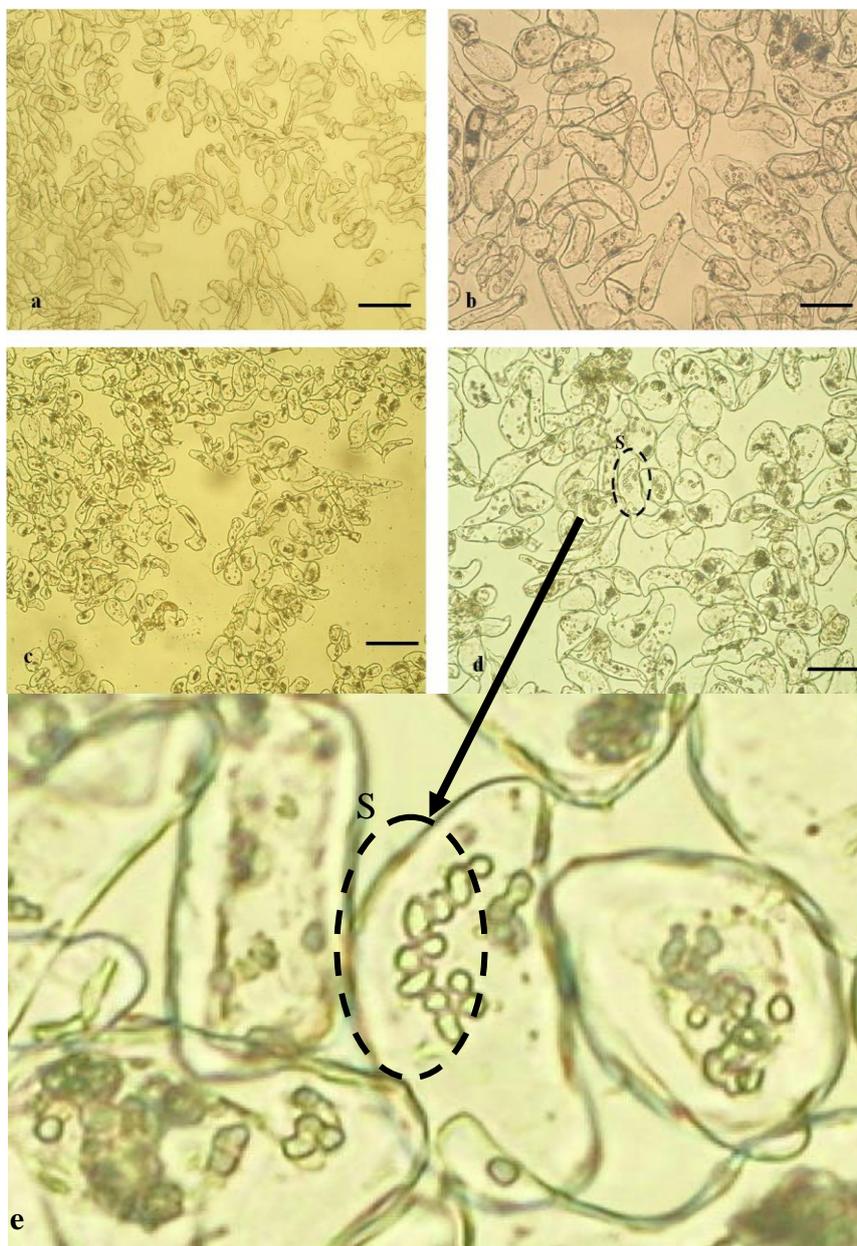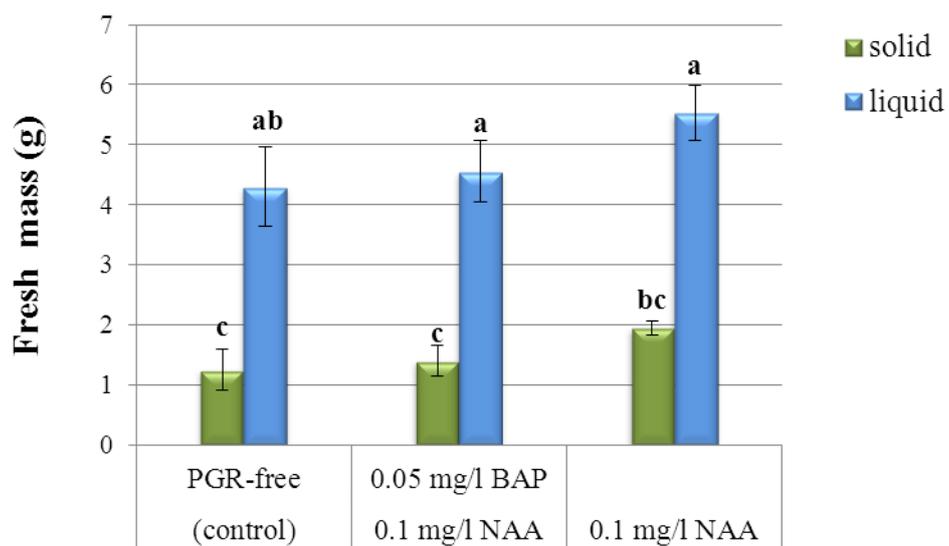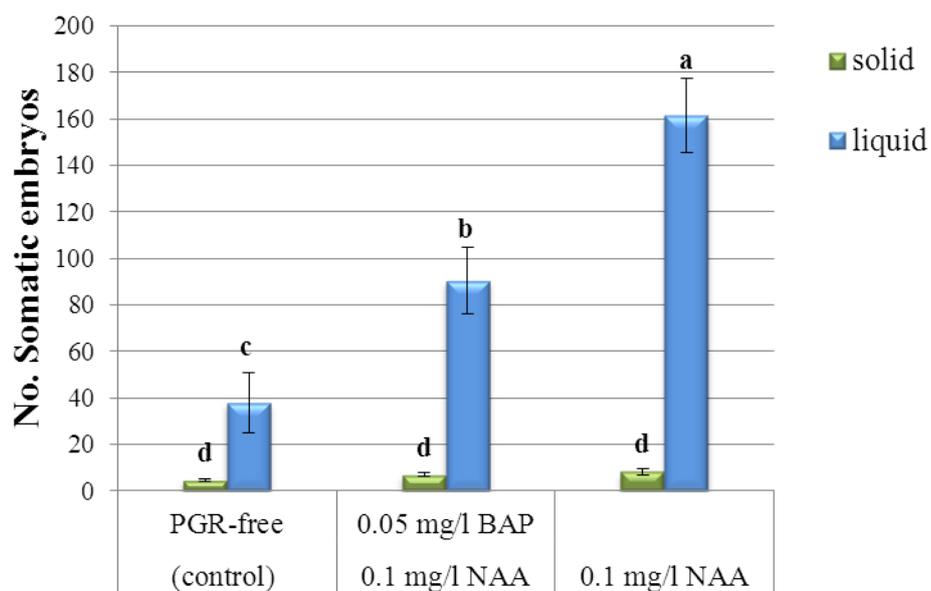
Figure 4. Microscopic view of date palm cv. Zaghlool cell suspension culture 16 weeks after transferring of friable embryogenic callus to a, b) Rasotherm flasks filled with liquid MS containing 1 mg l^{-1} 2,4-D under 4x,10x magnification, respectively, c, d) Rasotherm flasks filled with liquid MS containing 10 mg l^{-1} and 1.5 mg l^{-1} 2iP under 4x,10x magnification, respectively, E) Starch grains under 20x magnification, S= starch grains, for a, c: bar = 200 µM and for b, d: bar = 80 µM.

a.

b.

Figure 5. The effect of culture system and medium composition on a) The fresh mass resulted from 500 mg callus cv. Zaghlool after 6 weeks of transfer to solid or liquid medium with different medium compositions, b) The number of somatic embryos resulted from 500 mg callus after 6 weeks of transfer to solid or liquid medium with different medium compositions Means followed by different letters are significantly different using Tukey test at p=0.05.

Figure 6. Somatic embryo formation of cv. Zaghlool from 500 mg initial callus weight after 6 weeks of culture in: a) Solid PGR-free MS with 1.5 gl^{-1} AC, b) Cell suspension in Erlenmeyer flasks filled with liquid MS medium containing 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC, c) Cell suspension in Erlenmeyer flasks filled with PGR-free liquid MS medium and 1.5 g l^{-1}AC, d) Cell suspension in Erlenmeyer flasks filled with liquid MS medium containing 0.1 mg l^{-1} NAA, 0.05 mg l^{-1} BAP and 1.5 g l^{-1} AC

The large increase in the number of somatic embryos in liquid media may be due to the ease of available nutrients to the cells or perhaps due to the large surface area of the cells directly exposed to the nutrient medium (Duval et al., 1995). High numbers of somatic embryos can be produced in suspension cultures, which makes this technique ideal for large-scale micropropagation of healthy plant material. The liquid medium allows close contact of the tissue with the medium stimulating and facilitating the uptake of nutrients and phytohormones, leading to better growth (Mehrotra et al., 2007).

Liquid medium not only increased the number of resultant somatic embryos but also accelerated the formation of somatic embryos. The somatic embryos were formed within 3-6 weeks after inoculation in the liquid media (Figures 5b, 7b) while they were formed in 12-18 weeks on solid medium (Ibraheem et al., 2010). Similar to our results Zouine et al. (2005) reported that the initiation of somatic embryos in suspension culture within 2 months, while on solid medium somatic embryos were formed after 4 months. Recently, Al-Khayri (2012) found that cell suspensions accelerated significantly the appearance of somatic embryos in date palm cv. Barhi., Mehrotra et al. (2007) reported that the growth and multiplication rate is enhanced by forced aeration, since continuous shaking of the medium provides sufficient oxygen supply to the tissue, which ultimately leads to their faster growth. Furthermore, in addition to these advantages, the preparation of

liquid medium and handling of shake cultures is comparatively easier to the solid medium.

Callus induced from leaflet segments

To ensure the previous results and to examine any effect of the source of callus another comparative study between liquid and solid media was carried out. The friable embryogenic callus induced on leaf segments of converted somatic embryos was the starting material. According to the above results on shoot tip-induced callus, the treatment of BAP was eliminated.

The fresh mass increased significantly in the liquid medium supplemented with 0.1 mg l^{-1} NAA and 1.5 g l^{-1}AC compared to the solid medium (Figure 7a). It increased also in the liquid PGR-free medium but without significant effect compared to the PGR-free solid medium. Also for solid medium

adding 0.1 mg l^{-1} NAA enhanced the fresh mass but without significant effect compared to the PGR-free medium (Figure 7a).

Somatic embryos appeared in the 3rd week after transfer to the induction medium and increased in the second subculture (subculture=3 weeks). The number of somatic embryos varied according to the culture system and the medium composition (Figure 7b). The number of induced somatic embryos was enhanced significantly on liquid media (Figure 7b). The liquid medium enriched with 0.1 mg l^{-1} NAA and AC was the best treatment with about 65 embryos per callus. Also the PGR-free liquid medium enhanced the number of embryos significantly, compared to the solid media and the number of embryos was about 35 embryos per callus (Figure 7b).
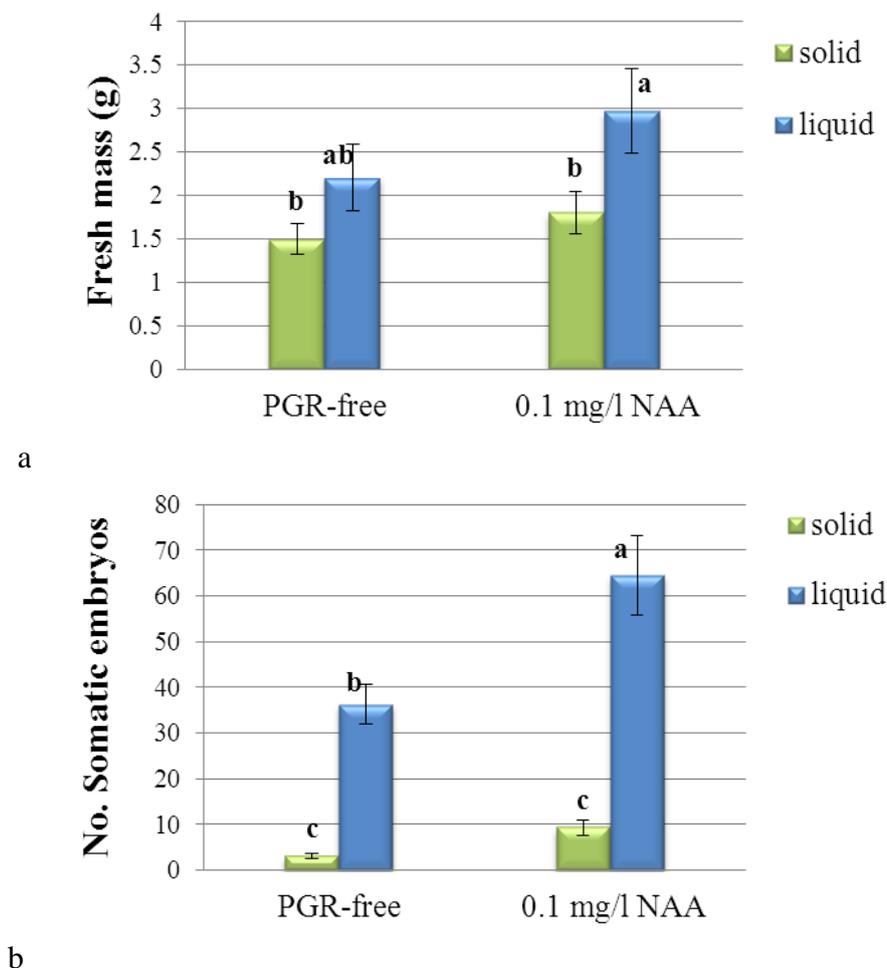

a

b

Figure 7. The effect of culture system and medium composition on somatic embryogenesis induced from callus of leaflet segments cv. Zaghlool: a) The fresh mass resulted from 500 mg callus after 6 weeks of transferring the calli to solid or liquid medium with 1.5 g l^{-1} AC with or without 0.1 mg l^{-1} NAA, b) The number of somatic embryos resulted from 500 mg callus after 6 weeks of transferring to solid or liquid medium with 1.5 g l^{-1} AC with or without 0.1 mg l^{-1} NAA Means followed by different letters are significantly different using Tukey test at p=0.05.

Generally, the number of somatic embryos was 7-12 times more in cell suspension cultures compared to solid cultures (Figure 7b). There are no published reports about using the callus induced from date palm converted somatic embryos as a source for cell suspension cultures. This method might be promising for commercial and scientific purposes because it enables the researchers to obtain callus and somatic embryos in a shorter time than the common pathways.

These results confirmed our previous results on the calli of shoot tip origin. The medium we developed previously for somatic embryo formation on solid media (Ibraheem et al., 2010) stimulated also the number of somatic embryos in liquid culture (Figure 5b, Figure 7b). However the number of somatic embryos formed from the leaflet segment calli was less than those of shoot tips calli. The number of induced embryos from 500 mg callus was about 160 from shoot tip calli and 65 from leaf segment calli. Nevertheless, this technique seems to be promising to have callus in a short time and to increase the number of produced somatic embryos by using liquid medium. The number of somatic embryos increased about 6-16 fold in liquid media compared to those developed in solid media as shown in Figure 5 and Figure 7.

Conclusions

Liquid culture system is a promising technique for rapid mass propagation of date palm. Somatic embryo production in liquid media is about ten times greater than that on solid media. The suspension cultures were superior for date palm callus growth than in RITA$^{®}$ bioreactors. Furthermore, the suspension cultures are technically easier and more economical than the bioreactors. Therefore we suggest the use of cell suspensions to produce high number of date palm somatic embryos and recommend it for a large scale production of date palm plantlets and should be studied for other cultivars. Adding low concentration of auxin 0.1 mg l^{-1}NAA and 1.5 g l^{-1} AC enhanced the number of induced somatic embryos in the liquid cultures of date palm.

Acknowledgment

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