Promising protocol for *in vitro* direct organogenesis of date palm cv. Ekhlass

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ABSTRACT

Over the last forty years, serious attempts and efforts were made to develop direct organogenesis method through axillary buds growth and development pathway. Direct organogenesis has been considered the protocol for distinguishing high genetic stability and true-to-type date palm vitroplants. To achieve this goal, specific poking and several incisions that were made mechanically at specific time on the shoot tips apices explant of date palm (*Phoenix dactylifera* L.) cv. Ekhlass eliminated apical dominance and promoted axillary buds growth.

The shoot tips were cultured initially for 2 or 4 weeks on a medium-free hormone. While, axillary buds proliferated on the explant re-cultured monthly, for three month, on modified MS (Murashige and Skoog 1962) medium (MMS) supplemented with BA (1.0 mg/L), 2iP (1.0 mg/L), Kin (1.0 mg/L), NOA (0.5 mg/L), solidified with gelrite (2.0 g/L), and incubated at 25±1°C under dark conditions. Propagules cultured on the previously described medium, omitting BA plus NOA and supplementing with 2iP, Kin and IAA at (0.2 mg/L), putrescine (50 mg/L) and adenine hemi-sulphate (160 mg/L) recorded significantly the highest axillary buds multiplication rate, and growth value, after incubation under 16-h photoperiod using fluorescent tubes with a light intensity of 1500 Lux for 8 weeks. Separated shoots, cultured on basal MS medium in addition to NAA (O.2 mg/L), spermidine (100 mg/L), sucrose (30 g/L) and solidified with phyto- agar (6.0 g/L), recorded the highest significant roots number, and roots length after 4 weeks of incubation. Healthy rooted plantlets, hardened by receiving pre-acclimatization treatment through transferring from agar rooting medium to 1/2 MS salts strength liquid medium and raised light intensity to 8000 Lux (natural light) for 4 weeks, had the highest survival percentage values in a mixture containing compost and perlite (1:1, v/v) after 3 months in acclimatization.

Keywords: *Phoenix dactylifera* L., tissue culture, micropropagation, axillary buds,polyamine (PAs).

INTRODUCTION

Expansion of date palm agriculture is faced with challenges stemming from propagation and genetic improvement limitations. The heterozygous nature of this dioecious species hampers the use of seeds which produce off type seedlings. On the other hand, the limited availability of offshoots and the difficulties of establishing propogules from offshoots render this traditional propagation method inadequate, particularly for large-scale propagation. Based on recent advances in plant tissue culture, micropropagation technique has been developed for the rapid mass propagation of date palm. Some limitations associated with genetic improvement have been circumvented by taking advantage of tissue culture applications and molecular methodologies. Somaclonal variation occurring is quite common in date palm micropropagated plants produced through indirect embryogenesis pathway, but it can be controlled by *in vitro* direct pathway practices (Jain *et al.,* 2011).

It is essential to provide the candidate famers, not only well adapted cultivars, but also they must be true-to-type tissue culture-derived date palms that do not present any abnormalities. This true-to-typeness is guaranteed when the tissue culture palms have been produced by strict organogenesis (without any callus formation). The most important abnormalities are infertility of the female flowers and dwarfism. For the cv. Barhee, the batches produced by some laboratories were 100% abnormal (Al-Wasel 2001). A mixed embryogenesis/ organogenesis method could also eliminate the risk of abnormalities development (Ferry *et al.,* 2000).

One of the main technological factors limiting the use of this technique is the production of abnormal plants, when plantlets are obtained by somatic embryogenesis. At the moment, the shoots that are supplied with a true-to-typeness guarantee are produced by organogenesis. However, this technique does not offer the same propagation speed. It is a high labor-consuming process and consequently a costly one. Furthermore, very few laboratories control organogenesis at an industrial scale. This slow development associated with a participative-approach strategy is usually better adapted to the small farmer capacity and, quite preferable to the uncontrolled, unreasonable and unsustainable date palm plantations growth that has been adopted in some countries over the last 30 years (Ferry, 2011).

Although research in date palm biotechnology is relatively limited, specially through direct pathways. It is evident that direct organogenesis is a promising technique and will reflect significant influence in date palm true- to- type plant production (Ibrahim and Hegazy, 1999 and 2001).

The aim of this work was to study the availability of micropropagate the high quality date palm cv. ‘’Ekhlass’’ through a complete promising protocol for axillary buds proliferation pathway.

MATERIALS AND METHODS

This work was carried out in the Plant Tissue Culture Dept. of the Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City Sadat City, during the period 2011- 2013.

Explant materials used in this study were obtained from 4 years- old offshoots of female date palm cv. “ Ekhlass” have high quality fruits grown at, Mr. Nabil Abdrabboh model farm, Al-Katta Desert, Gizza governorate, Egypt. By using a hatchet and a sharp knife, the leaves and fiber sheath were removed acropetally. Ethyl alcohol (70%) was sprayed over cutter and plant material during dissection process. When the final size were about 5 cm in width, and 10.0 cm in length, the selected plant materials were excised and inverted upside down and sprayed with alcohol. Then, soaked in sterilized antioxidant solution (150 mg/L each of citric acid and ascorbic acid). Then kept in the refrigerator at (5.0 C°) until the surface sterilization procedure is performed. Surface sterilization were implemented twice by soaked in commercial disinfectant Clorox (5.25 % NaOCl) solution 2% for 30 min, 2 drops/ 100 ml solution of Tween 20 (polyoxyethylenesorbitan monolaurate) as wetting agent were used. Then rinsed with sterile distilled water. Another post- treatment used was 0.1 % Mercuric chloride solution containing two drops of tween -20 per 100 ml for 10 minutes. Explants were then rinsed three times with sterile distilled water and finally soaked in sterilized anti-oxidant solution till final dissecting. Additional leaves were removed when the cluster of very little leaves of the apex is reached and obtaining the shoot tip with a base of meristele tissue, then cultured shoot tip with a good contact on the surface of the medium.

Nutrient Media

Shoot tips explant isolated from offshoots were cultured on modified MS basal medium (Murashige and Skooge, 1962) supplemented with [asparagen (100 mg/L), glutamine (200 mg/L), bioten (0.5 mg/L), adenine himi-sulphate (80 mg/L), thiamine-HCl (10 mg/L), Ca-pantothenate (10 mg/L), a ascorbic acid (75 mg/L), citric acid (75 mg/L), NaH2PO4. 2H2O (170 mg/L), activated charcoal (1.5 g/L) and raised sucrose up to (40 g/L)] was used. The pH of the solid media was adjusted to 5.6 and 5.2 for liquid medium with 0.1 M KOH or 0.1 M HCl prior to gelling agent addition. Media were dispensed either in jars (150 and 350 ml) in aliquots of 30 and 50 ml/jar respectively, covered with polypropylene closure. or into a glass tubes (25 x 2.5 cm; Borosil) capped with Bellco plastic caps containing 15 ml and autoclaved at 121°C and 1.2 Kg/cm^2 for 20 min.

1- Effect of right timing for induce shoot tip poking and incisions.

Shoot tips explant were cultured individually on modified MS medium (MMS) free-hormone solidified with gelrite (2 g/L). Poking and several incisions were done mechanically to shoot tips; directly with culture or after 2 or 4 weeks from culturing. Nine jars 150 ml (replicates) were used for each treatment. Cultures were incubated in total darkness at 25±1°C. Data of fresh weight (g) and degree of browning were recorded after 6 weeks for all treatments. Crude Protein (%),Total amino acids (%), Total soluble carbohydrates (%), Total soluble phenol (g / g protein) and PAL activity (nkat / g protein) were analyzed and recorded.

2- Effect of some growth regulators apical dominance break down and axillary buds growth:

Shoot tips resulted from the best previous treatment were subjected individually on MMS medium supplemented with cytokinins [BA (6-benzyladenine), Kin (6-Furfurylaminopurine) and 2iP (6-(ŷ,ŷ-dimethylallyl amino purine)]. Auxin [NOA (Naphthoxy acetic acid)] were used (mg/L) ; Control (free hormone), NOA (0.5), {NOA: BA, 0.5:1}, {NOA: Kin, 0.5:1}, {NOA: 2iP, 0.5:1} and {NOA: BA: Kin: 2iP, 0.5:1:1:1} and solidified with gelrite (2 g/L). Nine jars (replicates) were used for etch treatment. Cultures were incubated at the same conditions previously mentioned and recultured 3 times to the same medium 4 weeks intervals. Data of fresh wt. (g) and axillary bud proliferation %, total soluble phenols and phenylalanine ammonialyase (PAL) activity were recorded.

3- Effect of putrescine and adenine hemi-sulphate on axillary buds multiplication rate and growth value.

Grown shoot tips resulted from the best previous treatment were subjected individually on MMS medium omitted BA and NOA supplemented with 2iP, kin and IAA at the concentration (0.2 mg/L), putrescine (50, 100 and 150 mg/L), adenine hemi-sulphate (80 and 160 mg/L). Nine jars (350 ml) were used as replicates for etch treatment. Cultures were incubated under 16-h photoperiod using fluorescent tubes with a light intensity of 1500 Lux for 8 weeks and recultured monthely. Data of axillary bud formation, axillary buds multiplication rate, fresh weight(g) and growth value were recorded.

4- Effects of spermidine concentrations on rooting stage

Individually proliferated shoots were cultured on basal MS medium supplemented with sucrose (30 g/L) in addition to NAA (naphthalene acetic acid) at the concentration of 0.2 mg/L and different levels of spermidine (0, 50, 100 and 150 mg/L) and solidified with phyto- agar (6.0 g/L). Nine glass tubes (replicates) were used for each treatment. Cultures were incubated under the same conditions previously mentioned with raised light intensity up to 3000 lux. After 4 weeks, data of root formation %, no. of roots and root length (cm), total soluble phenols and phenylalanine ammonialyase (PAL) activity were recorded.

5- Acclimatization of plantlets using different growing mixture types:

Plantlets produced from rooting medium after 4 weeks were rinsed under tap water and the roots system immersed in fungicide (Tashgarin) solution (0.5 %, v/v) for 5 min. Then, planted in plastic pots (5 ×18 cm) filled with different growing mixture types as follows: compost and bark chips (1:1, v/v), compost and perlite (1:1, v/v), compost and coconut shell and finally, compost and rice shell (1:1 v/v). The plants were covered with transparent polyethylene sheet and sub-irrigated if needed. The potted plants were incubated for 30 days in phytotron at 25 ± 1ºC, relative humidity (70 %) and 16 h photoperiod with a light intensity of 1500 lux. Acclimatization was achieved through gradually removing the plastic sheet each day till it totally removed after 30 days. Plants were transferred to plastic greenhouse in tunnel under shade condition (black saran 63%) and were left to grow for another two months. Plants were sub-fertigated once a week with commercial fertilizer of NPK (Kristalon, 1.0 g/L) at a ratio of 20: 20: 20. After 3 months saplings survival %, no. of leaves/plant and leaf area (cm2) were recorded.

6- Acclimatization with using specific pre-treatments for hardening

Rooted plantlets produced in 4 weeks in the best previous rooting agar medium contained NAA 0.2 mg/L and spermidine 100 mg/L were subjected to pre-acclimatization process through hardening by reculture in 1/2 MS salt strength liquid medium for 4 weeks and received natural light intensity (8000 Lux) in the greenhouse. Acclimatization was achieved typically as previously mentioned on the best results of the growing mixture (compost and perlite). After 3 months saplings survival %, no. of leaves/plant and leaf area (cm2) were recorded.

Degree of browning: It was determined according to the rate of scalling by Pottino (1981), which included, no browning (1), average browning (2) and high browning (3).

Growth value: Explants growth value was determined according to the equation of Ziv (1992).

Ziv (1992). $GV = \frac{Fw_F - Fw_i}{Fw_i}$ Where's

GV = Growth value. FwF = Final fresh wt. Fwi = Initial fresh wt.

Chemical analysis:

Crude protein (usual micro kjeldahl methods) were determined according to the methods described by (A.O.A.C. 1990). Total amino acids were determined according to

the method of Rosein (1957). Total soluble carbohydrates was determined in the ethanolic extract using the phenol-sulfuric acid method according to Dubois *et al.,* (1956).

The colorimetric method of Folin-Denis as described by Swain and Hillis (1959) was employed for the determination of total soluble phenols in ethanolic extracts of leaf samples. Total tannins were determined calorimetrically as described by (A. O. A. C. 1995). Extraction and assay of phenylalanine ammonialyase (PAL) were done according to Lamb *et al.,* (1979).

Statistical analysis:

Data of all studied experiments were statistically analyzed by one factorial randomized complete design using the SAS (1988) package. The Least Significant Difference among levels of each treatment were compared using L.S.D. test at 5%, according to Steel and Torrie (1980).

RESULTS AND DISCUSSION

Over the last forty years, serious attempts and efforts were made to develop direct organogenesis method through axillary buds growth and development pathway. Direct organogenesis has been considered the protocol for distinguishing high genetic stability and true-to-type date palm vitroplants. As a result of reviewing a large numbers of date palm micropropagation studies, which demonstrated the *in vitro* pathways. It is predicted this report may consider as the first practical complete protocol on direct axillary buds proliferation.

1- Effect of right timing for induce shoot tip poking and incisions.

It could be noticed different behaviors from shoot tips grown after culture depending on timing to induce poking and incisions to the shoot tips, it was very critical for the obtained responses.

Data presented in Table (1) and Fig. (1- a) indicated that, inducing poking and incisions to the shoot tips cultured resulted in different response and the timing was very critical factor for eliminate oxidative browning which play an important role in shoot tips growth and subsequently axillary buds multiplication. Inducing poking to the shoot tips 2 weeks after culture resulted the lowest significant degree of browning and the highest significant value of total Amino Acids (5.92 %), crude Protein (37.31%), total Soluble carbohydrates (19.41 %), total soluble phenol (4.95 mg / g f. wt.) and PAL activity (112.12 nkat /g protein) as compared with the other studied timing 0 or 4 weeks. On contradictory, inducing poking and incisions to the shoot tips after 4 weeks from culturing induced severe oxidative browning and subsequently significant negative response and the lowest significant value of total Amino Acids (2.87 %), crude Protein (17.90 %), total Soluble carbohydrates (13.11 %), total soluble phenol (0.78 mg / g f. wt.) and PAL activity (57.24 nkat /g protein) as compared with the other studied timing 0 or 2 weeks. Results strongly indicated that oxidative browning may the critical point which could affect negatively all the parameters under study. Hegazy, 2003 reported that in date palm tissue culture, injury through separation of explant tissues is accompanied by release of discoloring substances in the medium which may have profound physiological disorders on the cultured tissues and accompanied by an increase in growth value and higher concentration of total soluble phenols. The inhibitory action of phenol may results from its oxidation to quinones by polyphenol oxidase and peroxidase and subsequent binding with proteins, such process may lead to the loss of various enzyme activities (Hu and Wang, 1983). In his regard, Zaid (1984) who found that addition of activated charcoal and PVP in the culture media and subculturing on fresh medium for short periods of incubation prevented explant browning.

2- Effect of some growth regulators on apical dominance break down and axillary buds growth:

Shoot tips received poking and several incisions 2 weeks after culturing obtained positive response Table (2) and Fig (1-b & c) when reculture three times under dark conditions on MMS in addition to NOA (0.5 mg/L) in combination with equal level of BA (1.0 mg/L), kin (1.0 mg/L) and 2iP (1.0 mg/L) were recorded the highest significant values of fresh wt. 17.13 (g) and higher significant percentage values of axillary bud growth (33.33%), this was accompanied by the highest significant values of total soluble phenols (4.77 mg/g f.wt) and phenylalanine ammonialyase (PAL) activity (99.47 nkat /g protein) as compared with the other studied individual growth regulators treatments. Total soluble phenols and PAL activity contribute to the formation of protective materials, i.e. lignin, suberin and flavonoids, which would in somehow affect the speed of plant development. In this concern, Beauchesne *et al.,* (1986) found that, at the bottom of the young leaves some very little axillary buds are often visible. Auxins at low concentration, enhanced date palm bud growth in vitro after four to six months, gave some signs of budding which is indicates giving true- to-type plantlets. Date palm shoot tips cultured on medium containing low auxin concentrations initiated leaves and in some cases roots while, high auxin concentrations resulted in the formation of callus (Tisserat, 1979). culturing explant *in vitro* necessitates a continuous supply of growth regulators to the culture medium i.e. auxins and cytokinins supplied either singly or in combination at diverse ratios, depending on the species and the type of explant (Ziv, 1991). Moreover, Zaid and Tisserat (1983) reported that, addition of growth regulators

to nutrient medium was not necessary to stimulate shoot proliferation, better shoot tip development occurred on 10 and 100 mg/L NAA. On the other hand, Tisserat (1984) reported that addition of cytokinin at any level to date palm tissue culture media did not enhance shoot differentiation.

3- Effect of putrescine and adenine hemi-sulphate on axillary buds multiplication rate and growth value.

Growing axillary buds upon transfer to light conditions for 8 weeks on previous medium omitted BA and NOA while supplemented with IAA, 2iP and Kin at equal concentrations (0.2 mg/L), in addition to putrescine (50 mg/L) and adenine hemi-sulphate (160 mg/L) resulted in significant axillary bud multiplication rate (2.11 buds /shoot tip) and the highest fresh weight (25.2 g) and growth value (727) as compared with the control and the other studied concentrations treatments (Table 3 and Fig. 1- d & e). It could be noticed that adenine sulphate at low level in combinations with all putrescine concentrations had negative response to induce axillary bud multiplication. Clusters of shoots Fig. (1- f) were transferred to the same medium for 4 weeks elongation. Similar results were published by Hegazy (2008) found on date palm floral buds ‘’Selmy’’ that embryos cultured on modified MS medium in addition to putrescine (100 mg/L) obtained significant values of multiplication rate and growth value as well as total soluble protein and PAL activity. Hegazy and Abo shamaa, (2010) on achieved direct date palm embryos cv. Medghool on modified MS medium contained putrescine (100 mg/L) . in this regard, Srivastava (2002) published that, PAs metabolism is affected by auxins, cytokinins, and gibberellins in several plant systems and that PAs are essential for many of the growth responses attributed to these hormones. The specific roles of PAs in these responses are unknown. In contradictory, Handa and Mattoo (2010) on tomato they found that the diamine putrescine generally contrast those with polyamines spermidine and spermine emphasizing that individual biogenic amines should be considered to have defined action in plant biology and that they differentially affect growth and development. In this regard, Al Kaabi *et al.,* 2007 reported that organogenesis and somatic embryogenesis are the two techniques currently used in various laboratories in the world for in vitro mass propagation of date palm. Organogenesis in date palm has a low efficiency due to the low number of explants that respond in vitro, the long time required for the initiation phase, the low multiplication rate and the strong influence of the variety (Beauchesne *et al.,* 1986).

4- Effects of spermidine concentrations on root growth characters and chemical analysis

Resulted data in Table (4) and Fig. (1- g) showed that, shoots cultured on basal MS medium supplemented with NAA (0.2 mg/L) in addition to spermidine (100 mg/ L) were recorded the highest significant values of root formation (100 %), roots no.(2.67) and root length (5.42 cm), total soluble phenols (5.94 mg/g f.wt) and AL) activity (124.66 nkat /g protein) as compared to the other studied concentrations treatments. Srivastava, (2002) reported the genetic analysis further indicated that high and low rooting responses were probably controlled by multiple genes. In addition, Sane *et al.,* (2006) on date palm they reported that, rooting without hormone resulted in the development of fine ramified roots that were unable to survive when planted in a nursery. However, Picoli *et al.* (2001) mentioned that failure of hyperhydric plants to grow when transferred to soil may often be due to malfunctioning of the leaf rather than the poor rootability. It could be noticed that high spermidine concentration had no beneficial effect on root growth characters and chemical analysis. This could be act as a reflect to the balance between its indigenous concentration and the exogenous concentration added . In his concern, Handa and Mattoo (2010) reported that Biogenic amines putrescine, spermidine and spermine are ubiquitous in nature and have interested researchers because they are essential for cell division and viability, and due to a large body of their pharmacological effects on growth and development inmost living cells. In addition, Srivastava (2002) published that, polyamines (PAs) are generally recognized as active regulators of plant growth. They are present in all cells, and their mMolar titer is responsive to physiological effects caused by many agents, such as hormones, light, and stress, but their precise mode of action in plant growth and development is still unclear. However, AL-Mayahi, (2014) Reported on date palm cv. Ashgar that rooting medium consisting of N6 medium supplemented with 0.2 mg L-1 NAA, copper sulphate and cobalt chloride both at 0.5 µM. Resulted in maximum induction of roots . Total phenol content increased at high concentration of Cu and Co.

5- Acclimatization of plantlets using different growing mixture types:

Data presented in Table (5) and Fig. (1-h) showed that, growing mixture containing compost and perlite (1:1, v/v) recorded the highest significant percentage values of plantlets survival (73.33 %) as well as higher leaves no. (4.7) and leaf area (23.8 cm2) as compared with the other studied growing mixture types. However, compost and rice shell (1:1, v/v) were recorded the lowest results in plantlets survival (43.33), leaves no. (3.2) and leaf area (17.4 cm2). In this regard, (Hegazy, 2008) suggested that the superiority of compost and perlite could be ascribed to their effects

on sparring more suitable conditions for the growing roots. Compost might increase the organic matter content, which in turn improved the soil physical condition. Perlite could hold three to four times its weight of water as well as it was most useful in increasing aeration in mixture.

6- Acclimatization with using specific pre-treatments for hardening

Results in Table (6) and Fig. (1-i) indicated that plantlets produced in 4 weeks on solid rooting medium and transfer for another 4 weeks to liquid 1/2 salt strength as well as received natural light intensity 8000 Lux in the greenhouse resulted in significant saplings survival (90 %) as compared with the control.

However, no significance could be obtained for leaves no. (4.9) and leaf area (27.11 cm2). Result obtained positive response to hardening and this may due to the good chance for roots to grow in liquid medium and avoid fine roots to destroyed during transfer in acclimatization if it is on solid medium as well as expose to natural light in the green house for 4 weeks could be help in transfer from heterotrophy to autotrophy. Moreover, reduce the transpiration as results to increase the wax film and improve stomata mechanism.

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Tables

Table1: Effect of right timing to induce shoot tip poking and incisions on fresh wt. (g), degree of oxidative browning as well as crude protein, total amino acids, total hydrolysable carbohydrates, total soluble phenols and phenylalanine ammonialyase (PAL) activity on Date palm cv. Ekhlass grown in vitro.

Treatment		Growth characters			Chemical analysis				
Free hormone Medium (MMS)	No. of weeks	Initial Fresh wt.. (g)	Final fresh Wt. (g)	Degree of browning	Total Amino Acids (%)	Crude Protein (%)	Total Soluble carbohydrates (%)	Total soluble phenol (mg / g f. wt.)	PAL activity (nkat /g protein)
Poking & incisions	0	0.98^{a}	1.83^{b}	2.2^{b}	4.11^{b}	23.21^{b}	16.33^{b}	3.15^{b}	87.02^{b}
	2	0.91^{b}	1.71^{c}	1.0^{c}	5.92^{a}	37.31^{a}	19.41^{a}	4.95^{a}	112.12^{a}
	4	0.93^{ab}	1.98^{a}	2.7^{a}	2.87^{c}	17.90^{c}	13.11^{c}	0.78^{c}	57.24^{c}

Means within each column followed by the same letter are not significantly different at P= 0.05

Table 2: Effect of plant growth regulators on fresh wt. (g) and axillary bud proliferation as well as total soluble phenols and phenylalanine ammonialyase (PAL) activity of date palm shoot tips ‘’ cultured in vitro for 12 weeks.

Treatment (mg/L)	Growth characters			Chemical analysis	
	Initial fresh wt. (g)	Final fresh wt. (g)	Axillary Bud growth %	Total soluble phenol (mg/g f.wt)	PAL activity (nkat /g protein)
MMS Free hormone (control)	0.93^{bc}	4.44^{d}	00.00^{b}	2.96^{d}	81.23^{d}
NOA (0.5) + BA	0.88^{d}	12.28^{b}	00.00^{b}	3.47^{c}	86.00^{c}
+ Kin	0.99^{a}	9.48^{c}	00.00^{b}	3.73^{c}	89.06^{c}
+ 2iP	0.95^{b}	10.52^{c}	00.00^{b}	4.17^{b}	94.15^{b}
+ BA (1) + Kin (1) + 2iP (1) + NOA (0.5)	0.92^{c}	17.13^{a}	33.33^{a}	4.77^{a}	99.47^{a}

Means within each column followed by the same letter are not significantly different at P= 0.05

Table 3: effect of growth regulators in addition to putrescine and adenine hemi-sulphate Concentrations on axillary buds multiplication rate, fresh weight (g) and growth value of date palm ‘’ Ekhlass ‘’ cultured in vitro for 14 weeks in dark followed by 8 weeks under light conditions.

Treatments (mg/L)				Growth characters			
Growth Regulators (mg/L)		Putrescine	adenine hemi-sulphate	Axillary buds			
				Formation %	Multiplication Rate	Fresh weight (g)	Growth Value
2iP + Kin + IAA	0.2	0	80	0.00[b]	1.00[b]	1.20[e]	38.5d
		50	80	0.00[b]	1.00[b]	8.22[d]	216.5cd
		100	80	0.00[b]	1.00[b]	21.53[b]	539.5ab
		0	160	0.00[b]	1.00[b]	12.72[c]	396.5bc
		50	160	33.33[a]	2.11[a]	25.20[a]	727.0a
		100	160	0.00	1.00[b]	24.45[a]	602.5ab

Means within each column followed by the same letter are not significantly different at P= 0.05

Table 4: Effects of NAA and spermidine concentrations on root growth characters and chemical analysis of date palm ‘’Ekhlass’’ shoots cultured in vitro for 4 weeks.

Treatment (mg/L)		Growth characters of roots			Chemical analysis	
NAA	Spermidine	Formation %	No.	Length (cm)	Total soluble phenol (mg / g f. wt)	PAL activity (nkat /g protein
Free hormone (control)		22.22 [c]	0.11 [c]	0.44 [c]	4.91c	59.97c
0.2	50	55.56 [b]	0.22 [c]	0.72 [c]	5.42b	108.28b
	100	100.00[a]	2.67 [a]	5.42 [a]	5.94a	124.66a
	150	66.67 [b]	1.22 [b]	2.56 [b]	5.43b	61.23c

Means within each column followed by the same letter are not significantly different at P= 0.05

Table 5: Effect of growing mixture types on survival percentage, number of leaves and leaf length of hardened Ekhlass plantlets after 3 months in acclimatization.

Treatments			Growth characters		
			Survival %	Leaves	
				No.	area (cm^2)
Compost	+ Bark chips	(1:1, v/v)	66.67^{bc}	4.2^{b}	22.5^{b}
	+Perlite	(1:1, v/v)	73.33^{a}	4.7^{a}	23.8^{a}
	+Coconut shell	(1:1, v/v)	50.00 ^{c}	3.8^{c}	20.1^{c}
	+Rice shell	(1:1, v/v)	43.33 ^{b}	3.2^{d}	14.7^{d}

Means within each column followed by the same letter are not significantly different at P= 0.05

Table 6: Effects of pre-acclimatization treatment on survival %, no. of leaves and leaves area (cm2) of date palm ‘’Ekhlass’’ shoots cultured in vitro

Treatments							
Pre-acclimatization on rooting medium				Acclimatization	Survival %	Leaves	
Solid full strength 4 weeks	1/2 salt Strength 4 weeks	Artificial light 1500 Lux	Natural light 8000 Lux	Compost + Perlite		No.	Area (cm^2)
+	-	+	-		73.33^{b}	4.7^{a}	23.80^{a}
+	+	-	+		90.00^{a}	4.9^{a}	24.11^{a}

Figures

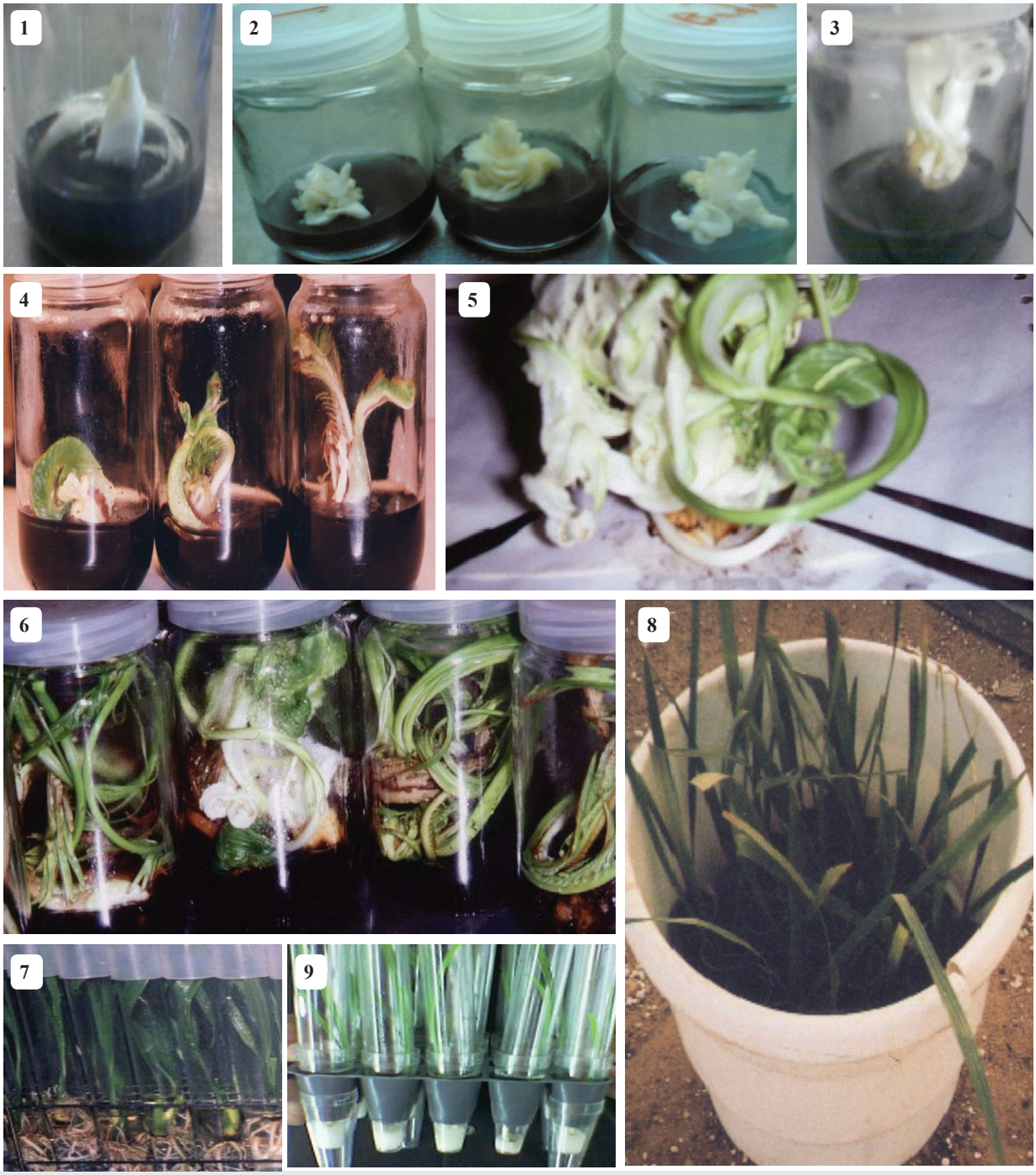

Fig.1. Complete promising protocol for *in vitro* direct organogenesis of date palm cv. Ekhlass a-Shoot tip explant after 2 weeks *in vitro*. b- Shoot tips growing after poking and incisions under dark condition c- shoot tip growth after 3 months and before transfer to light d- Shoot tips growing under light condition e- Shoot tip proliferation after 6 months f- Proliferated shoots growth in elongation stage. g- shoots formed well roots after a month h- Plantlets in pre-acclimatization in liquid 1/2 MS salts strength for a month. i- Healthy plantlets after 3 months in acclimatization.