Research in Biotechnology, 4(3): 20-28, 2013 ISSN: 2229-791X
www.researchinbiotechnology.com

Regular Article

The role of coconut water and casein hydrolysate in somatic embryogenesis of date palm and genetic stability detection using RAPD markers

Hussam S.M. Khierallah* and Nahla H. Hussein

Date Palm Research Unit, College of Agriculture, University of Baghdad, Iraq
*Corresponding author email : khierallah70@yahoo.com

The present investigation was conducted to determine the effect of various concentrations of coconut water (CW) and casein hydrolysate (CH) on callus growth, somatic embryogenesis and subsequent germination of somatic embryos in date palm Bream cultivar and detecting genetic stability using RAPD markers. Apical bud (2.0 cm) was excised, surface sterilized and cultured on modified Murashige and Skoog 1962 (MS) medium supplemented with 50 mg/L Picloram and 3.0 mg/L isopentenyladenine (2ip). Initial calli were transferred into a medium containing CW and CH. Callus growth was best achieved when 20% (v/v) CW was added giving a mean callus fresh weight of 1.95g compared with those supplemented with CH at 2.0 g/L which recorded 1.7 g. However, CW was more effective in enhancing somatic embryogenesis. Results showed that best somatic embryogenesis occurred on either 20%(v/v) CW or 2.0 g/L CH producing 65 and 47 embryos per culture, respectively. The subsequent germination of somatic embryos was also enhanced after the addition of CW and CH to previous culture medium. The highest percentage of embryo germination (76%) was achieved with 10% (v:v) CW and 60% was achieved with 2 g/L CH while it was only 45% in the medium lacking CW and CH. The highest percentage of embryo germination (76%) was achieved with 10% (v:v) CW and it reached 60% with 2 g/L CH while it was only 45% in the medium lacking CW and CH. Randomly Amplified Polymorphic DNA (RAPD) markers conducted in this investigation confirmed the resemblance among the tissue culture plantlets as well as with donor tree.

Key words: *Phoenix dactylifera* L.; coconut water; casein hydrolysate; somatic embryogenesis; *in vitro.*

Date palm (*Phoenix dactylifera* L., $2n = 2x = 36$) is a dioecious, perennial, monocotyledonous fruit tree belongs to Arecaceae family, and is of a major socio-economic importance in West Asia and Africa as a fruit tree and also as an ornamental plant (Morton 1987). The tree plays an important role in the development of sustainable agriculture in many drought and saline affected regions of the world (Wellmann *et al*. 2007, Kurup *et al*. 2009). Date palm has been cultivated for at least 7,000 years and is believed to have originated in Mesopotamia (Wrigley 1995). Extensive efforts have been made to propagate date palms through tissue culture (Omar *et al*. 1992, Zaid and de Wet 2002, Al-Khayri 2005). Generally, propagation by either offshoots or tissue culture results in true-to-type plants but some off-

Received: 2.3.2013; Revised: 2.6.2013; Accepted: 3.6.2013

types with abnormal phenotypes also have been reported in tissue cultured plants which may be due to somaclonal variations (Al-Wasel 1999). *In vitro* production of date palm via indirect organogenesis or somatic embryogenesis requires the application of relatively high concentrations of 2,4-D or NAA for the initiation process (Tisserat 1979, Bhaskaran and Smith 1992). However, these auxins are known to be associated with genetic instability in plants (Phillips *et al*. 1994, Cullis 1999). The use of natural compounds instead of plant growth regulators in culture medium may reduce or omit the possibility of genetic instability in plants. Organic additives such as casein hydrolysate and coconut water have been used to increase embryogeneic callus growth and somatic embryogenesis in several plant species and date palm as well (Hegazy *et al*. 2009, Khayri 2010, 2011). Since long lived plants may develop mutants even in apical meristem (Klekowski 1985) and during the last few years, variations have been detected among *in vitro* date palm cultivars Barhi, Medjool and Khalas such as Dwarfism, delay in fruiting and fruiting set failure (McCubbin *et al*. 2004, Al-Kaabi *et al*. 2005). All these cases would greatly affect the utilization of tissue culture techniques. Various techniques were used to confirm the true to typeness produced *in vitro*. Among the different techniques used in generating molecular markers, for direct detection of genomic variation at DNA level, Random Amplified Polymorphic DNA (RAPD) markers have been successfully used for the cultivar analysis and species identification in most plants, due to the technical simplicity and speed of the methodology (Bader *et al*. 2007, Al-Khalifah and Askari 2007).

The objective of the present study was to examine the effect of various concentrations of coconut water (CW) and casein hydrolysate (CH) on callus growth and somatic embryogenesis in date palm Bream cultivar employing PCR-RAPD markers for the early detection of genetic variations in date palm *in vitro* cultures.

Materials and Methods

Culture Initiation

Young offshoots of date palm cv. Bream (2 – 3 years old) were chosen and detached from the mother tree. Offshoots were dissected acropetaly until the shoot tips appeared. Shoot tips of 3 cm (apical meristem with soft inner leaves) were excised with immature fiber 2 cm in diameter and then dipped in autioxidant solution consisted of 150 mg/L cirtic acid plus 100 mg/L ascorbic acid (Tisserat 1991). Explants were surface sterilized in 50% (v:v) commercial bleach (sodium hypochlorite) plus 300 mg/L $KMNO_4$ containing eight drops of tween 20 as emulsifier for 20 minutes under vacuum, and rinsed three times with sterile distilled water. They were transferred to Petri dishes and all leaf primordia were removed except two pairs surrounding the apical meristem. The medium used in the initiation stage was composed of Murashige and Skoog 1962 (MS) salts plus the following (mg/L): thiamine – HCl, 1.0; pyridoxine – HCl, 1.0; adenine sulfate $2H_2O$, 50; myo-inositol, 100; $NaH_2PO4.$ $2H_2O$, 170; glutamine, 200; sucrose, 30000 activated charcoal, 2500; polyvenypyroledone (PVP), 2000 and agar-agar 7000. Callus initiation medium was supplemented with 50 mg/L Picloram and 3.0 mg/L isopentenyladenine (2ip). The pH of the medium was adjusted to 5.7 with 0.1 N NaOH or HCl, before the addition of agar. Media were heated until boiling and dispensed into 150×25 mm culture tubes with 25 ml each, then covered with aluminium plugs. All tubes with media were autoclaved at 121°C and 1.04 kg/cm² for 15 minutes. Apical meristems were divided longitudinally into four equal segments aseptically inside laminar air flow cabinet and cultured on callus initiation medium for 24 weeks and recultured at 4 weeks intervals (Fig. 1-A and B). Initial calli were then inoculated into 250 ml polycarbonate jars containing 40 mL of embryogenic callus proliferation medium (Fig. 1-C). The medium consisted of MS salts with the same supplements described for callus induction medium for eight

weeks. Embryogenic calli were then transferred to MS medium containing various concentrations of coconut water (0, 5, 10 or 20%) (v:v) or casein hydrolysate (0, 0.5, 1.0, or 2.0 g/L) after cytokinin exclusion. Coconut water was prepared according to Al-Khayri (2010). Embryos germination percentage was recorded after 4 weeks of transferring the globular embryo masses into to a medium containing 0.1 mg/L NAA and 0.05 mg/L BAP. All cultures were incubated in a culture room under low light intensity (1000 lux) for 16 hours daily at 27 + 1°C for four weeks. Two subcultures at four week intervals were done and data were recorded. Ten replicates of each treatment were used. Data were subjected to analysis of variance (ANOVA) and the means were compared at 5% significance level using least significant difference (LSD) test.

RAPD analysis

Total DNA was extracted from germinating embryos (figure 3-F) according to Benito *et al*. (1993) with some modifications. RAPD analysis was carried out according to Williams *et al*. (1990) with a few modifications. A total of 12 random decamer primers (Operon Technologies Inc., Alameda, California, USA) were used for RAPD amplification. PCR reactions were carried out in 25 µl volume containing 25 ng of total genomic DNA from each sample, 0.2 µl of a single primer, 100 mM of each dNTPs, 1X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$) and 1 unit DNA *Taq* polymerase (Roch, Mannheim, Germany, 1999). Amplification was performed using a thermocycler (PE 9600) programmed for RAPD: 1 cycle at 94ºC for 4 min, and 35 cycles with the following cycle profile: 1 min DNA denaturation step at 94 ºC, 2 min annealing step at 36 ºC, 2 min extension step at 72 ºC and last cycle at 72 ºC for 7 min, and an optional soak period at 4 ºC. Amplification products were loaded on 1% agarose gel and stained with ethidium bromide (0.5 mg/ml). Amplification for each primer was performed at least twice and only reproducible products were taken. DNA was visualized on a UV transilluminators (Gel documentation system ATTO, Japan). Fragment lengths were estimated by comparison with standard size markers (100bp DNA Ladder).

Results

The role of coconut water and casein hydrolysate

Addition of CW to the culture medium affected the proliferation of embryogenic callus. Results shown in table (1) indicate that the addition of CW increased significantly ~~the~~ both fresh and dry weight of embryogenic callus and the number of globular embryos as well. The highest fresh and dry weights were achieved at 20% of CW (1.95 and 0.48 g respectively). The same concentration gave the highest number of globular embryos (65 embryos). Results indicated that the addition of CH increased significantly both fresh and dry weight of embryogenic callus and the number of globular embryos as well (Table 2). The highest fresh and dry weights were achieved at 2.0 g/L of CH (1.70 and 0.43 g respectively). The same concentration gave the highest number of globular embryos (47embryos). The subsequent germination of somatic embryos was also affected by CW and CH added to the previous culture medium as shown in fig. 3-D. The highest percentage of embryo germination (76%) was achieved with 10% (v:v) CW while it was only 45% in the medium lacking CW (figure 1). On the other hand, the addition of CH also increased embryo germination percentage. The highest percentage of embryo germination (60%) was achieved with 2 g/L CH while it was only 45% in CH free medium (fig. 3-E).

Genetic stability of tissue culture-derived plants

A preliminary experiment was conducted to generate RAPD pattern with 12 primers to identify those that would be suitable in the present study to ensure reproducibility of RAPD marker data, the primers generating no or faint (nonreproducible)

bands were discarded (OPB.03 , OPB.05, OPC.08 and OPD.09). Eight primers showed clear and good amplification results. Most of them, about 7, generated monomorphic banding pattern for all samples tested (Fig. 2-a), while only one of the twelve primers (OPD.01) showed polymorphic banding pattern. (Fig. 2-b). The number of amplified fragments generated by these primers varied from 3 in OPE.03 to 8 in OPA.01 with an average of 5.38 bands for each primer. Molecular weight of the scored bands ranged from 0.48 kb to 4900 kb. Polymorphisms were detected by presence or absence and of amplified fragments for each primer used. As shown in figure 2 (b) there are genetic changes in the DNA amplification pattern by using OPA.01 primer for sample 2 (two polymorphic bands) and sample 9 (one polymorphic bands) when compared to the intact tree and other tested plantlets. Table 3 summarized the genetic variation revealed by OPA.01 primer.

Table 1. Effect of CW on embryogenic callus fresh, dry weight and number of globular embryos for date palm cv. Bream

% Coconut water	Embryogenic Callus		Globular embryo .No
	Fresh weight (g)	Dry weight (g)	
0	0.46	0.09	20
5	1.14	0.24	46
10	1.59	0.36	54
20	1.95	0.48	56
$LSD_{(0.05)}$	0.27	0.06	7.18

Table 2. Effect of CH on embryogenic callus fresh, dry weight and number of globular embryos for date palm cv. Bream

Hydrolysate Casein (g/L)	Embryogenic Callus		Globular embryo .No
	Fresh weight (g)	Dry weight (g)	
0	0.46	0.09	20
0.5	0.94	0.13	33
1.0	1.45	0.35	38
2.0	1.70	0.43	47
$LSD_{(0.05)}$	0.24	0.06	4.48

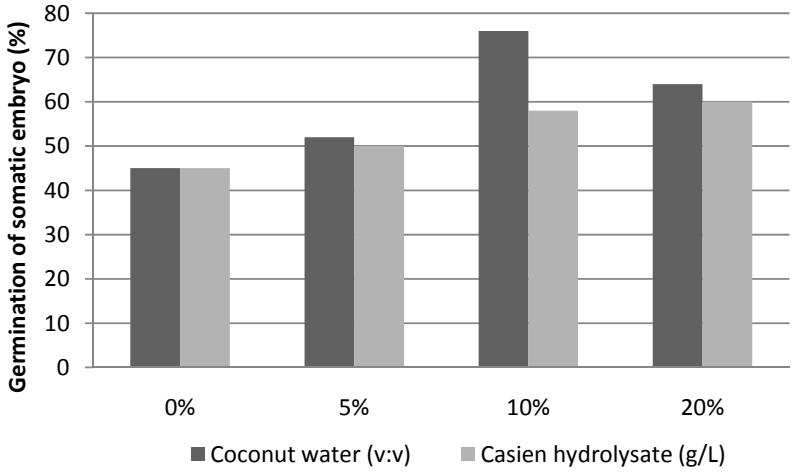

Figure 1. Effect of various concentrations of CW and CH on germination percentage of somatic embryos of date palm cv. Bream *in vitro*

Table 3. Polymorphisms revealed by RAPD markers using the primer OPA.01 (+) presence of the amplified band (-) absence of the amplified band.

Primer	No. Bands	Molecular Weight (bp)	Intact tree	Samples tested									
				2	3	4	5	6	7	8	9	10	11
OPA.01	8	4800	+	-	+	+	+	+	+	+	+	+	+
		4700	+	-	+	+	+	+	+	+	+	+	+
		1900	-	-	-	-	-	-	-	-	+	-	-
		1600	+	+	+	+	+	+	+	+	+	+	+
		1540	+	+	+	+	+	+	+	+	+	+	+
		1280	+	+	+	+	+	+	+	+	+	+	+
		1200	+	+	+	+	+	+	+	+	+	+	+
		1000	+	+	+	+	+	+	+	+	+	+	+

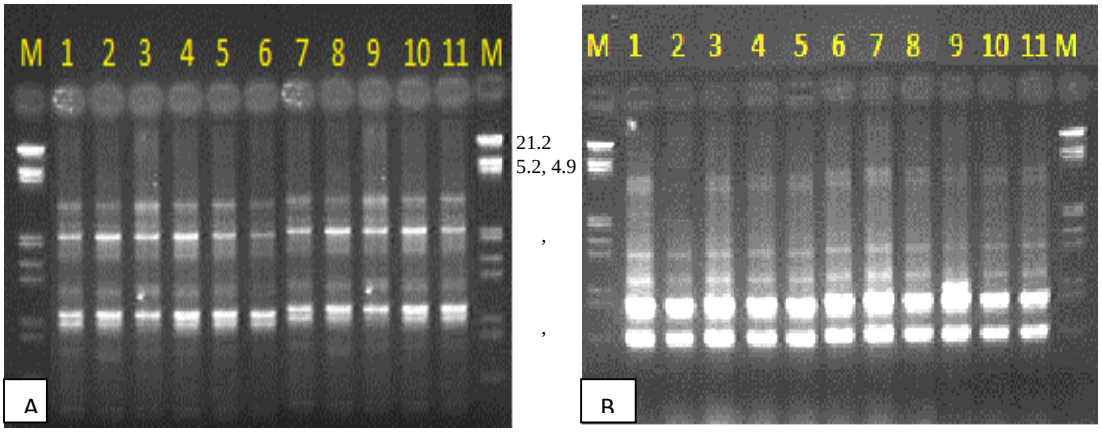

Figure 2. (A) Monomorphic banding patterns of samples tested reveled by OPD.06 primer for Bream, (B) Polymorphic banding patterns using the primers (OPA.01) Numbers on the left indicate the fragment size of molecular weight markers (lane M) in kb. The lanes 1 are the banding pattern of the intact trees, while the lanes 2 to 11 are the banding pattern of the samples selected randomly from germinating somatic embryos.

Discussion

Results indicated that supplementation of organic additives CW and CH improved callus growth and somatic embryogenesis in date palm tissue cultures. It was found that CW (the liquid endosperm of *Cocos nucifera* fruits) when added to a medium containing auxin, it can induce cell division (Goerge *et al*. 2008). Coconut water was shown to have cytokinin activity by Kuraishi and Okumura (1961) and recognised natural cytokinin substances have since been isolated [9-β-D-ribo-furanosyl zeatin, zeatin and several unidentified ones but the levels of these compounds in various samples of coconut water have not been published. An unusual cytokinin-like growth promoter, 2-(3-methylbut-2-enylamino)-purin-6-one was isolated by Letham (1982). Because coconut water contains natural cytokinins, thus adding it to culture medium often has the same effect as adding a recognised cytokinin. This means that a beneficial effect on growth or morphogenesis is often dependent on the presence of an auxin (Goerge *et al*. 2008). Increasing the number of globular embryos by adding casein hydrolysate may be attributed to that casein as a simple protein decomposes into glucosamine and galactosamine or mannosamine as well as simple sugars linked with proteins by covalent bonds or by glycoside bond with hydroxyl group of the amino acids serine and threonine (Al-Dalaly and Al-Rekaby 1995). It was found that the initiation of embryonic cells occurred by organizing the activity of

proteins through translation of specific genes which control two mechanisms: first is the transfer of signals across the cell surface by repeated replacement of leucine receptors by Serine-threonine receptors and second replacement these particles through plasmodesmata and this will result in an abundance of these amino acids which leads to the activation of somatic embryos formation (Goerge *et al*. 2008).

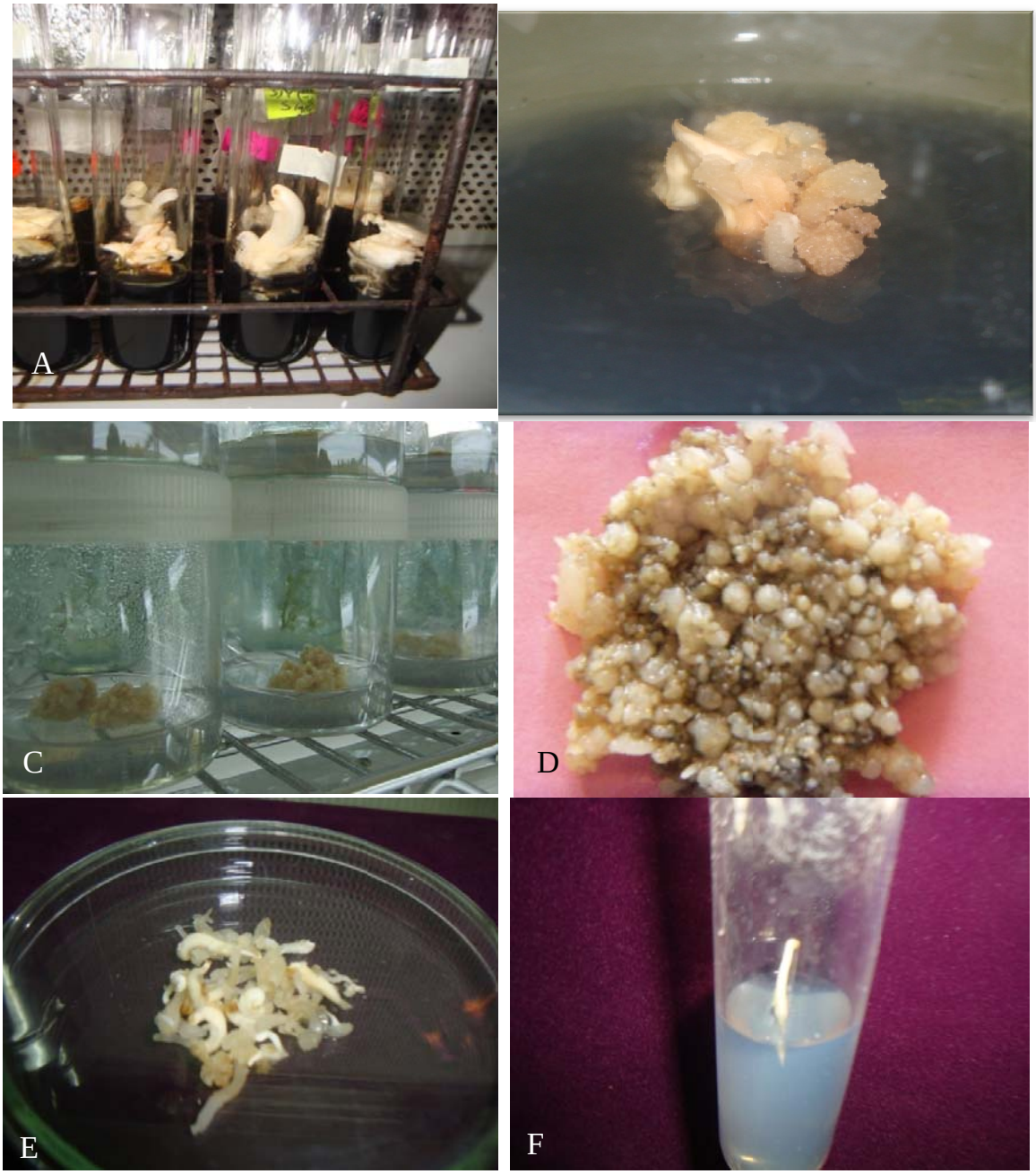

Figure 3. A. Date palm cv. Bream apical bud segments after 4 weeks culturing on callus induction medium suplemented with 50 mg/L picloram plus 3 mg/L 2ip; B. after 16 weeks C. Embryogenic callus proliferation on medium suplemented with 20% (v:v) CW; D. Globular embryos of Bream derived from medium containing CW; E & F. Somatic embryos germination on medium suplemented with 0.1 mg/L NAA plus 0.05 mg/L BAP.

The findings of this study indicated that genetic variations may exist in *in vitro* cultures of date palm derived plantlets even when natural products used instead of plant growth regulators in the culture media. Although plant tissue culture has been considered as a mean of vegetative propagation in which a rapid and identical (phonetically and genetically) clones are produced, somaclonal variations in some plant species may result from changes in nuclear, mitochondrial, chloroplast genomes, epigenetic variation as well. It is likely that this is a consequence of tissue culture and could have been induced in the callus production stage due to the use of high concentration of 2,4-D. These changes have been explained as the most suitable adaptation mechanism for the new environmental conditions exploiting the plasticity of the plant genome (Parfitt and Arulsekar, 1987). The probable causes include one or more of several processes such as changes in karyotype chromosome number, structure), point mutation, somatic crossing over, sister chromatid exchange, DNA methylation, activation of transposable elements and epigenetic variations (Brar and Jain 1998). Somaclonal variations can be characterized based on morphological, biochemical (isozymes) and DNA markers such as Randomly Amplified Polymorphic DNA (RAPDs), Restriction Fragment Length Polymorphism (RFLPs), Amplified Fragments Length polymorphism (AFLPs) and others (Brar and Jain 1998). DNA molecular markers have been successfully used to screen date palm plants derived from tissue culture and detecting genetic instability (Corniquel and Mercier 1994). RAPD technique has been reported to be useful for studying genetic variation in date palm (Sedra *et al*., 1998) and the detection of genetic stability of tissue culture derived plants (Al-Khalifah and Askari 2007, Ali *et al*. 2007). In conclusion, inclusion of organic additives such as CW and CH to date palm culture medium improved callus growth and somatic embryogenesis. RAPD variations could be exist referring to genetic instability of tissue culture derived plants at early stages.

References

Al-Dalaly BK and Al-Rekaby K. 1995. Food Chemistry, (in Arabic) Al-Kendy Publisher, Baghdad, Iraq. pp. 234.

Ali TA, Jubrail JM and Jassim AM. 2007. The use of RAPDs technique for the detection of genetic stability of the regenerated plantlets (Barhi cv.) in Iraq. Acta Hort. 736:105–112. http://www.actahort.org/books/736/736_11.htm

Al-Kaabi HS, Zaid A and Ainsworth C. 2005. Plant-off-types in tissue culture-derived date palm (*Phoenix dactylifera* L). Proceeding of The International Workshop on True-To-Typeness of Date Palm Tissue Culture-Derived Plants, Morocco, 23 - 25 May, 2005. http://www.inra.org.ma/publications%5Couvrages%5Ctruetype.pdf

Al-Khalifah NS And Askari E. 2007. Early detection of genetic variation in date palms propagated from tissue culture and offshoots by DNA fingerprinting. Acta Hort. 736:105–112. http://www.actahort.org/books/736/736_8.htm

AL-Khayri JM. 2005. Date palm *phoenix dactylifera* L. In: Jain S. M., Gupta PK(eds.) Protocols for somatic embryo-genesis in woody plant. Springer, Netherland, pp 309-319.

Al-Khayri JM. 2010. Somatic Embryogenesis of Date Palm (*Phoenix dactylifera* L.) Improved by Coconut Water. Biotech-nology, 9 (4): 477-484.

Al-Khayri JM. 2011. Influence of yeast extract and casein hydrolysate on callus multiplication and somatic embryo-genesis of date palm (*Phoenix dactylifera* L.) Sci. Hort., (130):531-535.

Al-Wasel ASA. 1999. Phenotypic comparison of tissue culture derived and conventionally propagated (by offshoots) date Palm (*Phoenix dactylifera*

L.) cv. Barhi trees. I. Vegetative characteristics. In: The Inter. Conf. Date Palm, Nov. 1999. Assiut Univ. Egypt. pp. 97-106.

Benito C, Figueiras A, Zaragozal C, Gallego F and Dela Penal A. 1993. Rapid identification of *Trijticeae geno* types from single seed using the polymerase chain reaction. J. Plant. Mol. Biol., 21(1): 181-183.

Bhaskaran S and Smith RH. 1992. Somatic embryogenesis from shoot tip and immature inflorescence of (*Phoenix dactylifera* L.) cv Barhee. Plant Cell Rep. 12:22-25.

Brar DS and Jain SM.1998. In: Jain, S.M.; Barar, D.S. and B.S. Ahloowalia (Eds) Somaclonal Variation and Induced Mutation in Crop Improvement. Kluwer Academic Publishers London. UK, p 15-37.

Corniquel B and Mercier L. 1994. Date palm (*Phoenix dactylifera* L.) cultivar identification by RFLP and RAPD. Plant. Sci., 101: 163-172.

Cullis CA. 1999. Environmental Stress- A Generator of Adaptive Variation. In: Lerner, H.R. (Ed). Plant Adaptation to Stress Environments. Marcel Dekker, New York. pp. 149-160.

George EF, Michael A and Klerk GD. 2008. Plant Propagation by Tissue Culture. Third Edition. Springer, Netherland pp. 118-182.

Hegazy AE, Nasr MI, Ibrahim IA and El-Bastawissy HH. 2009. Micropropagation of date palm cv. Malakaby through embryogenesis: 3. Effect of tryptone, yeast extract casein hydrolysate and pineapple extract. J. Agric. Sci. Mansoura Univ. 34, 1561-1576.

Klekowski EJ. 1985. Mutations, apical cells, and vegetative reproduction. Proceedings of the Royal Society of Edinburgh. Section B. Biological Sciences p. 67-73

Kuraishi S and Okumura FS. 1961. A new green-leaf growth stimulating factor, phyllococosine, from coconut milk. Nature 189, 148-149.

Kurup SS, Hedar YS and Al-Dhaheri MA. 2009. Morphophysiological evaluation and RAPD markers-assisted character-ization of date palm (*Phoenix dactylifera* L.) varieties for salinity tolerance. J. Food Agric Environ 7:503–507.

Letham DS. 1982. A compound in coconut milk which actively promoted radish cotyledon expansion, but exhibited negligible activity in tissue culture bioassays for cytokinins, was identified as the 6 oxypurine, 2-(3-methylbut-2-enylamino)-purin-6-one. Plant Sci. Lett. 26, 241-249.

McCubbin MJ, Zaid A and Van Staden J. 2004. A southern african survcy conducted for off-types on date palm produced using somatic embryogenesis. Emir. J. Agric. Sci. 2004. 16(1): 8-14. http://ejfa.info/index.php/ejfa/article/viewFile/5213/2671

Morton J F. 1987. Date In: Fruits of warm climates. Julia F Morton, Miami, pp 5–11.

Murashige T and Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures physiol. Plant. 15:473-497.

Omar MS, Hameed MK and Al-Rawi MS. 1992. Micro propagation Of Date Palm (*Phoenix dactylifera* L.) In: Bajaj, Y.P. S. (Ed): Biotechnology In Agriculture and Forestry. Vol. 18 High- Tech. and micro propagation II. Springer–Verlag, Berlin, Heidelberg, 471-492.

Parfitt DE and Arulsekar S. 1987. In: Bonga, J.M. and D.J. Durzan (Eds) Cell and Tissue Culture in Forestry Volume 1. General Principles and Biotechnology, Martinus Nijhoff Publishers. Boston. USA. pp 286-297

Phillips RL, Kaeppler SM and Olhoft. P. 1994. Genetic instability of plant tissue culture: Breakdown of Normal Control.

Proceeding of the National Academy of Science USA. 91: 5222-5226.

Sedra MH, Las Hermes P, Trouslot P, Combes MC, and Hamon S. 1998. Identification and genetic diversity analysis of date palm (*Phoenix dactylifera* L.) varieties from morocco using RAPD markers. Euphytica 103:75-82.

Tisserat B. 1979. Propagation of date palm (*Phoenix dactylifera* L.) *in vitro*. J. of Exp. Bot. 30: 1275-1283.

Wellmann A., Escobar Araya H and Johnson DV. 2007. Datepalm cultivation in Chile and Peru (South America): current status and future prospects for development. Acta Hort. 736:71–85.

Wrigley G. 1995. Date palm. In: Smartt J, Simmonds NW (eds). Evolution of Crop Plants, second edition. Longman Group, Essex, England, pp. 399-403.

Zaid A and Wet PF. 2002. Date Palm propagation chapter 1-5, p.18 -138.