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INFLUENCE OF SOME FACTOR ON SOMATIC EMBRYOS INDUCTION AND GERMINATION OF DATE PALM CV BARHI BY USING CELL SUSPENSION CULTURE TECHNIQUE

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Abstract

This study was done to examine different concentrations of mercuric chloride ($Hgcl_2$) for Explants sterilization and different concentration of NAA) mg/L with 2iP for their effect on initiation primary callus and subsequent embryogenesis in *Phoenix dactyliferae* cultivar Barhi. Leaf primordial and shoot tips were excised from 2-3 years old offshoot. The result showed that contamination percentage decreased with an increase in concentration of $Hgcl_2$ for two explants where in the control (0.0)% gave highest value 75% and 62.5% of contamination for leaf primordial and shoot tip respectively while the addition (0.1)% gave a significant decrease 37.5 and 0% for explants respectively. The Explants were inoculated onto Murashiege and Skoog, 1962 (MS) medium supplemented with 2iP 3mg/L and containing 0, 25, 50 or 100 mg/L of NAA, the addition (50) mg/L of NAA gave highest value 68.8% in percentage of callus initiation with significant difference from addition 0% gave lowest value (18.8)%, as well Explants of shoot tip gave significant difference was 53.1% while leaf primordial gave 28.1%, Primary callus was transferred to fresh MS medium containing (0, 5, 10 and 20) mg/L of NAA with 3mg/L 2iP, Results were recorded after 12 weeks. A highest value of embryonic callus induction percentage or fresh weight and dry weight 87.5% or 1.56g and 0.47g for the character respectively for the treat 20 mg/L while control treatment 0.0 gave the lowest value 12.5% or 0.37g and 0.14g for same character respectively. Inoculated 1g fresh biomass into a 250 ml Erlenmeyer flask containing 50 ml MS liquid medium supplemented with NAA 20mg/L and agitated at 150 rpm by shaker to making suspension culture the measurements of packed cell volume (pcv) or fresh and dry weight taken weekly for 12 weeks, maximum values were reached in the eighth week of PCV and fresh weight but the dry weight reached to highest value in seventh week, then the curve began to decline gradually until it reached stability in the last two weeks. The cell suspension was Diluted to different initial cell density of 100, 500, 10000, 50000 and 100000 cells /ml with hemocytometer. The initial cell number of (10000) cells gave significant different of plating efficiency PE (11.6)% while (100) cells gave lowest value 1.8% of PE.

Key words: somatic embryos, Date Palm, cell suspension.

Introduction

Date palm (*phoenix dactyl liferae* L.) belong to order palm, family Arecaceae which includes more than 200 Genus and about 4000 species, it is one of the most useful families after Gramineae family (Mater, 1991), because the importance in the value of its products used in different industries and the basically the nutritional value of it is fruits including vitamins and nutrients such as phosphorus, iron, vitamin B5, B6 important to treat colitis and muscle spam, beside that nutrition experts point out that eating dates by pregnant and breast feeding activates the cells and enriches the mother's milk and baby will gain health and immunity. Traditionally the date palm was propagated *via* seeds or offshoots, but the plantlets produced from seeds are not identical and lesser in quality than mother plant while the propagation of offshoots is the best method, but number produced from the mother tree is limited especially in rare cultivars, thus scince the 1970 S Attempts of researchers began to used plant tissue culture technique in vegetative propagation of palms for providing large numbers of offshoots in a relatively short period, first attempts by Schroeder, (1970) and Reuveni *et al.,* (1972). For cultivated specific parts of date palm, there are two methods of propagation, the first is direct organogenesis (Al-Maari and Al-Ghamdi, 1997; Al-

Khateeb, 2002) and the other method is somatic embryogenesis from embryonic callus was mentioned by many researchers (Reuveni, 1979; Mater, 1983; Omar, 1988 and Al-Musawi, 2001), its presents a great potential for the rapid propagation and genetic resource preservation of this species. Therefore it becomes the most common micro propagation method in commercial plant tissue culture lab's over the last few years, the efficiency of the embryo formation method increased for the many variety of date palm by using cell suspension culture technique, some researchers went to develop this technique (Fki *et al*., 2003; Zouine and El-Hadrami, 2004; San *et al.*, 2006 and Saker *et al.,* 2007) this technique allows for equal exposure of the cultured cells to the nutrients as well as oxygen, simultaneously reducing the inhibitory role of phenolic substances from cells by diluting them in the liquid medium (Boufis *et al*., 2007) that will prevent of some determinants in formation of embryogenic like A synchronization in growth and development of embryos and decrease the proportion of germination (Othmani *et al.,* 2008; Badawy *et al.,* 2009 and Al-Khayri, 2012). The balance between Cytokinin and Auxin plays an important role in determining the shape of cellular differentiation and organs formation where the high concentration of the Aux and low of ck in media lead to callus induction, while when we done this in opposite way, this will stimulates induction of buds and later grows into branches (Miller and Skoog, 1957). EL-Hammady *et al.,* (1999) obtain the highest percentage of embryonic callus of Barhi when adding NAA at (20 or 50) mg/L with 2ip 2 mg/L, mohsen, (2004) was found supply media with Auxin at (20) mg/L gave significant difference in fresh callus weight of barhi after one month of culture 590 mg. Asemota *et al.,* (2010) found the induction of embryonic of callus by using NAA at 100 mg.L with sucrose 30g.l to media.

Plants growing in the external environments contaminated with microorganisms. Microbes are common cause for various diseases. These microbes can compete for nutrients, increase culture mortality, tissue necroses, reduced shoot proliferation and rooting (oyebanji *et al.,* 2009). Sterilization is one of the reliable means to control the pathogenic. A disinfectant is defined as a chemical that kills or destroys nearly all disease-producing *via* microorganisms (Baddour, 2008). Disinfectants can act on microorganisms in two different ways: growth inhibition (bacteriostasis, fungistasis) or lethal action (bactericidal, fungicidal or virucidal effects), as the objects of treatment. Disinfection is a sterilization process which makes an object free from viable organisms. Disinfectants are acting on bacterial wall (Fougeres, 1989), cytoplasmic membrane (Dauphin, 1988) energy metabolism (Guellouzh, 1987) and bacterial spores (Russel, 1983). There are different types of disinfectant are used, physical and chemical such as, fungicides, alcohols, $HgCl_2$ and NaOCl. Mercuric chloride ($HgCl_2$) is a wide range of disinfectant. Mercury is an extremely hazardous chemical element because of its volatility in the metal state and ability to form numerous toxic volatile organic compounds under the action of bacteria present in aquatic ecosystems (Ostrovskii *et al.*, 2000). Chlorine is electronegative, therefore the chloride compound oxidizes the peptide linkages, thus denatures the protein of microbes (Barrette *et al.*, 1986 and Jaya *et al.*, 2009). As it is having strong sterilization efficiency, mercuric chloride is most commonly used in laboratory to kill the microbes on the explants. This disinfectant is toxic not only for microbes as well as other superior organisms. It may be fatal if swallowed, causes severe irritation to eyes, skin and respiratory tract, causes allergic skin reaction, affects kidney and central nervous system.

Material and Methods

Offshoots (2-3 years old) of Barhi cultivar were chosen and detached from mother palm. Leaves were dissected acropetally. Shoot tips of 2 cm in length with leaves primordial (apical meristem with soft inner leaves). Explants were dipped in antioxidant solution consisted of 150mg/L ascorbic acid plus 100 mg/L ascorbic acid (Tisserat, 1991). Explants were surface sterilized *via* different concentration (0.0 or 0.025 or 0.05 and 0.1)% of mercuric chloride solution containing few drops of tween-20 for 15 minutes under vacuum and rinsed three times with sterile distilled water. They transferred to Petri dishes where leaf primordial were removed except the two pairs surrounding the apical Meristem which then divided longitudinally in to four equal segments and cultured in jars aseptically. The medium of initiation stage was composed of Murashige and Skoog, (1962) (MS) salts plus the following (in mg/L); thiamine-Hcl 1.0; pyridoxine-Hcl 1.0; adenine sulfate.$2H_2O$ 40; myo-inositol 100; $NaH_2PO_4.2H_2O$ 170; sucrose 3000 ; activated charcoal 2000 and agar-agar 7000. The pH of the medium was adjusts to 5.7 with 0.1 N NaOH or HCl, before addition of agar. The medium was dispensed into culture jars with aliquots of 25 ml in each of 25 ml in each and then covered with polypropylene caps and autoclaved under 1.04Kg/cm^2 at 121°C for 15 minutes callus initiation medium was supplemented with (0.0, 25, 50, or 100 mg/L of NAA and 3 mg/LN6-2-isopentyl adenine (2ip). Primary callus was obtained after 20 weeks of growth in full darkness. The primary callus was later transferred in medium supplemented 0.0, 5, 10 and 20 mg /l NAA with

Table 1: Effect of different concentration of $HgCl_2$ in percentage of contamination for Different Explants *in vitro* culture of date palm CV Barhi.

Concentration	Explants		Mean of con.
	Leaf primordial	Shoot tip	
0	75.0	62.5	68.8
0.025	62.5	37.5	50
0.05	50.0	25	37.5
0.1	37.5	0	18.8
LSD	N.S		33.46
Mean of Explant	56.2	31.2	
LSD	23.66		

same component as the medium above where it was obtained embryo callus. Results of percentage of explants contamination and percentage of callus, dry and fresh weight of callus were recorded after 12 weeks. For the establishing cell suspension culture transferred 1 g of friable callus from semisolid media and inoculate into a 250 ml Erlenmeyer flask containing 50 ml MS liquid medium supplemented with 30 g/L sucrose, 1.5 mg / L 2ip and 10 mg/L NAA without agar. Incubated cultures at 16_ h photoperiod of cool - white florescent light, 40 mmol/ m^2/s, ans 23+ c and agitated at 150 rpm *via* shaker. Maintain cultures by regular sub culturing at 2- week interval estimated PCV packed cell volume by place 5 ml cell suspension in a sterile graduate centrifuge tube and centrifuge at 2000*. For 5 min to record the PCV as percentage cell mass of the total centrifuged volume and then they were tacked the measurements of fresh and dry weight for same samples. The measurements are taken weekly for 12 weeks. Plot the PCV values in relation to time, to construct a growth curve reflecting various phases of cell growth. Plating efficiency (P.E) was estimated *via* diluted cell suspension concentration to give initial cell density of 100, 500, 1000, 5000, 10000, 50000 and 100,000 cells/ mL with hemocytometer, after that mixed samples of cell suspensions with melted agar medium after letting the autoclaved medium to cool down to 30-35°C. The medium used is similar to cell suspension medium, but contains 7 g/L agar and is dispensed in 15×100 mm Petri dishes, at 20 ml per dish, the cell suspension was mixed and molten and evenly spread in the plate to in the plate to solidify, forming affixed thin layer of cells. Assess the recovery potential of cell suspension to form cell colonies in relation to the initial cell concentration, count the colonies using an illuminated colony counter. The plating efficiency is determined using the following equation:

$$PE = \frac{\text{Final number of colonies per plate}}{\text{Initial number of cellular units per plate}} \times 100$$

Table 2: Effect addition of different concentrations of NAA on callus initiation percentage (%) for two Explants in vitro culture of date palm C.V BARHI.

Concentration	Explants		Mean of con.
	Leaf primordial	Shoot tip	
0	0.0	37.5	18.8
25	37.5	37.5	37.5
50	50.0	87.5	68.8
100	25.0	50.0	37.5
LSD	N.S		32.79
Mean of Explant	28.1	53.1	
LSD	23.18		

Result and Discussion

The result showed that contamination decreased with an increase in concentration of $HgCl_2$ for two explants where in the interaction between concentration and explants (Table 1) we found control (0.0)% gave highest value 75% of contamination for leaf primordial while the addition (0.1)% gave least value 0% for shoot tip. The treatment of addition of 0.1% exhibited a significant decrease in contamination 18.8% while the control (0.0) gave 68.8%. The shoot tip Explants gave a significant decrease in contamination (31.2%) compared with the leaf primordial (56.2%).

The results showed there is no significant different in the interaction between Explants and concentration (Table 2). The addition of (50)mg /L of NAA gave highest value 68.8% in percentage of callus initiation with significant difference from addition 0% gave lowest value (18.8)%, as well shoot tip gave significant difference was 53.1% while leaf primordial gave 28.1%.

The percentage of embryonic callus or fresh and dry weight increased with concentration (Table 3) where the treatment 20 mg/L gave highest value 87.5% or 1.56g and 0.47g for the character mentioned above respectively while control treatment 0.0 gave the lowest value 12.5% or 0.37g and 0.14g for same character respectively.

The results in table 4, showed, there was no increase in the PCV or dry and fresh weight for the first and second weeks this represented in curve lag phase and then the curve began to increase to appear exponential

Table 3: Effect addition of different concentrations of NAA on induction Embryo callus percentage or fresh and dry weight (%) for in vitro culture of date palm C.V BARHI.

Concentration mg.L	Embryonic callus	Fresh weight	Dry weight
0	12.5	0.370	0.143
25	37.5	1.080	0.277
50	62.5	1.350	0.387
100	87.5	1.560	0.470
LSD	45.39	0.722	N.S

Table 4: The cell suspension culture growth of date palm, showing packed cell volume (PCV%) or dry and fresh weight in relations to time as it pertains to each of the growth phase (lag phase, exponential phase, linear phase progressive deceleration phase and stationary phase).

Weeks	PCV	Fresh weightmg	Dry weightmg
First week	2.20	5.400	1.000
Second week	2.33	5.800	1.067
Third week	3.00	6.600	2.000
Fourth week	3.80	8.200	3.200
Fifth week	4.80	10.530	4.000
Sixth week	5.80	11.200	5.467
Seventh week	7.40	13.800	6.333
Eighth week	9.00	14.000	5.133
Ninth week	8.60	13.600	5.000
Tenth week	8.40	13.200	4.200
Eleventh week	8.38	13.000	4.067
Twelfth week	8.38	13.000	3.933
LSD	0.559	1.867	0.854

phase until they reached a maximum value in the eighth week of PCV and fresh weight but the dry weight reached to highest value in seventh week and followed by the increase stoops and the cells entre in the linear phase and then deceleration in the recent weeks (Fig. 1).

The initial cell number of (10000) cells gave significant different in the plating efficiency PE (11.6)% and followed by the treatment 100,000 cells gave 8.60% while the PE in (100) cells gave lowest value 1.8% (Table 5).

Conclusion

The experiment of table 1 showed that $HgCl_2$ at concentration of 0.1% good sterilization where contamination decreased for different explants, may be back to the effectiveness in killing bacteria by destroy

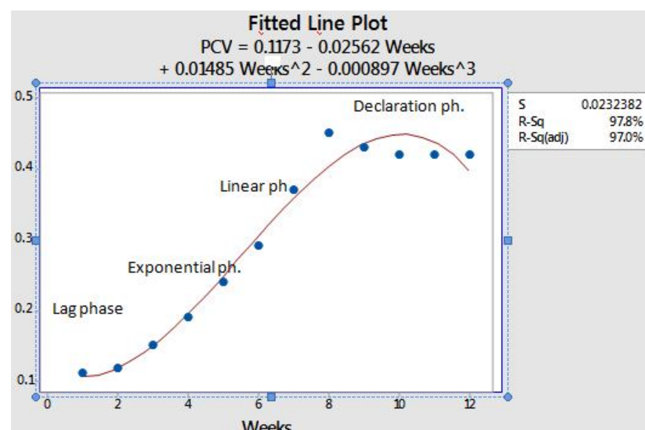

Fig. 1: Showing packed cell volume (PCV%) or dry and fresh weight in relations to time as it pertains to each of the growth phase (lag phase, exponential phase, linear phase progressive deceleration phase and stationary phase).

Table 5: The cell suspension culture growth of date palm, expressed in the number of colonies, recovered after plating on semi sold medium at various cell densities.

Initial cell numbers	Plating efficiency PE %
100	1.80
500	3.10
1000	4.70
5000	7.20
10000	11.60
50000	8.10
100000	8.60
LSD	3.38

bacteria wall and cytoplasm membrane at this concentration. Khierallah, (2007) found when sterilization of immature inflorescence with $HgCl_2$ for 15 minute, contamination has decreased to (0,5)% Barhi and Maktoom respectively but that cause damage for explants while contamination (10, 15)% when sterilization for 10 minute with a quick response to survival explants. jan *et al.*, (2013) found that the use $HgCl_2$ with (0.1)% this increase proportion of living parts non - contamination to 37.77% while the treatment with Nacol 1.5% lowest survival rate 7.77% .

The study has proved (Table 2 and 3) that NAA with 2iP improved percentage of pro-callus and embryo callus induction and increasing dry and fresh weight of callus that may be cells have ability to dedifferentiation, in another meaning transformation from functional to meristematic situation (callus), but that stimulated it by NAA is play important role in stimulating genes that control their gene expression which make in many processes such as cell division chloroplast development and etc. (George *et al.*, 2008).

Abo- EL-NIL, (1986) explained when using NAA with more concentration 10 mg/L this led to stimulation induction of callus at high rate especially when there is 2iP at 3mg /L. Asemota *et al.,* (2007) found adding NAA and 2, 4-D at 15 mg/L gave highest mean of fresh weight of date palm callus 800 mg/L.

The packed cell volume or fresh and dry weight (Table 4) was maximum value in seventh and eighth week but in the subsequent weeks the values declined, that may be consumption of nutrients in the media by cells and accumulation of metabolic products, while in the 7th and 8th weeks nutrient was maximized. Naik and Al-khayri, (2012) found a highest PCV % 12.1 in the eighth week while in the 1 or 2 and 3 weeks there was no obvious increase in PCV % but in 9 or 10 or 11 and 12 weeks the PCV began to decline from 9 to 12 weeks.

The plating efficiency (PE) table 5, was maximized at initial cell number (10000) cells that may be return to

effect of this density of cells in helping to manufacture of important compounds necessary for their division without a competition among cells, simultaneously this compounds must reach the internal concentration of it to a certain level before the cell began to division. where in low densities of cells the accumulation of this compounds be slower, on the other hand in the density over 10000 cells was got a competition between cells on the material elements and accumulation of metabolic materials affects in negatively on their division (Salman, 1988).

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