Study of some essential requirements of vitamins for *In Vitro* micropropagation of Date palm (*Phoenix dactylifera L.*) *cv. Barhee*

Sabeh D. Alutbi
Biology Department
Science college

Kadem I. Abbas
Horticulture Department
Agriculture college

****Ahmed A. Saad***
Horticulture Department
Agriculture college

Abstract

This study was conducted to investigate the effects of vitamins: Biotin, Pyridoxine, C-Pantothenate, and Riboflavin on the embryogenic callus and somatic embryos of date palm (*Phoenix dactylifera* L.) CV. Barhee.

The results showed that the best significant effect at level 2 mg/L for each vitamin on embryogenic callus was 4.9 , 4.6 , 4 , 3.9 gm. Respectively, whereas the less values at control treatment were 3.2 , 3.6 , 2.9 , 3 gm respectively.

There are significant values for the maturation period of the somatic embryos at level 2mg/L for the four vitamins were 25 , 26 , 26 , 29 day respectively as compared with control treatment.

The best significant effect for interaction between biotin and pyridoxine on the number of somatic embryos at level 2 mg/L for both was 28 embryos whereas for interaction between pantothenate and riboflavin also at 2mg/L for both was 27.6 embryos and the less values are at control treatment; Finally the best significant effect for interaction between biotin and pyridoxine on the fresh weight of somatic embryos at level 2mg/L for both was 0.280 whereas the less values at control treatment were 0.086 , 0.084 gm respectively.

Key words : vitamins, micropropagation, date palm.
*Part of PhD. thesis

Introduction :-

Date palm (*Phoenix dactylifera L.*), is monocotyledonous plant and belongs to the family Arecaceae; It was known since 4000 years B.C. in the native region near the arab gulf, especially in the south of Iraq (Al–Baker, 1972).

Shroeder (1970) was the first one in obtaining a plantlet by a tissue culture of date palm.

Tissue culture researches were to continue by somatic embryogenesis and organogenesis (Al–Wasel, 2001) and most of them were concentrated in the preparation of culture media on plant growth regulators and carbohydrates, especially the differences in their concentrations. (Zaid and Tisserat, 1984; Eshraghi et al, 2005).

Vitamins, the complex organic compounds in very low concentrations are necessary for metabolism in the maintenance of physiological processes like growth and development.

Most common vitamins are the thiamine–HCl and meso–lnositol were used in tissue culture of date palm for the initiation of callus (Reynolds & Murashige, 1979; Tisserat, 1981) whereas others used pyridoxine, Niasin and Ca–pantothenate for the initiation of callus also (Rhiss et al, 1979; Poulain et al, 1979).

Hassan (1987) studied the effect of five vitamins : Pyridoxine , riboflavin , thiamine , Meso–lnositol , Ca–pantothenate in groups with the removal of one vitamin in each trial; The results showed no significant differences in the effect of the removal of the vitamin on the growth of apical and axillary buds and the weight of callus with different responses between Barhee & Hillawi cultivars.

Dass et al, (1989) used the following vitamins (mg/L) :-Pyridoxine–HCl, 1; Thiamine–HCl, 1; Meso–lnositol, 100; Ca–pantothenate, 1, biotin, 0.01 in three different kinds of MS media for the establishment of embryogenic cultures from shoot tips of date palm CV. Muscat.

Alkhayri (2001) found that at the treatment which consisted of 0.5 mg/L thiamine and 2mg/L biotin, the optimum callus growth and the highest numbers of embryos; whereas the greatest elongation of embryos was at the treatment which consisted 0.5 or 2 mg/L thiamine in combination with 1mg/L biotin.

Inspite of importance of vitamin thiamine-HCI in micropropagation of date palm *In Vitro*, only few papers were concerned with the vitamins, however, thiamine-HCI is normally used in culure media; The goal of the current study to determine of the best kind and level of four vitamins: Pyridoxine, Biotin, Riboflavin and Ca–pantothenate, individually or in combinations to supplement the growth of the callus and the development of embryos of date palm CV. Barhee.

Materials & Methods

1. Plant material :-

This work is carried out in Date Palm center, University of Basrah (2005-2007) to obtain embryogenic callus and somatic embryos from subcultured callus which was intiated by the culturing excised shoot tips of date palm CV. Barhee on MS medium (Murashige & Skoog, 1962) supplemented

with 2,ip(2, Isopentenyl adenine) 3mg/L and 2,4–D(2,4-Dichlorophenoxy acetic acid) (50mg/L) which was substituted later by Naphalene acetic acid, NAA, (30mg/L).

2- **Preparation of culture media (1liter) :-**

The culture medium consisted of MS basal salts containing (mg/L): Sucrose (30000), NaH_2PO_4 (170), Meso–Inositol (100), adenine sulphate (40), Thiamine–HCl (0.5), activated charcoal (3000), Agar (7000).

Media were adjusted to PH 5.8 before the addition of agar, dispensed in flasks (50ml per flask) and test tubes (20ml per tube), capped with cotton plugs and aluminum foil and autoclaved for 15 min. at $121^{\circ}C$, and 1.05 kg/cm^2.

2. Effect of vitamins (Pyridoxine–HCl with biotin and Ca–pantothenate with riboflavin.

(A) Callus growth : A factorial experiment was carried out to study the effect of the addition of biotin with pyridoxine–HCl and Ca–pantothenate with riboflavin at levels 0,1,2,3 mg/L for each vitamin, to medium as mentioned in paragraph (2) and the medium was supplemented with NAA (1 mg/L), 2ip (1 mg/L), glutamine (200 mg/L) and glycine (2 mg/L).

5 replicates were used for each treatment, callus fresh weight was measured after aperiod of 60 days for 100 mg embryogenic callus culture.

(B) Required period for somatic embryos maturation (days):

The same treatments of callus growth (5 replicates of each treatment) were carried out, a period for first appearance of somatic embryos in each treatment was determined.

(c) Number of somatic embryos (embryo) :

The same treatment of callus growth (5 replicates of each treatment) were carried out; the number of somatic embryos (≥ 5mm length) was determined.

(D) Fresh weight of somatic embryo (gram) :-

10 somatic embryos (≥ 5mm long) were choosed randomly, mean of weights for somatic embryos was considered as embryo fresh weight.

Statistical analysis :-

All experiments are carried out as factorial using completly Randomized Design (C.R.D), The significance of difference among means was tested by Revised Least Significant Difference (R.L.S.D) at $P \leq 5\%$ (Al–Rawi & Kalafullah, 1980).

Results

(1) **The effect of vitamins on fresh weight of embryogenic callus** :-

(A) The effect of biotin & pyridoxine–HCl :-

The results of statistical analysis [Table 1] showed that the best effect of biotin was 4.9 gm. at level 2 mg/L which differs significantly from the rest studied levels (0,1,3 mg/L) were (3.2, 4.4, 4.3 gm) respectively, also the best effect of pyridoxine–HCl was 4.6 gm at level 2 mg/L which differs significantly from the rest studied levels (0,1,3 mg/L) were (3.6 , 4.3 ,

4.2gm) respectively. The best value of interaction between biotin and pyridoxine–HCl was (5.5gm) at level 2 mg/L for both vitamins and the less value was (2.4gm) at control treatment for both [Table 1 , Fig.1].

(B) The effect of riboflavin & Ca–pantothenate :-

The results of statistical analysis showed the best value were (3.9 gm) , (4 gm) at level 2 mg/L for both riboflavin and Ca–pantothenate, where as the less value were (3) , (2.9 gm) at control treatments of riboflavin & Ca–pantothenate.

The value of interaction effect (4.5 gm) at level 2 mg/L for both vitamins differs significantly from that (2.3 gm)/L at control treatments for both vitamins [Table 2 , Fig. 2].

(2) **The effect of vitamins on required period (days) for somatic embryos formation** :-

(A) The effect of pyridoxine–HCl and biotin :

The results showed significant differences among levels (0,1,2,3 mg/L) of each vitamin, thus required period was 25 days for biotin and 26 days for pyridoxine–HCl at the level 2 mg/L for both vitamins where as control treatment was (31 days) for both.

The interaction value was (20.6 days) at level 2 mg/L for both vitamins [Table 3].

(B) The effect of Ca–pantothenate and riboflavin :

The results showed the significant effect of Ca–pantothenate and riboflavin were (25.7 , 28.9 days) respectively at level 2 mg/L for both vitamins.

The best interaction value for both vitamins was (22 days) at level 2 mg/L for both vitamins [Table 4].

(3) **The effect of vitamins on the number of somatic embryos** :-

(A) The effect of biotin & pyridoxine–HCl :-

The results showed significant superiority of interaction value (28 embryos) at levels (2 mg/L) for both biotin & pyridoxine–HCl among other treatments, where as the less value was (12 embryos) at levels (0 mg/L) for both biotin and pyridoxine–HCl. [Table 5].

(B) The effect of Ca–pantothenate and riboflavin :

The results showed a significant interaction value (27.6 embryos) at level 2 mg/L for both Ca–pantothenate and riboflavin, where as (10.6 embryos) at control treatment for both vitamins.

The results also showed that the effect value of Ca–pantothenate and riboflavin were 25 , 21.8 embryos respectively at the level 2 mg/L for both [Table 6].

(4) The effect of vitamins on the fresh weight of cylindrical somatic embryos gm) :

(A) The effect of biotin & pyridoxine–HCl :

The results showed a significant differences superiority of the combination (2 mg/L for both biotin and pyridoxine–HCl) (0.281 gm) at $P \leq 5\%$ than other treatments. Where as the less value at the control treatment was (0.086 gm). [Table 7].

(B) The effect of Ca–pantothenate and riboflavin :

The results showed the best value of interaction was 0.280 at combination 2 mg/L Ca–pantothenate with 2mg/L riboflavin where the less value was (0.084gm) at control treatment for both two vitamins.

The effect of Ca–pantothenate & riboflavin at level 2mg/L for both was (0.198 , 0.173 gm) respectively.[Table 8].

Discussion

1. **The effect of vitamins on callus growth** :-

There is a difference between primary callus and embryogenic callus for vitamin requirements.

Most literatures concerning procallus initiation in date palm were included the addition of 0.5 mg/L thiamine–HCl and 100 mg/L Meso–lnositol to culture media. (Reynolds & Murashige, 1979; Vermandi and Navaro, 1997).

The results of current study have showed that the addition of 0.5 mg/L thiamine and 100 mg/L Meso–Inositol only have no significant effect for obtaining embryogenic callus as well as somatic embryos and plantlets, however, the results showed the superiority of vitamin biotin among vitamins (pyridoxine–HCl , Ca–pantothenate , riboflavin) in aggrement with Alkhayri (2001).

The role of biotin in kreb's cycle and the formation of aspartic acid and pyriodoxin–HCl and enzymatic cofactor in decarboxylation and transamination reactions contributes in protein synthesis (Mohammed,1985).

The significance increase in fresh weight of embryogenic callus may be due to the production of ATP (Source of energy) in kreb's cycle and to the activity of protein synthesis, as steps of preparing to cell division and expansion. Mohammed (1985) stated that riboflavin inhibits auxin formation and this may interprete the lower effect of riboflavin among other vitamins in the proliferation of embryogenic callus since callus proliferation induced by auxin , the plant growth regulator of cell division and growth.

The result also showed the significant effect of the level 2mg/L of vitamins among other levels (0 , 1 , 3 mg/L) for the growth of embryogenic callus of date palm was in aggrement with alkhayri (2001) that found that 2mg/L biotin and 0.5mg/L thiamine–HCl were the optimal levels for date palm callus growth.

The results also showed that the use of vitamins in combination was better than in individual, this in aggrement with Kackar et al (1989).

In this study the addition of vitamins to callus cultures causes the disappearence of the verification from callus tissues, however verification was normally observed by Tisserat (1981).

2. **The effect of vitamins on the period of formation, fresh weight, and number of somatic embryos** :-

Barhee is aless cultivar in somatic embryos formation among date palm cultivars via tissue cultures.

Although intact plant could synthesize its vitamins, enzymes and cofactors (Mohammed, 1985). Embryogenic callus is not like intact plant, specially in first stages those achieved in dark, therefore the addition of vitamins to media is necessary, however current study showed a significant effect of vitamins, specially biotin and Ca–pantothenate in comparison with control treatment, this may be due to the role of biotin in ATP production and Ca–pantothenate in carbohydrate metabolism, then, enhancement of meristematic activity which may causing the increase of the fresh weight, number and shorten the formation period of somatic embryos ; these results are in aggrement with Dass et al (1989), who used biotin and Ca–pantothenate in modified MS medium for the development of somatic embryos of date palm.

The results also showed the best level of vitamins was 2 mg/L for the formation of somatic embryos, this results was in aggrement with Alkhayri, (2001).

Riboflavin treatment was significantly better than control treatment ; this may be due to the role of riboflavin in inhibition of auxin activity that favourates somatic embryos formation than callus proliferation ; Mohammed (1985) Stated the effect of riboflavin in auxin inhibition.

References :-

1. Al–Baker, A (1972). The date palm. "Areview of its past and present status ; and the recent advances in its culture, industry and trade, Al–Ani press, Baghdad PP. 1085. (in Arabic).
2. Al–khayri, J. (2001). Optimization of biotin and thiamine requirement for somatic embryogenesis of date palm (*Phoenix dactylifera L.*), *In Vitro* cell. Dev. Biol. Plant. 37 : 453–456.
3. Al–Rawi, K. ; Khalafullah, A. (1980). Agricultural experiments design and analysis. Ministry of higher education and scientific research. University of Mosul. 488PP. (in Arabic).
4. Al–Wasel, A.S. (2001). Phenotypic comparison of tissue culture derived and conventionally propagated by offshoots date palm (*Phoenix dactylifera L.*) CV. Barhee Trees 1–vegetative characteristics. J. KSU. 13, Agric. Sci. (1):65–73.
5. Dass, H.; Koul, R.; Joshi, S. and Bhansali, R.(1989). *In Vitro* regeneration of date palm plantles. Current Sci.58:22–24.
6. Eshraghi, P.; Zarghami, R.; Mirabdulbaghi, M. (2005). Somatic embryogenesis in two lranian date palm cultivars. Afric. J. of Biotchnol. 4(11) : 1309–1312.
7. Hassan, AbdulRazaq. (1987). The effect of inorganic salts and vitamins on

initiation and growth of date palm callus *In Vitro* MSc. Thesis–Agriculture college, Basrah University, Iraq, 100PP.

8. Kackar , N.; Solank, K. Joshi, S. (1989). Micropropagation of date palm (*Phoenix dactylifera L.*). CV. Khadrawy using tissue culture technique. Annals of Arid Zone 28 :1&2:137–141.
9. Mohammed, A. (1985). Plant physiology, (part two) Ministry of higher education and scientific research, University of Mosul, PP:966–973.
10. Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 437–497.
11. Poulain, C.; Rhiss, A. and Beauchesne (1979). Multiplication vegetative en culture *In Vitro* du palmier dattier (*Phoenix dactylifera L.*) C.R. Acad. Sc. : 1151–1154.
12. Reynolds, J. and Murashige, T. (1979). Asexual embryogenesis in callus cultures of palms, *In Vitro* , 15(5) : 383–387.
13. Rhiss, A., Poulain, C.; Beauchesne, G. (1979). La culture *In vitro* appliuee ala multiplication vegetative du palmier–dattier (*Phoenix dactylifera L.*). Fruits, 34 : 551–554.
14. Shroeder, C. (1970). Tissue culture of date shoots and seedlings. Date Growers' lnst. Rep., 47: 25–27.
15. Tisserat, B. (1981). Production of free–living palms through tissue culture. Date palm J., 1: 43–45.
16. Veramendi, J. and Navarro. L. (1997). Influence of explants sources of adult palm (*Phoenix dactylifera L.*). on embryogenic callus formation. Hort. Sci. J. 72(5) : 665–671.
17. Zaid, A. and Tisserat , B. (1984). Survey of the morphogenetic potential excised palm embryo in *In Vitro*. Crop Res., 24: 1–9.

Table 1 : Effect of Biotin & Pyridoxine–HCl and their interaction on the fresh weight of embryogenic callus (gm), days after culture (100mg callus).

Biotin (mg/L)	Pyridoxine (mg/L)				Mean
	0	1	2	3	
0	2.409	2.849	3.662	3.785	3.176 d
1	3.330	4.927	4.767	4.599	4.406 b
2	4.425	4.895	5.552	4.729	4.900 a
3	4.445	4.640	4.529	3.636	4.313 c
Mean	3.652 d	4.328 b	4.628 a	4.187 c	

Different letters indicate significant difference at $P \leq 5\%$.

R.L.S.D for (Pyridoxine & Biotin interaction) = 0.019

Table 2 : Effect of Ca–pantothenate and Riboflavin and their interaction on the fresh weight of embryogenic callus (gm), days after culture (100mg callus).

Ca-Pantothenate (mg/L)	Riboflavin (mg/L)				Mean
	0	1	2	3	
0	2.285	2.478	3.279	3.463	2.877 d
1	2.344	3.285	3.655	4.095	3.345 c
2	3.741	3.931	4.454	4.189	4.079 a
3	4.102	3.705	3.370	3.485	3.916 b
Mean	3.118 d	3.350 c	3.940 a	3.808 b	

Different letters indicate significant difference at $P \leq 5\%$.

R.L.S.D (Ca–pantothenate & riboflavin interaction) = 0.002.

Table 3 : Effect of the Biotin and Pyridoxine and their interaction on the period of somatic embryos formation (day).

Biotin (mg/L)	Pyridoxine (mg/L)				Mean
	0	1	2	3	
0	31.27	30.80	31.27	30.33	30.91 d
1	30.60	23.80	23.73	26.73	26.21 b
2	27.93	25.67	20.67	27.60	25.46 a
3	27.80	28.20	29.13	31.20	29.08 c
Mean	29.40 d	27.11 b	26.20 a	28.96 c	

Different letters indicate significant difference at $P \leq 5\%$.

R.L.S.D (Biotin and Pyridoxine interaction) = 0.177.

Table 4 :The effect of Ca–pantothenate and riboflavin and their interaction on the period of somatic emberyos formation (day).

Pantothenate (mg/L)	Riboflavin (mg/L)				Mean
	0	1	2	3	
0	35.00	33.47	32.67	29.60	32.69 d
1	30.67	29.80	27.33	29.67	29.37 b
2	28.93	27.82	22.00	24.07	25.70 a
3	25.80	26.80	33.40	35.00	30.25 c
mean	30.10 d	29.47 b	28.85 a	29.59 c	

Different letters indicate significant difference at $P \leq 5\%$.

R.L.S.D (interaction of pantothenate) = 0.004.

Table 5 :The effect of Biotin and pyridoxine and their interaction on the number of somatic embryos (embryo)

Biotin (mg/L)	pyridoxine (mg/L)				Mean
	0	1	2	3	
0	11.93	12.02	14.33	14.53	13.25 d
1	14.67	16.67	21.93	22.07	18.67 c
2	22.00	24.20	28.07	20.80	23.77 a
3	20.00	20.00	20.13	15.13	18.82 b
Mean	16.98 d	18.27 b	21.12 a	18.13 c	

Different letters indicate significant difference at $P \leq 5\%$.

R.L.S.D for interaction of Biotin with pyridoxine = 0.282.

Table 6 :The effect of interaction of Ca–pantothenate with riboflavin on the number of somatic embryos (embryo).

Pantothenate (mg/L)	Pyridoxine (mg/L)				Mean
	0	1	2	3	
0	10.60	13.27	15.47	15.93	12.64 d
1	17.47	19.13	21.00	21.67	19.82 b
2	22.93	24.40	27.60	25.40	25.08 a
3	25.53	24.73	23.00	9.93	19.36 c
mean	19.13 c	20.38 b	21.77 a	18.23 d	

Different letters indicate significant difference at $P \leq 5\%$.

R.L.S.D of (interaction of Ca–pantothenate with riboflavin) = 0.091.

Table 7 :The effect of biotin & pyridoxine–HCl and their interaction on fresh weight of somatic embryos (gm).

Biotin (mg/L)	pyridoxine (mg/L)				Mean
	0	1	2	3	
0	0.086	0.093	0.121	0.141	0.110 d
1	0.101	0.105	0.139	0.151	0.124 c
2	0.140	0.143	0.281	0.223	0.197 a
3	0.189	0.161	0.150	0.124	0.156 b
Mean	0.129 c	0.126 c	0.173 a	0.160 b	

Different letters indicate significant difference at $P \leq 5\%$.

R.L.S.D of (interaction of biotin and pyridoxine) = 0.021.

Table 8 :The effect Ca–pantothenate & riboflavin and their interaction on the fresh weight of somatic embryos (gm) 30 days after culture.

Pantothenate (mg/L)	Riboflavin (mg/L)				Mean
	0	1	2	3	
0	0.084	0.085	0.121	0.133	0.106 d
1	0.102	0.103	0.137	0.149	0.123 c
2	0.136	0.151	0.280	0.223	0.198 a
3	0.188	0.161	0.152	0.126	0.157 b
mean	0.127 c	0.126 c	0.173 a	0.158 d	

Different letters indicate significant difference at $P \leq 5\%$.

R.L.S.D of (interaction of Ca–pantothenate & riboflavin) = 0.034.

دراسة بعض المتطلبات الضرورية من الفيتامينات في إكثار نخلة التمر (*Phoenix dactylifera L.*)صنف البرحي خارج الجسم الحي

صبيح داود العطبي
قسم علوم الحياة / كلية العلوم

كاظم ابراهيم عباس
قسم البستنة / كلية الزراعة

احمد عبد الله سعد
قسم البستنة / كلية الزراعة

الخلاصة

أجريت هذه الدراسة لمعرفة تاثير الفيتامينات : البايوتين ، البايريدوكسين ، البنتوثينيت-كالسيوم والرايبوفلافين على الكالس الجنيني والأجنة الحضرية لنخلة التمر صنف البرحي. فاظهرت النتائج أن أفضل تأثير معنوي عند التركيز 2 ملغم/لتر لكل منها كان 4.9 غم ، 4.6غم ، 4غم ، 3.9غم على التوالي بينما اقل قيم عند المعاملة المحايدة كانت 3.2غم ، 3.6غم ، 2.9غم ، 3غم على التوالي وكذلك توجد قيم معنوية لفترة نضوج الأجنة الخضرية أيضاً عند تركيز 2 ملغم/لتر لكل من الفيتامينات الاربعة كانت 25 ,26 , 26 , 29 يوم على التوالي مقارنة بالمعاملة المحايدة.

كان أفضل تأثير معنوي للتداخل بين البايوتين والبايريدوكسين على عدد الأجنة الخضرية عند التركيز 2 ملغم/لتر لكليهما كان 28 جنين بينما للتداخل بين البنتوثينيت والرايبوفلافين أيضاً عند التركيز 2 ملغم/لتر لكليهما كان 27.6 جنين واقل قيم عند المعاملة المحايدة ، وأخيراً أفضل تأثير معنوي للتداخل بين البايوتين والبايريدوكسين على الوزن الطري للأجنة الخضرية عند التركيز 2 ملغم/لتر لكليهما كان 0.281 غم وللتداخل بين البنتوثينيت والرايبوفلافين عند التركيز 2 ملغم/لتر لكليهما كان 0.280 بينما كانت اقل قيم عند المعاملة المحايدة 0.086 ، 0.084 غم على التوالي.

الكلمات الدليلية: الفيتامينات ، الاكثار الدقيق ، نخلة التمر ، نخلة التمر ،

*مستل من اطروحة دكتوراه

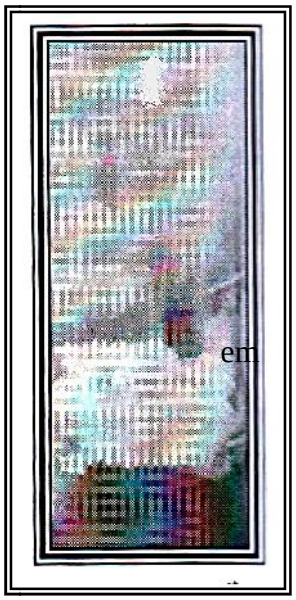

Fig 1. Effect of biotin and pyridoxinee on fresh weight of embryogenic callus(em) of date palm CV. Barhee.

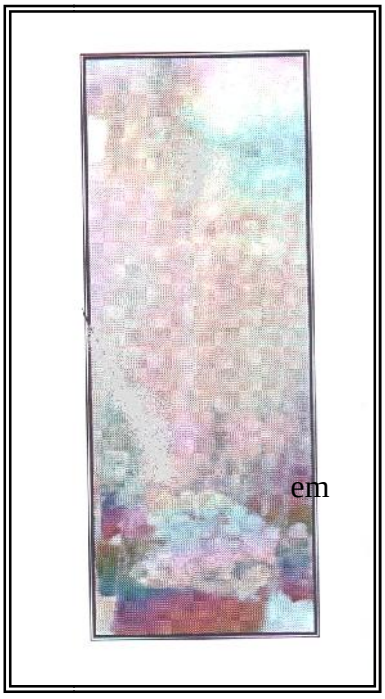

Fig 2. Effect of Ca–pantothenate and riboflavin on the embryogenic callus(em) of date palm CV. Barhee.