Response of four medical plants of *Euphorbia* species in callus initiation *in vitro.*

استجابة اربعة نباتات طبية من جنس *Euphorbia* لاستحثاث الكالس في خارج الجسم الحي

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Abstract

Callus cultures were initiated for four *Euphorbia* species. Nodule explants cultured on Murashige and Skoog (MS) medium, supplemented with different concentrations 0,0.5,1,1.5,2 mg/l of the auxin 2,4-Dichlorophenoxy acetic acid 2,4-D. Half of the cultures were incubated for16 hrs/day photoperiod, while the other half was incubated under complete darkness. The incubation temperature was 25±1ºC. Observations on number of nodule explants initiated callus were recorded at 2,4,6,8 weeks of culture. For callus maintenance, 50mg of callus produced were re-cultured on MS medium supplemented with different concentrations of 2,4-D 0,0.4,0.8,1.2,1.6 mg/l. Callus fresh and dry weights were recorded after 4 weeks. Results showed that nodule explants of *Epeplus* and *Ehirta* incubated under light conditions achieved the highest response to callus initiation 75-100% compared with the other species under experimental conditions. *E.Helioscopia* incubated under light conditions achieved the lowest response for callus initiation 25-75%. Results also showed significant differences between *Euphorbia* species in fresh and dry callus weights, *E. Ehirta* produced the highest fresh and dry weight of callus reaching 1.410 and 0.046 mg respectively. The amount of fresh and dry weight of callus produced under dark conditions was significantly higher than that produced under light conditions.

Key words: medical plants, *Euphorbia,* callus

المستخلص

استحث الكالس من اجناس *Euphorbia* وهي *E. helioscopia* ، *E. peplus* ، *E. granulata* و*E. hirta* وذلك بزراعة العقل بطول 1 سم على الوسط الغذائي MS المضاف له منظم النمو 2,4-D بالتراكيز 0.0, 0.5 , 1 , 1.5 , 2 ملغم/ لتر . حضنت نصف الزروعات في الضوء 16 ساعة/ يوم والنصف الثاني حضن في الظلام الكامل وبدرجة حرارة 25±1 م. اخذت الملاحظات عن نسبة استحثاث الكالس للاجزاء المزروعة كل اسبوعين ولغاية الاسبوع الثامن . اخذ 50 ملغم من الوزن الطري للكالس لكل جنس وزرع على وسط غذائي جديد يحتوي على الـ 2,4-D بالتراكيز 0.0, 0.4 , 0.8 , 1.2 ,1.6 ملغم/ لتر. قدر الوزن الطري والجاف للكالس بعد اربعة اسابيع من الزراعة. اظهرت النتائج تفوق الجنسين *E. peplus* و*E. hirta* في نسبة استحثاث الكالس المحضن في الضوء وبلغت 75 - 100% مقارنة بالجنسين الاخرين في حين اظهر الجنس *E. helioscopia* اقل استجابة لاستحثاث الكالس في الضوء وبلغت 25 – 75 % . بينت النتائج اختلافات معنوية في معدل الوزن الطري والجاف للكالس بين الاجناس المدروسة وكذ لك بين ظروف التحضين . تفوق الجنس *E. hirta* معنويا في معدل الوزن الطري والجاف للكالس على بقية الاجناس وبلغ 0.046,1.41ملغم على التوالي . اظهرت النتائج ايضا بان معدل الوزن الطري والجاف للكالس المنتج في الظلام قد تفوق معنويا على الكالس المنتج في الضوء .

الكلمات المفتاحية : نباتات طبية ، *Euphorbia* ، كالس

Introduction

The genus *Euphorbia* is one of the largest and most complex genera of flowering plants. High morphological plasticity and diversity of this genus make taxonomical studies on *Euphorbia* attractive for botanists. The species of *Euphorbia* have their own economic value and hence contribute to the floristic wealth of tropical and subtropical countries of the world. Some of the *Euphorbia* is used in folk medicine to cure skin diseases, gonorrhea, migraines, intestinal parasites, and warts [1]. The genus *Euphorbia* has been the source of large number of biological active compounds such as, tannins, flavonoids, unsaturated sterols/ triterpenes, carbohydrates, lactones and proteins/ amino acids that were reported as major active constituents of some *Euphorbia* species [2-6]. Over seventy jatrophane, modified jatrophane, segetane, pepluane, and paraliane diterpenoids, fifty of them reported for the first time, were extracted, purified and characterized from *E.dendroides, E.characias, E.peplus, E.helioscopia*, and *E.papalias*. These compounds showed interesting pharmacological activities [7].

Chemical investigation of *E.peplus* revealed almost identical profiles of secondary metabolites affording ingenanes, jatrophanes, and tetracyclic diterpene with new carbon skeleton for the name pepluane is proposed [8]. Also [9] found that cytotoxicities and anti-herpes simplex activities of nine diterpenes isolated from *Euphorbia* species were determined. Biotechnological tools are important for multiplication and genetic manipulation of the medicinal plants through callus inductions, cell suspension in bioreactors, *in vitro* regeneration of plantlets and genetic transformations [10,11]. Improved cell and tissue culture technologies would help in producing the active compounds *in vitro* with better productivities without cutting down the natural resources. There is sufficient progress at research level to suggest that the tissue culture of Euphorbiaceous can and should be further developed [12]. The aims of this investigation are to study the response of the species of *Euphorbia* for callus induction by using nodule explants incubated in the dark and light conditions to reveal the reflection of the genotypes *in vitro*, and to use this strategy in future to improve *in vitro* production of secondary metabolites by adding biotic and a biotic factors to the medium of callus production to enhanced accumulation of such products.

Material and methods

Stem explants were excised, rinsed with tap water for 30 min, then transferred to a laminar air flow-cabinet where they were subsequently rinsed with sodium hypochlorite(2% NaOCl) for 15 min [13,14], followed by three rinses in sterile distilled water. For callus initiation, nodal explants 1 cm were cultured on MS [15] medium supplemented with 1mg/l BA (Benzyl adinine) and 2,4-Diclorophenoxyacetic acid 2,4-D at different concentrations 0, 0.5, 1, 1.5 , 2 mg/ l individually. The pH was adjusted to 5.8 using NaOH or HCl (1 N), then 9 g/l of the agar type agar-agar was added to the medium. Autoclaving was carrying out at 121ºC under 1.04 Kg/cm² pressure, for 15 min. The medium was left at room temperature to cool and become ready to culture explants. Cultures were divided to two groups: the first group was incubated at 25± 1 ºC under light conditions (2000 lux,16 hrs / day). The second group was incubated at 25± 1ºC under darkness conditions. Each treatment was consisted of three replicates. Number of sprouts initiated callus was recorded after 2, 4, 6, 8 weeks. After eight weeks, the callus was removed from the explants using forceps and scalpel, then pieces weighing about 50 mg were sub cultured on fresh medium supplemented with different concentrations of 2,4-D (0, 0.4, 0.8, 1.2or 1.6mg /l) according to the results of initiation. The data were recorded after four weeks. Callus fresh weight was determined, and then oven dried at 40ºC for 48hrs for callus dry weight measurements. The experiments were analyzed using SAS [16] program factorial experiments. The least significant differences LSD test was used to compare between means.

Result and discussion

Callus initiation

Results of callus initiation showed different responses between *Euphorbia spp.* And the concentrations of 2,4-D incubated in dark and light conditions Table(1). Control treatment without 2,4-D of *E.helioscopia* induced 25% in third and fourth two weeks of incubation at light conditions only, while the other species failed to produce callus from nodule explants incubated in dark or light conditions. Nodules incubated in dark conditions showed responses for callus induction in *E.peplus* reached 25 and 75 % on MS supplemented with 1.5,2 mg/l 2,4-D, respectively in the first two weeks. While in light conditions the response reached 25, 50 and 50% on MS medium with 1, 1.5, 2 mg/l of 2,4-D, respectively. As well as in *E.hirta* reached 50% on MS with 1.5 mg/l 2,4-D. At the second two weeks showed responses for callus induction in all species of *Euphorbia*. The maximum callus initiation of nodules incubated in dark was recorded in *E.peplus* reached 100% on MS supplemented with 2mg/ml 2,4-D, and in *E.hirta* reached 75% on MS with 1,2 mg/l 2,4-D. As well as in *E.granulata* and *E.helioscopia* reached 75% on MS with 1.5 mg/l 2,4-D. Whereas in light conditions the response of *E.hirta* reached 100% on MS supplemented with 2 mg/l. In *E.peplus* reached 75% on MS with 1, 1.5 and 2 mg/l. While in *E.hirta* reached 75% on MS supplemented with all concentrations of 2,4-D. In *E.granulata* reached 75% on MS with 1.5 mg/l 2,4-D, and in *E.helioscopia* reached 75% on MS with 0.5 mg/l. However the maximum callus induction of nodules in light were recorded in *E.peplus* 100% on MS supplemented with 1, 1.5 and 2 mg/l 2,4-D. In the meantime *E.hirta* reached 75% on MS supplemented with all concentrations of 2,4-D. In *E.granulata* reached 75% on MS with

1.5 and 2 mg/l 2,4-D and in *E.helioscopia* reached 75% on MS with 1 mg/l 2,4-D.The final two weeks the response for callus induction in dark conditions in *E.hirta* reached 75% in all concentrations of 2,4-D. In *E.peplus* reached to 100% on MS with 0.5 and 2 mg/l 2,4-D. In *E.granulata* recorded 75% callus induction on MS with 1, 1.5and 2 mg/l 2,4-D. While *E.helioscopia* recorded 75% on MS with 0.5 mg/l 2,4-D .From above we can conclude that *E.hirta* prefers light conditions while the other species prefer dark conditions for best callus initiation

Callus production

Results displayed in Table 2 illustrate the mean of fresh weight of *E.hirta* and *E.peplus* were significantly increased compared with the other species, reached 1.174 and 1.156 mg respectively. While *E.helioscopia* recorded the minimum mean of fresh weight reached 0.496 mg and showed significant differences from other species. The Concentrations of 2,4-D also showed significant differences. The highest callus fresh weight reached 1.200 and 1.060 mg at the concentrations of 2,4-D 0.4 and 0.8 mg/l, respectively. While the minimum mean of callus fresh weight were recorded in control treatment reached 0.230 mg and showed significant differences from other concentrations of 2,4-D.The interaction between the species of *Euphorbia* and 2,4-D concentration recorded highly significant differences. The maximum callus fresh weight in *E.peplus* reached 1.77 mg with 0.4 mg/l 2,4-D.

Table (1): Callus initiation from nodule explants of *Euphorbia* species

Conc. of 2,4-D mg/l	Callus fresh weight (mg)				
	E.helioscopia	*E. peplus*	*E.granulata*	*E.hirta*	Mean
0	0.000	0.000	0.000	0.920	0.230
0.4	0.800	1.770	1.260	0.970	1.200
0.8	1.680	0.850	0.580	1.140	1.060
1.2	0.000	1.630	1.000	1.210	0.960
1.6	0.000	1.530	0.640	1.630	0.950
Mean	0.496	1.156	0.696	1.174	--
LSD (P≤0.05)	2,4-D = 0.136	*Euphorbia* species = 0.121		2,4-D x *Euphorbia* species = 0.272	

Table (2): Effect of 2,4-D concentrations and *Euphorbia* species on callus fresh weight incubated under dark conditions

Weeks	2,4-D mg/l	*E. Helioscopia*		*E.peplus*		*E.Granulata*		*E.hirta*	
		Light	Dark	Light	Dark	Light	Dark	Light	Dark
2	0	-	-	-	-	-	-	-	-
	0.5	-	-	-	-	-	-	-	-
	1	-	-	+	-	-	-	-	-
	1.5	-	-	++	+	-	-	++	-
	2	-	-	++	+++	-	-	-	-
4	0	-	-	-	-	-	-	-	-
	0.5	+	++	++	+++	-	-	++	++
	1	++		+++	++	++	++	+++	+++
	1.5	-	+++	+++	+	+++	+++	+++	+++
	2	-	-	+++	++++	++	++	++++	+++
6	0	+	-	-	-	-	-	-	-
	0.5	++	+++	++	+++	+	-	+++	+++
	1	+++	+	++++	+++	++	++	+++	+++
	1.5	+	++	++++	+++	+++	+++	+++	+++
	2	+	++	++++	++++	+++	++	+++	+++
8	0	+	-	-	-	-	-	-	-
	0.5	++	+++	++	++++	+	++	++++	+++
	1	+++	+	++++	+++	++	+++	++++	+++
	1.5	+	++	++++	+++	+++	+++	++++	+++
	2	+	++	++++	++++	+++	+++	++++	+++

\- Indicate response 0%
\+ Indicate response 25%
++ Indicate response 50%
+++ Indicate response 75%
++++ Indicate response 100%

The results of callus production of *Euphorbia* species from nodule explants in light were revealed in Table (3) The mean callus fresh weight significantly increased in *E. hirta* compared with the other species, reached 1.64 mg. Whilst *E. peplus* recorded the minimum mean of fresh weight on nodule explants reached 0.390 mg and showed significant differences from other species except *E. helioscopia*. The concentrations of 2,4-D showed significant differences, reached 1.14 mg of mean callus fresh weight at the concentration of 1.6 mg/l. While the minimum mean of callus fresh weight were recorded in control treatment and showed significant differences from other concentrations of 2,4-D.The interaction between the species of *Euphorbia* and 2,4-D concentration recorded the maximum callus fresh weight in *E.hirta* reached 2.70 and 2.530 mg callus fresh weight with 1.6 and 1.2 mg/l 2,4-D, respectively, and showed significant differences from all other combinations.

Table (3): Effect of 2,4-D concentrations and *Euphorbia* species on callus fresh weight incubated under light conditions (16 hrs/ day).

Conc. Of 2,4-D mg/l	Callus fresh weight (mg)				
	E.helioscopia	*E. peplus*	*E.granulata*	*E.hirta*	Mean
0	0.000	0.000	0.000	0.000	0.000
0.4	0.730	0.520	0.820	1.550	0.905
0.8	0.650	0.410	1.020	1.450	0.882
1.2	0.570	0.550	0.650	2.530	1.075
1.6	0.580	0.470	0.810	2.700	1.140
Mean	0.506	0.390	0.660	1.646	--
LSD P≤0.05)	2,4-D = 0.188	*Euphorbia* species = 0.168		2,4-D x *Euphorbia* species = 0.376	

The species of *Euphorbia* showed significant differences in callus dry weight incubated in dark conditions Table (4).The maximum mean of dry weight showed significant differences in *E.peplus* compared with *E.helioscopia* and *E.granulata* species, which reached 0.044 mg. While *E.granulata* recorded the minimum mean of dry weight reached 0.021 mg and showed significant differences from *E.peplus* and *E.hirta*. The concentrations of 2,4-D showed significant differences, reached 0.047 mg of mean callus dry weight at the concentration of 0.4 mg/l. While the minimum mean was recorded in control treatment 0.008 mg and showed significant differences from the other concentrations. The interaction between the species of *Euphorbia* and 2,4-D concentrations recorded the maximum callus dry weight in *E.peplus* reached 0.083 mg with 1.2 mg/l 2,4-D and showed significant differences from most combinations.

Table (4): Effect of 2,4-D concentrations and *Euphorbia* species on callus dry weight incubated under dark conditions.

Conc. Of 2,4-D mg/l	Callus dry weight (mg)				
	E.helioscopia	*E. peplus*	*E.granulata*	*E.hirta*	Mean
0	0.000	0.000	0.000	0.034	0.008
0.4	0.060	0.045	0.032	0.050	0.047
0.8	0.073	0.038	0.024	0.032	0.042
1.2	0.000	0.083	0.023	0.026	0.033
1.6	0.000	0.052	0.027	0.054	0.033
Mean	0.026	0.044	0.021	0.039	--
LSD (P≤0.05)	2,4-D = 0.009	*Euphorbia* species = 0.008		2,4-D x *Euphorbia* species = 0.019	

The measurements of callus dry weight revealed high differences among the species as shown in Table (5). The maximum mean of callus dry weight was significantly increased in *E.hirta* compared with the other species, reached 0.052 mg of mean callus dry weight of nodule explants and showed significant differences from other species. While the minimum mean was recorded in *E.peplus* reached 0.018 mg and showed significant differences from other species. The concentrations of 2,4-D showed significant differences, reached 0.041 mg of mean dry weight at the concentration of 1.6 mg/l and differs significantly from all other combinations. While the minimum mean was recorded in control treatment 0.011 mg and also showed significant differences from the other concentrations. The interaction between the species of *Euphorbia* and 2,4-D concentrations recorded the maximum callus dry weight in *E.hirta* reached 0.074 mg with 1.2 mg/l 2,4-D and showed significant differences from most interactions .

Table (5): Effect of 2,4-D concentrations and *Euphorbia* species on callus dry weight incubated under light conditions 16 hrs/day).

Conc.of 2,4-D mg/l	Callus dry weight (mg)				
	E.helioscopia	*E.peplus*	*E.granulata*	*E. hirta*	Mean
0	0.000	0.000	0.000	0.043	0.011
0.4	0.047	0.032	0.023	0.033	0.034
0.8	0.038	0.017	0.033	0.043	0.033
1.2	0.031	0.021	0.023	0.074	0.037
1.6	0.055	0.021	0.021	0.068	0.041
Mean	0.034	0.018	0.020	0.052	--
LSD (P≤0.05)	2,4-D = 0.005	*Euphorbia* species = 0.005		2,4-D x *Euphorbia* species = 0.011	

Conversely, the results in Table (6) indicated significant differences between the four studied species of *Euphorbia* in callus fresh and dry weight. *E.hirta* produced the highest fresh and dry weight of callus reached 1.41 and 0.046 mg respectively, and showed significant differences from the other species.While the lowest mean of fresh weight of callus reached 0.501mg in *E.helioscopia* and the lowest callus dry weight was 0.021mg in *E.granulata.* The result also showed significant differences between incubation conditions. The maximum mean of callus fresh and dry weight were 0.881and 0.035 mg on nodule explants incubated in dark conditions. The interaction between *E.spp* and incubation conditions revealed significant differences in fresh and dry weight of callus. *E.hirta* produced the highest mean of fresh weight of callus incubated in light and dark conditions reached 1.646 and 1.174 mg respectively .While the maximum mean of dry callus produced from. *E.hirta* and *E.peplus* were 0.052 and 0.044 mg respectively, and significant differences with the most of interaction .*E.peplus* produced lowest mean of fresh and dry weight of callus in light conditions reached 0.390 and 0.018 mg respectively, while *E.helioscopia* produced minimum mean of fresh callus weight in dark conditions reached 0.496 mg and significant differences of most of interaction.

Table (6): Effect of incubation conditions and *Euphorbia* species of callus fresh and dry weight.

Euphorbia spp	Callus fresh weight (mg)			Callus dry weight (mg)		
	Light	Dark	Mean	Light	Dark	Mean
E.hetioscopia	0.506	0.496	0.501	0.034	0.026	0.030
E.peplus	0.390	1.156	0.773	0.018	0.044	0.031
E.granulata	0.660	0.696	0.673	0.020	0.021	0.021
E.hirta	1.646	1.174	1.410	0.052	0.039	0.046
Mean	0.801	0.881		0.031	0.035	
LSD (P≤0.05)	*E.spp*= 0.142,Incubation conditions = 0.042			*E.spp*=0.006 Incubation conditions = 0.003		
	E.spp×Incubation conditions = 0.192			*E.spp*×Incubation conditions = 0.012		

According to the results of tissue culture stated above, we can conclude that there are differences within studied species on the base of response to callus initiation *E.hirta* achieved the highest response for callus induction reached 75-100 % in period eight weeks according to the concentration of 2,4-D supplemented to MS medium. While *E.helioscopia* achieved the lowest response for callus induction reached 25-50 % in the period eight weeks according to the concentration of 2,4-D supplemented to MS medium. Also these results revealed on callus production in the second experiment especially on *E.peplus* nodule explants incubated in dark and *E.hirta* nodule explants incubated in light that achieved highest callus production compared with the other species. [14] Stated that the highest frequencies of callus induction in *E.helioscopia* observed on MS media supplemented with 3.0 mg/l 2,4-D.The differences of these results may be due to the differences in genotypes of studied species in the base of the response to callus initiation and production, as well as due to endogenous concentration of growth hormones. Similar results were reported in different studies [14,17 – 20]. Furthermore, in this study we stated a technique that may be used by any taxonomist to preserve and regenerate plants (unavailable in studied area or preserve important rare or endangered plants), as well as this technique may be used in studies of production of secondary metabolites from callus and cell suspension.

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