Tissue culture and evaluation of some active constituents of *Ruta graveolens* L. I: Effect of plant growth regulators and explant type on growth of *Ruta graveolens* L. callus cultures

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ABSTRACT

The effect of plant growth regulators and explant type on growth of Ruta graveolens L. callus cultures was studied. The explants used were stem, leaf, shoot tip and root. The most successful concentrations of plant growth regulators on four MS basal solid media were I (MS+ 1 mg/l 2,4-D), II (MS+1mg/l 2,4-D+ 1mg/l kinetin), III (MS+0.5mg/l 2,4-D+ 0.5 mg/l kinetin) and IV (MS+1mg/l 2,4-D+ 5mg/l kinetin). The highest survival values in callus formation were those of stem and root cultures on medium III. Also, leaf and shoot tip cultures on medium II showed higher survival percent age amongst their cultures. The morphological characters of callus cultures were described.

The effect of plant growth regulators and explant type on growth dynamics of callus (fresh weight, dry weight, callus index and callus vigor) was studied. Stem cultures on media I II, shoot tip on media II, III and IV, root cultures on media II and IV produced significant increase in the previous values, while leaf cultures on medium III produced significantly increased fresh weight and callus index values although dry weight and callus vigor values showed insignificant differences between different leaf cultures.

Key words: *Tissue culture, growth regulators, Ruta graveolens, callus.*

INTRODUCTION

Ruta graveolens L. (Rutaceae) (Chopra *et al.*, 1958) is an important medicinal plant known as common rue (Bedevian, 1936), herb of grace (Philips and Foy, 1990) and sadhab (Chopra *et al.*, 1956). The herb and its oil are used in traditional medicine as antispasmodic anthelmentic, and bacteriostatic (Ibn Sina, 1953). It is used also to treat rheumatism, diarrhea, amenorrhoea, skin disease and toothache (Brown, 1995; Hoffman, 1996; Kapoor, 1990; and Kowalchik *et al.*, 1998). Many authors have realized the interest of *Ruta graveolens* L. tissue cultures because of its very satisfying growth *in vitro* and accumulation of secondary products in its tissue (Bajaj, 1989). Tissue culture studies on

Ruta graveolens L. abroad has received a good deal of attention with different types of media, hormones and additives (Baumert *et al.*, 1994, Bohlmann *et al.*, 1995; Eilert *et al.*, 1983; Eilert and Wolters, 1989; Stakhova, *et al.*, 1990).

The key for establishing tissue cultures of the plants is the choice of the optimum culture medium components, proper explant source and plant growth regulators concentrations (Dixon and Gonzales, 1994).

No work has been undertaken with tissue culture of *Ruta graveolens* L. in Egypt, so far. Therefore, this work is planned to study the effect of plant growth regulators and explant type on callus formation to serve as a basis for *in vitro* production of secondary metabolites.

MATERIALS AND METHODS

Plant material

Seeds of *Ruta graveolens* L. were obtained from plants grown in the Experimental Farm of the Ministry of Agriculture, authenticated by Prof. Dr. Nabil El-Hadidy, Prof. Taxonomy and Flora, Faculty of Science, Cairo University.

Culture medium

Murashige and Skoog basal medium (Murashige and Skoog, 1962) consisting of the following: commercially available powdered MS medium (Duchefa), sucrose (Adwic A.R.E), agar (Oxoid Bacteriological Agar No.1, Oxoid Ltd, UK). Plant growth regulators as kinetin, 2,4-D, (Sigma), Dil HCl and/or KOH solutions for pH adjustment were prepared according to Egyptian Pharmacopoeia (1984).

Materials for sterilization of the seeds

- Savlon solution:
  An antiseptic solution containing 0.3% w/v chlorohexidine gluconate and 3% w/v cetrimide.
- Chlorox: 1.5% sodium hypochlorite.
- Tween 80: used as a wetting agent.

Preparation of the culture medium

Media for growth of undifferentiated callus of *Ruta graveolens* L. comprised (4.4 g/l) full-strength Murashige and Skoog basal medium mixtures, 30 g/l sucrose, different concentrations of kinetin and 2,4-D, pH 5.7-5.8, 4 g/l Agar, which steam sterilization in autoclave under pressure of 1 Kgf/Cm^2 (121°C) for 20-30 min.

Washing and sterilization of the seeds

The seeds were sterilized by immersion in 10% savlon for 5 minutes with shaking, then washed three times with distilled water. They were immersed in chlorox solution with 1-2 drops of tween 80 and shaking for 10 min, then washed three times with sterile distilled water.

Cultivation of the sterilized seeds

The seeds were cultured in jars containing sterilized solid MS control media without plant growth regulators, and incubated at 22 28 °C and 16 h/ day photoperiod (2000-2500 Lux) for 6-8 weeks. The plantlets grown were used to obtain explants for callus cultures.

Induction of callus

Explants of different organs (shoot tip, leaf, stem and root) from the 6-8 weeks old seedlings were cultured on the prepared sterile MS media supplemented with different concentrations of plant growth regulators and incubated in 16 h/ day light (2000- 2500 Lux) over a period of 8 weeks.

Callus formation

Many trials for callus formation in the presence of different concentrations of 2,4-D and kinetin were done.

Callus development

It was studied by measurements of growth dynamics by statistical analysis. The variance separation of means among treatments was determined using LSD (least significant difference) at 5% level.

Measurements of growth dynamics

These measurements were recorded for the different cultures (Bekhit, 1996).

- Survival percentage

The number of formed calli in each 100 explant was recorded. The values represented the mean of 4 records. This percentage was calculated at the end of the incubation period (i.e., 8 weeks).

- Fresh weight determination

The calli collected after 8 weeks of culturing were washed with distilled water to remove the adhering material, dried on filter paper, weighed and weights were expressed in grams. In each weight, 4 replicates were used i.e. 4 jars each containing 6 explants. The weight resembled that of the callused explants in each jar, i.e., the dead explants were not weighed.

- Dry weight determination

The calli were air dried, heated in oven at 60°C untill constant weight. In each weight, the same 4 replicates used in fresh weight determination were used here. The weights were expressed in grams.

- Callus index determination

Callus index = (n x G/N) x 100

Where

n = total number of callused explants.

G = average weight of callus rating on explants.

N = total number of cultured explants.

- Growth vigor

The vigor of the callus cultures was recorded as the following (according to Bottino, 1981):

1: a number given to dead explants (no. callus growth).

2: a number given to intermediate sizes (below callus growth).

3: a number given to medium-sized callus (average callus growth).

4: a number given to intermediate sizes (above average callus growth).

5: a number given to maximum callus growth.

- Fresh weight of one callus/ week

Weight of one callus was recorded in each week starting from the 2nd week. The weight was an average of 4 replicates and expressed in grams.

- Growth value of calli or increase value (IV)

$$IV = (G_e \quad G_s) / G_s$$

Where

G_e = mass (mg) of callus at the end of each incubation period.

G_s = the starting mass (mg) of the callus.

N.B. The starting mass used as G_s was that of the callus after 2 weeks from the culture, to decrease the error in weighing small weights as possible.

- Callus growth curve determination:

Four replicates of the grown cultures from different explants, on MS with different concentrations of P.G.R. (Kinetin and 2,4-D) were harvested every week for 8 weeks period.

IV was determined as an average value of 4 replicates and used for drawing callus growth curve. Each replicate contained 6 explants.

Statistical analysis of the data was carried out according to randomized complete block design for comparing among means using least significant differences (LSD) according to Snedecor and Cochran (1974).

RESULTS AND DISCUSSION

A- Effect of plant growth regulators and explant type on callus formation

The most successful media supplemented with plant growth regulators are recorded in Table (1). Morphological characters of callus cultures are described in Tables (2) and (3) and Figs (1,2,3 and 4).

Table(1):The most successful media supplemented with P.G.R. in callus formation.

Medium	P.G.R.
I	MS + 1 mg/L 2,4-D
II	MS + 1 mg/L 2,4-D + 1 mg/L Kinetin
III	MS + 0.5 mg/L 2,4-D + 0.5 mg/L Kinetin
IV	MS + 1 mg/L 2,4-D + 5 mg/L Kinetin

Table (2): Morphological characters of the stem and leaf cultures.

	Stem cultures		Leaf cultures	
P.G.R.	Characters	Fig. (1)	Characters	Fig. (2)
I	Yellow, compact, non-differentiated callus.	A	Greenish yellow, friable callus, with adventitious roots, buds and shoots.	A
II	Greenish yellow, friable callus, with adventitious roots, shoots and buds	B	Greenish yellow, friable callus, with adventitious roots, buds and shoots	B
III	Greenish, friable callus, with adventitious buds and roots	C	Greenish, friable callus, with adventitious roots and buds.	C
IV	Greenish, friable callus, with adventitious shoots, showing vitrification	D	Greenish, friable callus, with adventitious shoots and roots and showing vitrification.	D

Table (3): Morphological characters of the shoot tip and root cultures.

	Shoot tip cultures		Root cultures	
P.G.R.	Characters	Fig. (3)	Characters	Fig. (4)
I	Yellow, compact callus, with adventitious buds.	A	Yellow, compact, non-differentiated callus.	A
II	Greenish yellow, friable callus, with adventitious roots, shoots and buds.	B	Greenish yellow, friable callus, with adventitious shoots, buds and roots.	B
III	Greenish, friable callus, with axillary and adventitious shoots.	C	Yellowish, compact callus, with adventitious shoots and showing vitrification.	C
IV	Greenish, friable callus, with axillary and adventitious shoots, showing vitrification.	D	Greenish, friable and non-differentiated callus.	D

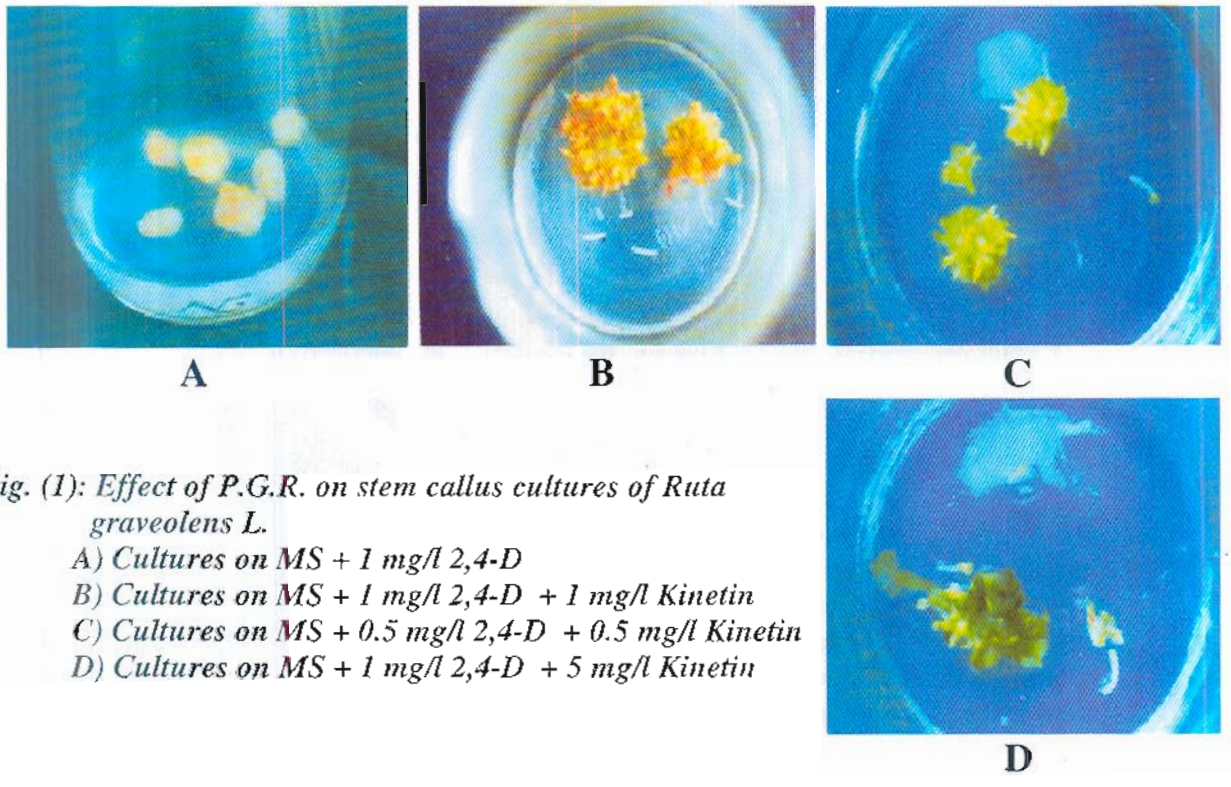

Fig. (1): Effect of P.G.R. on stem callus cultures of Ruta graveolens L.
A) Cultures on MS + 1 mg/l 2,4-D
B) Cultures on MS + 1 mg/l 2,4-D + 1 mg/l Kinetin
C) Cultures on MS + 0.5 mg/l 2,4-D + 0.5 mg/l Kinetin
D) Cultures on MS + 1 mg/l 2,4-D + 5 mg/l Kinetin

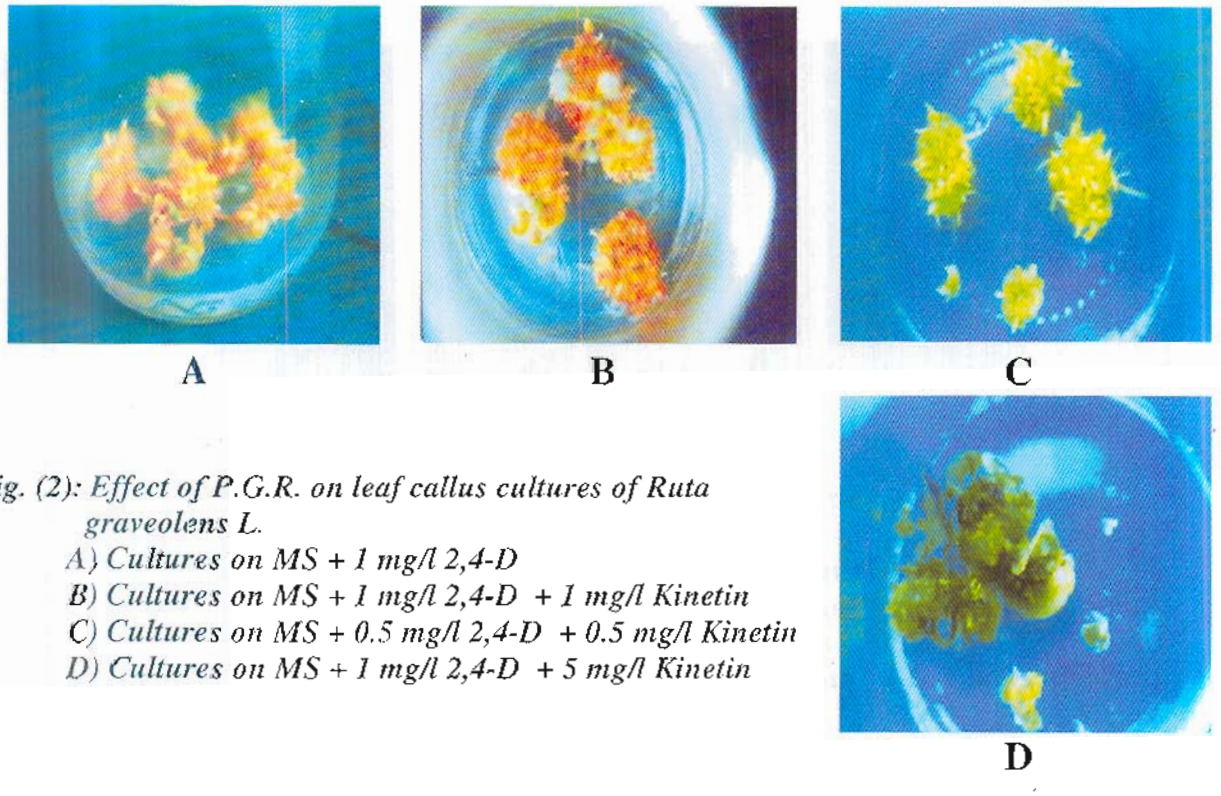

Fig. (2): Effect of P.G.R. on leaf callus cultures of Ruta graveolens L.
A) Cultures on MS + 1 mg/l 2,4-D
B) Cultures on MS + 1 mg/l 2,4-D + 1 mg/l Kinetin
C) Cultures on MS + 0.5 mg/l 2,4-D + 0.5 mg/l Kinetin
D) Cultures on MS + 1 mg/l 2,4-D + 5 mg/l Kinetin

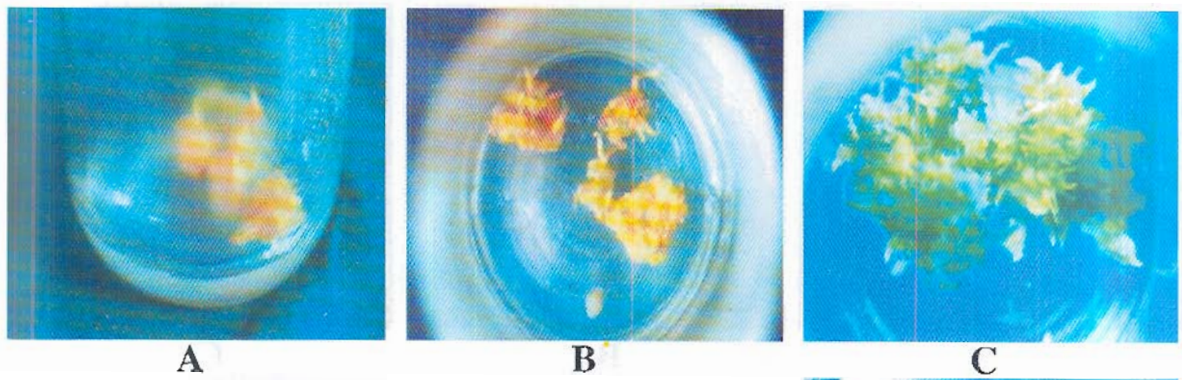

Fig. (3): Effect of P.G.R. on shoot tip callus cultures of Ruta graveolens L.
A) Cultures on MS + 1 mg/l 2,4-D
B) Cultures on MS + 1 mg/l 2,4-D + 1 mg/l Kinetin
C) Cultures on MS + 0.5 mg/l 2,4-D + 0.5 mg/l Kinetin
D) Cultures on MS + 1 mg/l 2,4-D + 5 mg/l Kinetin

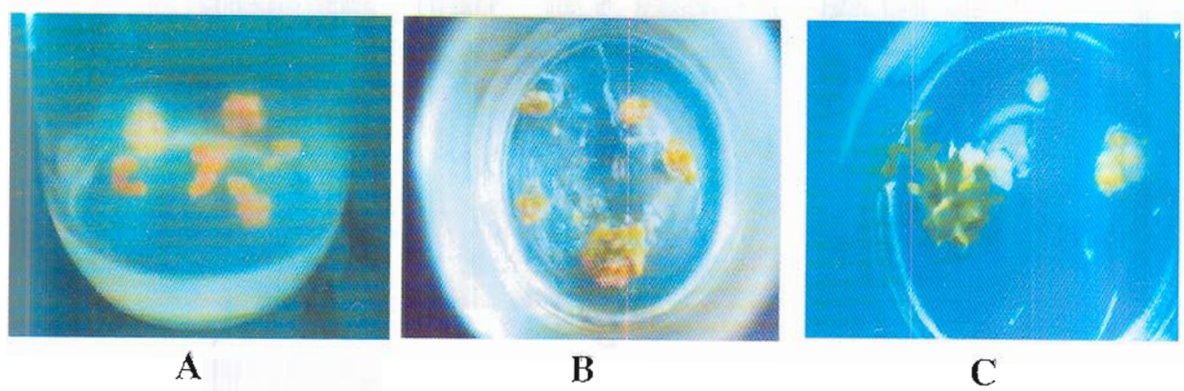

Fig. (4): Effect of P.G.R. on root callus cultures of Ruta graveolens L.
A) Cultures on MS + 1 mg/l 2,4-D
B) Cultures on MS + 1 mg/l 2,4-D + 1 mg/l Kinetin
C) Cultures on MS + 0.5 mg/l 2,4-D + 0.5 mg/l Kinetin
D) Cultures on MS + 1 mg/l 2,4-D + 5 mg/l Kinetin

B- Effect of plant growth regulators, explant type and incubation period on callus development

Growth dynamics of callus, survival percentage (Table 4), dry weight, callus index, callus vigor (Table 5), fresh weight (Table 6) and growth values of callus cultures (Table 7 and 8) and (Figs 5, 6,7,8) were studied.

Table (4): Effect of different P.G.R. and explant were studded type on survival percent of Ruta graveolens L. callus cultures.

P.G.R.	Survival%			
	Stem	Leaf	Shoot tip	Root
I	87.71	65.06	77.63	93.27
II	78.47	77	87.22	86.49
III	72.22	61.73	60.12	73.08
IV	14.78	54.76	59.52	87.96

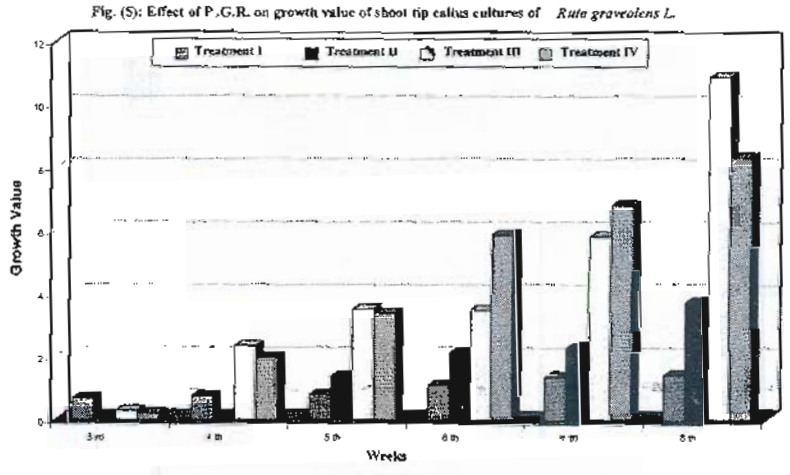

Fig. (5): Effect of P.G.R. on growth value of shoot tip callus cultures of Ruta graveolens L.

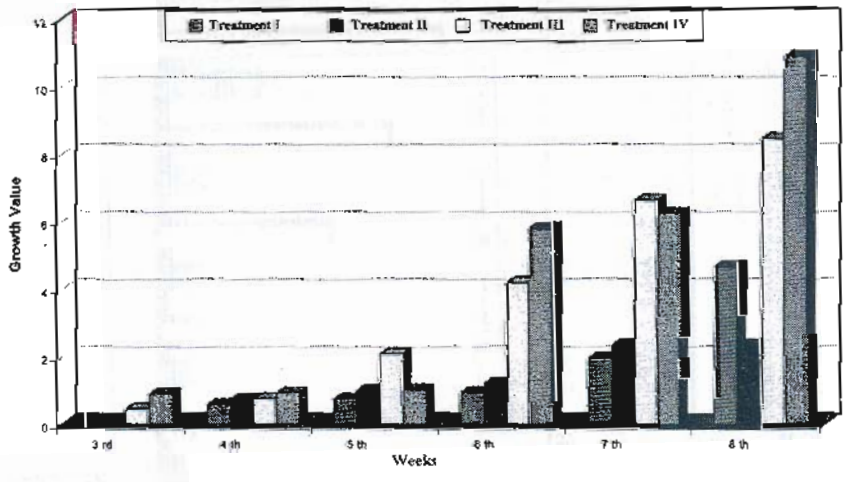

Fig. (6): Effect of P.G.R. on growth value of leaf callus cultures of Ruta graveolens L.

Table (5): Effect of P.G.R. and explant type on growth of callus of Ruta graveolens L. grown in vitro after 8 weeks from culture.

P.G.R.	Explant type															
	Fresh weight / jar (g)				Dry weight / jar (g)				Callus index				Callus vigor			
	Stem	Leaf	Shoot tip	Root	Stem	Leaf	Shoot tip	Root	Stem	Leaf	Shoot tip	Root	Stem	Leaf	Shoot tip	Root
I	1.323	1.367	0.464	0.424	0.081	0.093	0.027	0.021	22.048	22.758	7.730	6.005	3.583	3.498	1.915	2.708
II	1.294	2.049	2.720	0.514	0.095	0.145	0.153	0.04	21.568	34 140	45.323	8.555	2.375	2.628	3.125	1.958
III	0.616	1.016	2.637	1.254	0 053	0.095	0.275	0.125	7.790	16.940	43.950	20.923	1.918	2.418	3.250	2.793
IV	0	1.561	3.373	0 555	0	0.152	0.316	0.047	0	26.005	56.221	7.693	0	2.583	3 500	2.625
LSD	0.066	0.066	0.054	0 175	0.005	NS	0.236	0.082	0.548	0.651	0.319	12 033	0.956	NS	0 433	NS

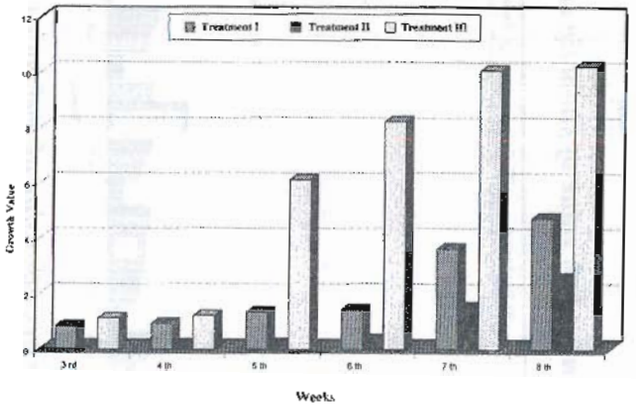

Fig. (7): Effect of P.G.R. on growth value of stem callus cultures of Ruta graveolens L.

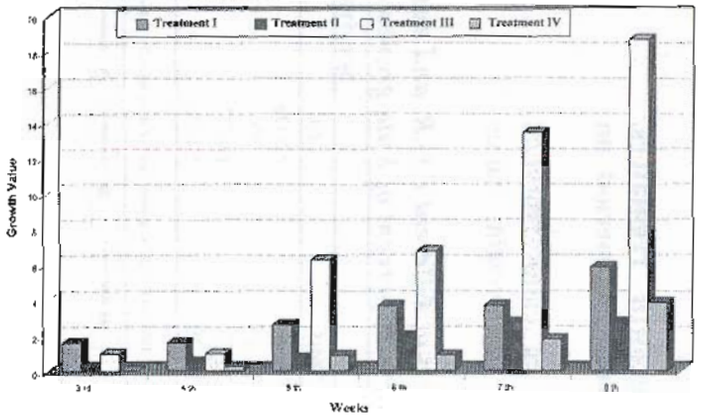

Fig. (8): Effect of P.G.R. on growth value of root callus cultures of Ruta graveolens L.

Table (6): Effect of P.G.R. and incubation period on fresh weight of callus of Ruta graveolens L. cultures.

P.G.R.	Explant type: Stem callus cultures							Leaf callus cultures						
week	2nd	3rd	4th	5th	6th	7th	8th	2nd	3rd	4th	5th	6th	7th	8th
I	0.040	0.073	0.077	0.093	0.097	0.184	0.229	0.048	0.050	0.075	0.083	0.092	0.140	0.269
II	0.081	0.090	0.097	0.099	0.131	0.131	0.290	0.106	0.106	0.114	0.213	0.233	0.353	0.356
III	0.015	0.033	0.034	0.109	0.142	0.170	0.173	0.028	0.040	0.048	0.085	0.143	0.210	0.260
IV	0	0	0	0	0	0	0	0.033	0.062	0.063	0.066	0.223	0.236	0.390
LSD	0.017	0.027	0.006	NS	0.005	NS	0.024	NS	0.035	0.047	0.043	0.074	0.030	0.093

P.G.R.	Explant type: Shoot tip callus cultures							Root callus cultures						
week	2nd	3rd	4th	5th	6th	7th	8th	2nd	3rd	4th	5th	6th	7th	8th
I	0.081	0.135	0.140	0.144	0.167	0.191	0.196	0.010	0.027	0.027	0.037	0.048	0.049	0.071
II	0.138	0.140	0.153	0.326	0.427	0.453	0.653	0.025	0.025	0.038	0.043	0.075	0.093	0.093
III	0.047	0.060	0.156	0.210	0.210	0.318	0.555	0.017	0.033	0.034	0.124	0.132	0.247	0.337
IV	0.072	0.087	0.213	0.315	0.496	0.563	0.673	0.024	0.024	0.030	0.045	0.045	0.066	0.115
LSD	0.008	0.021	0.041	0.026	0.254	0.036	0.052	0.005	0.006	0.008	0.025	0.077	0.077	0.216

Table (7): The effect of P.G.R. on growth values of stem and leaf callus cultures of Ruta graveolens L.

P.G.R.	Stem callus cultures						Leaf callus cultures					
	Weeks						Weeks					
week	3rd	4th	5th	6th	7th	8th	3rd	4th	5th	6th	7th	8th
I	0.831	0.931	1.331	1.428	3.600	4.712	0.053	0.574	0.747	0.932	1.942	4.658
II	0.101	0.190	0.218	0.360	1.516	2.565	0	0.730	1.005	1.196	2.326	2.359
III	1.120	1.211	6.086	8.208	10.052	10.201	0.455	0.755	2.073	4.182	6.618	8.455
IV	0	0	0	0	0	0	0.886	0.924	1.011	5.794	6.214	10.908

Table (8): The effect of P.G.R on growth values of shoot tip and root callus cultures of Ruta graveolens L.

P.G.R.	Shoot tip callus cultures						Root callus cultures					
	Weeks						Weeks					
week	3rd	4th	5th	6th	7th	8th	3rd	4th	5th	6th	7th	8th
I	0.671	0.739	0.783	1.075	1.367	1.431	1.602	1.602	2.610	3.625	3.675	5.819
II	0.014	0.107	1.366	2.095	2.284	3.735	0	0.520	0.730	1.980	2.710	2.710
III	0.273	2.332	3.492	3.492	5.802	10.872	0.895	0.968	6.225	6.697	13.411	18.668
IV	0.197	1.952	3.360	5.869	6.792	8.308	0.011	0.253	0.874	0.874	1.779	3.842

MS+ 1mg/l 2,4-D+ 5mg/l kinetin. Finally, root cultures was shown to produce best cultures with significant values on MS+0.5 mg/l 2,4-D +0.5 mg/l kinetin and highest dry weight on the previous medium and on MS+1mg/l 2,4-D+ 5mg/l kinetin. This shows that the different explants required an appropriate balance with equal ratios of exogenous auxin and cytokinin (2,4-D and kinetin) to produce high callus growth. These results were somehow in accordance with those obtained by Abou Mandour (1982), in which MS supplemented with 2,4-D, kinetin and NAA produced high callus growth.

Data analysis of fresh weights of one callus/week were recorded (Table 6) for an incubation period of 8 weeks. The analysis of data concerned with the effect on fresh weight of one callus indicated that stem cultures on MS+1mg/l 2,4-D+ 1mg/l kinetin produced significant increase in callus fresh weight in the 2^{nd}, 3^{rd}, 4^{th} and 8^{th} weeks, while cultures on MS+ 0.5 mg/l 2,4-D + 0.5mg/l kinetin produced highest values in 5^{th}, and 6^{th} weeks. Also stem cultures on MS+ 1mg/l 2,4-D produced significant increase in callus fresh weight in the 3^{rd} week only. Leaf cultures on MS+ 1mg/l 2,4-D + 1mg/l kinetin showed significant increase in fresh weight from the 2^{nd} to the 8^{th} weeks, while cultures on MS+ 1mg/l 2,4-D +5mg/l kinetin produced also high fresh weight values in 6^{th} and 8^{th} weeks. Shoot tip cultures on MS+ 1mg/l 2,4-D + 1mg/l kinetin produced significant increase in callus fresh weight in the 2^{nd}, 3^{rd}, 5^{th}, 6^{th} and 8^{th} weeks, while cultures on MS+ 1mg/l 2,4-D+ 5mg/l kinetin showed significant increase in callus fresh weight during the 4^{th}, 5^{th} , 6^{th}, 7^{th} and 8^{th} weeks . Also cultures of shoot tip on MS 2,4-D + 1mg/l produced high fresh weight in the 3^{rd} week only. The root cultures on MS+ 0.5mg/l 2,4-D + 0.5mg/l kinetin produced significant increase in callus fresh weight from the 3^{rd} to the 8^{th} week, while high values were produced by cultures on MS+ 1mg/l 2,4-D + 1mg/l kinetin and on MS+ 1mg/l 2,4-D + 5mg/l kinetin in the 2^{nd} week. Also, cultures on MS media supplemented with 1mg/l 2,4-D + 1mg/l kinetin produced significant increase in fresh weight of callus in 4^{th} and 6^{th} weeks.

From Tables (7 and 8) and Figs (5, 6,7 and 8) callus growth curves indicated the slow growing callus cultures and these included stem, leaf, and root cultures on MS+ 1mg/l 2,4-D and on MS+ 1mg/l 2,4-D + 1mg/l kinetin and root on MS+ 1mg/l 2,4-D+ 5mg/l kinetin. All cultures of different explants (stem, leaf, shoot tip, root) were highly fast growing on MS+ 0.5mg/l 2,4-D + 0.5mg/l kinetin and also stem, leaf and shoot tip cultures were highly fast growing on MS+ 1mg/l 2,4-D + 5mg/l kinetin. It was evident from such results that differentiated callus cultures were fast growing as compared to non-differentiated cultures and that agreed with Ramawat *et al.*, 1985.

From the previous results, we can conclude that the best explants were leaf, stem and shoot tip on nutrient medium II and root on medium III to hsdue good callus cultures suitable to obtain secondary metabolites from *Ruta graveolens* L. through tissue culture technique after 8 weeks under 2500 lux light intensity for 16 hours daily and 25 2 °C.

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الملخص العربي

زراعة الأنسجة وتقييم المواد الفعالة لنبات السذب

(روتا جرافيولنس ل.)

الجزء الأول: تأثير منظمات النمو والأجزاء النباتية على نمو الكالوس لنبات السذب

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١- زراعة الكالوس: تمت زراعة أجزاء نباتية مختلفة من الأوراق، الساق، القمة النباتية والجذور لنبات السذب فى ظروف معقمة على البيئة الأساسية لموارشيج وسكوج. كما تم وضعها فى الحضانة على نفس البيئة المزودة بتركيزات مختلفة من منظ مات النمو لمدة ثمانية أسابيع عند درجة حرارة ٢٢-٢٨م فى ظروف إضاءة لمدة ١٦ ساعة يوميا (٢٠٠٠ – ٢٥٠٠ لوكس) وتبين أن انجح بيئات تعطى أعلى نسبة نمو للكالوس هى كالأتى:

I بيئة موارشيج وسكوج+ ١ مجم/ لتر ٤,٢- ثنائى كلوروفينوكسي حمض الخليك.

II بيئة موارشيج وسكوج+ ١ مجم/ لتر ٤,٢- ثنائى كلوروفينوكسي حمض الخليك+ ١مجم/ لتر كينيتين.

III بيئة موارشيج وسكوج+ ٠,٥ مجم/ لتر ٤,٢- ثنائى كلوروفينوكسي حمض الخليك+ ٠,٥ مجم/لتر كينيتين.

IV بيئة موارشيج وسكوج+ ١ مجم/ لتر ٤,٢- ثنائى كلوروفينوكسي حمض الخليك+ ٥مجم/لتر كينيتين.

٢- نمو الكالوس: أسفر نمو الكالوس من أجزاء نباتية مختلفة عن النتائج الآتية:

* كانت أعلى نسبة نمو للكالوس من زراعات أجزاء من الساق والجذور على بيئةI وزراعات أجزاء من الأوراق والقمم النباتية على بيئة II.

* أوضحت دراسة الخواص المورفولوجية عن تكون زراعات متكشفة وغير متكشفة.

٣- تقدم نمو الكالوس: وقد تم تقييم نمو الكالوس بالقياسات الآتية: الوزن الطازج الوزن الجاف، معامل نمو الكالوس وشدة نمو الكالوس. أيضا تم دراسة العوامل التى تؤثر فى تقدم نمو الكالوس خلال ثمانية أسابيع.

أ- أوضحت التحاليل الإحصائية للقياسات بعد مرور ثمانية أسابيع أن أعلى زيادة معنوية للقياسات كانت فى زراعات الساق على بيئة I، زراعات الساق والأوراق والقمم النباتية على بيئة II وزراعات القمم النباتية والجذور على بيئة III و IV.

ب- تبين من التحاليل الإحصائية للأوزان الطازجة للكالوس خلال ثمانية أسابيع وجود زيادة معنوية للأوزان فى مختلف الزراعات.

ج- أظهر منحنيات نمو الكالوس أن الزراعات كانت ذات نمو سريع واشتملت على زراعات كل الأجزاء على بيئة III وزراعات كافة الساق، الأوراق والقمم النباتية على بيئة IV. كما أن هناك زراعات بطيئة النمو واشتملت على زراعات الساق، الأوراق والجذور على بيئة I وزراعات القمم النباتية على بيئة II.