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In vitro propagation, callus induction, and evaluation of active compounds on *Ruta graveolens*

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Abstract

Ruta graveolens belongs to Rutaceae family. It is a medicinal plant native to Jordan and is classified as an aromatic plant. *R. graveolens* plant is at risk of extinction, due to over-collection, overgrazing and deforestation. Therefore, this study was conducted to investigate factors affecting *R. graveolens in vitro* propagation. Callus induction was conducted from leaf discs on MS (Murashige and Skoog) media supplemented with 4.0 mg/l NAA. Callus tissue was maintained by frequent subculture on MS media supplemented with 1.0 mg/l 2,4-D. Rapid multiplication of *R. graveolens* was achieved by culturing microshoot on MS medium containing TDZ, 2-iP, zeatin or GA_3 at various concentration. Zeatin at 0.5 mg/l resulted in the highest shoot number and length. Crude extracts of callus, *in vitro* and *ex vitro* of *R. graveolens* were prepared by different solvents like methanol and ethanol. High performance liquid chromatography (HPLC) technique was used for quantitative analysis of five active compounds in three culture types. *In vitro* culture extracted by ethanol was contained the maximum content of metabolites compared to callus and *ex vitro* cultures.

Key words: *Ruta graveolens*, callus, crude extraction, methanol extract, microshoot.

Introduction

Ruta graveolens, known as rue, is a herb of grace and sadhab [1] and it belongs to Rutaceae family. *R. graveolens* is an important medicinal plant, which grows throughout Jordan and it is native to the Mediterranean region [2]. *R. graveolens* has been extensively used in traditional medicine to treat hysteria, gastrointestinal disorders, eye problems, menstrual problems, leucoderma, vitiligo, psoriasis, multiple sclerosis, cutaneous lymphomas, rheumatic, arthritis, eczema, ulcers, arthritis, and fibromyalgia [3]. *R. graveolens* has been used by local people in Jordan as an aphrodisiac, fertility promoting, antispasmodic, diuretic, sedative and analgesic agent and externally for its antirheumatic effect [2].

The seeds of *R. graveolens* show a very low germination percentage. Moreover, seeds do not produce homogeneous populations resulting in great variability in important features like sweetening levels and composition. Due to the above-mentioned difficulties, tissue culture is the only alternative for rapid mass propagation of true to type homogeneous *R. graveolens* plants. Plant tissue culture has some advantages over other propagation systems, as it provides rapid clonal propagation that can be performed throughout the year [4-6]. Also, clonal propagation through *in vitro* culture of *R. graveolens* can enhance the multiplication rate for this species. Thus, the development of micropropagation of *R. graveolens* could offer considerable advantages for production and conservation of this species [7]. Plants produce a variety of compounds such as morphine, caffeine, nicotine, and rubber. These compounds are the products of secondary metabolism, which occurs irregularly or rarely among plants [8]. Secondary metabolites or secondary compounds are compounds that are not required for normal growth and development. Most plants have not been examined for secondary compounds and new compounds are discovered almost daily. *In vitro* culture of plants has gained considerable importance during recent years in view of their possible application to the production of known and new aromatics [9].

Various compounds could be isolated from *R. graveolens* using HPLC technique. Secondary metabolites were identified in *R. graveolens* in callus cultures, *in vitro* culture, serum, dermis, plants, by HPLC methods and other recently published methods [10-12]. Also, furanocoumarin compounds such as psoralen, bergapten, xanthotoxin, and isopimpinellin isolated from three varieties of *Apium graveolens* were detected by normal-phase HPLC equipped with a variable wavelength detector set at 250 nm [13]. Therefore, this investigation was initiated to develop an efficient protocol for clonal propagation and to detect possible chemical compounds presented on callus, *in vitro* and *ex vitro* tissues of *R. graveolens*.

Materials and Methods

Seed establishment and micro propagation: Seed were collected from five years old *R. graveolens* plants that were obtained from the Al-Sareeh, Irbid, Jordan (about 600 m above sea level, 32.3306° N latitude and 35.8951° E longitude). Seeds were surface sterilized and germinated on agar water media, and shoots were cultured on MS medium [14] supplemented with 0.5 mM myo-inositol, 0.34 mM thiamine HCl, 2.4 mM pyridoxine HCl, 4.1 mM nicotinic acid and 3% sucrose. Once shoots were developed, microshoots were subcultured onto MS medium supplemented with 30 g/l sucrose and 0.3 mg/l BAP, 0.05 mg/l IBA and 1.0 mg/l GA_3. The microshoots were sub-cultured every 4-6 weeks using the same medium mentioned above for at least six times, and kept under 24±2ºC with a 16 hour photoperiod and photosynthetic photon flux density (PPFD) of 50 µmol $m^{-2}s^{-1}$ supplied by cool white fluorescent lamps.

In vitro shoot proliferation: Microshoots with apical meristem (10 mm in length) were subcultured on MS medium. For shoot proliferation, microshoots were subcultured on MS medium supplemented with 6-(γ,γ– dimethylallylamine) purine (2-iP), zeatin or thidiazuron (TDZ) at 0.0, 0.1, 0.2, 0.3, 0.4, 0.5 or l.0 mg/l. Data were collected after six weeks on number of newly formed shoots per initial microshoot, maximum shoot length, fresh weight, and dry weight. Each treatment consisted of 20 replicates, each experiment was repeated twice.

Callus induction and culture: Callus was induced from leaf discs, pieces of leaves inoculated into MS medium [14] supplemented with different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) or naphthalene acetic acid (NAA) at 0.0, 0.3, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 or 5.0 mg/l. After callus formation, calli were separated from leaves. Data was recorded after four weeks of incubation periods on callus induction percentage, texture, color and regeneration percentage. Callus fresh weight was recorded for three selected replicates. Callus samples were dried at 70°C for callus dry weight data.

Extraction of secondary metabolites: Healthy callus, *ex vitro* and *in vitro* plants of *R. graveolens* were dried at 80°C. The dried leaves and callus were powdered at the extraction day by using mortar and pestle. Powder of *ex vitro* and *in vitro* leaves and dried callus were dissolved in absolute ethanol and methanol (Sigma, USA) separately at 10:1 (v/w) solvent to dry weight [15]. The mixture was placed at room temperature for two days with occasionally shaking to dissolve secondary metabolites properly. The extracts were filtrated on Whatman no. 1 filter paper. In order to remove the solvents, the filtrate was placed in water path at 40°C to dryness.

High performance liquid chromatography (HPLC) analysis: Chromatographic quantification of psoralen, xanthotoxin, bergapten, and impratorin was performed according to procedure developed by Ekiert and Gomolka [16], and rutin was evaluated according to Hamad [17]. Chromatographic condition and compound identification mobile phase was gradient blend of methanol and acetonitrile in water. The HPLC system consisted in a HPLC from Merck-Hitachi, Germany® lachrom model, with interphase D-7000, diode array detector (L-7455), auto sampler L-7200, pump L-7150, solvent D-gazer L-7612, and D-7000 HSN software was used. Supelcosil® LC-8 (4.6 mm/25cm) analytical HPLC column and solvent system methanol-water as a mobile phase and flow rate 1 ml/min were used for rutin, psoralen, santhotoxin, bergapten and impartorin compound. A UV monitor operated at 310 nm was used as detector for above standard. The wavelength range was 300-360 nm and the total run time 50 min.

Experimental design and statistical analysis: Data were subjected to one-way analysis of variance ANOVA; differences between individual means were determined by least significant difference (LSD) test at 0.05 level of probability. The layout of the different experiments was a complete randomized design (CRD). Collected data were statistically analyzed using SAS software version 9.1 [18].

Results

In vitro establishment: *R. graveolens* cultures were established successfully. The sterilization procedures seemed to give satisfactory results. All established cultures gave healthy shoots when subcultured on the multiplication medium, which contained MS medium supplemented with 0.4 mg/l TDZ which enhanced shoot proliferation in *R. gravelones* (Table 1). Average number of shoots at the end of the culture period varied significantly with TDZ concentration. Increasing TDZ concentration from 0.0 (control) to 1.0 mg/l increased the mean number of proliferated shoots from 2.7 to 3.65, respectively, and maximum shoot lengths were also obtained on 0.4 mg/l TDZ. Increasing TDZ concentrations increased shoot lengths significantly, as well as fresh and dry weight (Table 1).

Table 1. Mean number of shoots/explants, shoot length and microshoot fresh and dry weight of *Ruta graveolens* after six weeks growth period after thidiazuron (TDZ) treatment.

TDZ (mg/L)	Number of new shoots	Maximum shoot length (cm)	Microshoot fresh weight (g)	Microshoot dry weight (g)
0.0	2.700 ^{bc}	1.710 ^{c}	0.061 ^{c}	0.011 ^{c}
0.1	2.200 ^{bc}	2.865 ^{ab}	0.429 ^{abc}	0.052 ^{bc}
0.2	2.000 ^{c}	2.245 ^{bc}	0.160 ^{bc}	0.029 ^{c}
0.3	3.700 ^{abc}	3.005 ^{a}	0.507 ^{ab}	0.070 ^{bc}
0.4	5.200 ^{a}	3.135 ^{a}	0.801 ^{a}	0.094 ^{ab}
0.5	3.850 ^{ab}	3.060 ^{a}	0.654 ^{a}	0.093 ^{ab}
1.0	3.650 ^{abc}	2.780 ^{ab}	0.599 ^{a}	0.142 ^{a}

Means followed by the different letters within the column are significantly different according to LSD test at P≤0.05.

Medium supplemented with 2-iP significantly increased the number of new shoots (Table 2). Maximum number of new microshoots was produce on MS medium supplemented with 0.2 mg/L 2-iP, which had shown superior responses (8.25 microshoot/explants). In this study, 2-iP gave higher shoot proliferation than other growth regulator (Table 2).

Table 2. Mean number of shoots/explant, shoot length and microshoot fresh and dry weight of *Ruta graveolens* after six weeks growth period after isopentenyl adenine (2-iP) treatment.

2-iP (mg/L)	Number of new shoots	Maximum shoot length (cm)	Fresh weight (g)	Dry weight (g)
0.0	2.700 ^{c}	1.710 ^{c}	0.061 ^{c}	0.011 ^{b}
0.1	6.750 ^{ab}	3.225 ^{ab}	0.379 ^{ab}	0.056 ^{ab}
0.2	8.250 ^{a}	3.005 ^{ab}	0.615 ^{a}	0.092 ^{ab}
0.3	3.550 ^{bc}	2.840 ^{b}	0.216 ^{bc}	0.038 ^{ab}
0.4	8.150 ^{a}	3.725 ^{a}	0.568 ^{a}	0.125 ^{a}
0.5	6.400 ^{ab}	3.210 ^{ab}	0.326 ^{abc}	0.060 ^{ab}
1.0	5.200 ^{abc}	2.635 ^{b}	0.317 ^{abc}	0.126 ^{a}

Means followed by the different letters within the column are significantly different according to LSD test at P≤0.05.

Zeatin increased the formation of microshoots where maximum number of shoot was obtained on medium supplemented with 0.5 mg/l zeatin (Table 3). The maximum shoot length was 4.18 cm at 0.5 mg/l, while 0.1 mg/l zeatin showed less shoot length in comparison to the other concentrations. In addition, maximum fresh and dry weight were 1.32 and 0.28 g, respectively, at 0.2 mg/l zeatin (Table 3).

Table 3. Mean number of shoots/explant, shoot length and fresh and dry weight of *Ruta graveolens* after six weeks growth period after zeatin treatment.

Zeatin (mg/L)	Number of new shoots	Maximum shoot length (cm)	Microshoot fresh weight (g)	Microshoot dry weight (g)
0.0	2.700 ^{b}	1.710 ^{d}	0.061 ^{d}	0.011^{c}
0.1	5.950 ^{ab}	2.820 ^{c}	0.364 ^{cd}	0.072 ^{bc}
0.2	9.550 ^{a}	3.265 ^{bc}	1.316 ^{a}	0.280 ^{a}
0.3	8.000 ^{ab}	3.670 ^{ab}	0.509 ^{bcd}	0.089 ^{bc}
0.4	10.450 ^{a}	4.140 ^{a}	0.952 ^{abc}	0.167 ^{ab}
0.5	11.250 ^{a}	4.180 ^{a}	1.241 ^{ab}	0.183 ^{ab}
1.0	7.350 ^{ab}	3.685 ^{ab}	0.753 ^{abc}	0.176 ^{ab}

Means followed by the different letters within the column are significantly different according to LSD test at P≤0.05.

Callus induction and culture: To find out the optimum medium for callus growth and development, fresh weight of callus was evaluated after four weeks of growth periods. Calli were initiated from leaves on MS media supplemented with different concentration of 2,4-D or NAA at 0.3, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0 mg/l (Figs 1 and 2). Results showed significant differences ($p < 0.05$) between the five tested media of either 2,4-D or NAA pertaining to their effect on callus growth. Initially small yellowish calli developed on the cut ends within seven days of inoculation, and subsequently covered the entire surface of the explants with 2,4-D (Fig. 1) or NAA (Fig. 2). Maximum callus fresh weight was obtained on MS medium enriched with 4 mg/l NAA, which yielded compact, organogenic and greenish calli. Using 2.0 mg/l 2,4-D resulted in soft, friable and yellowish calli. Of the various concentrations studied, 2.0 mg/l 2,4-D induced maximum callus fresh and dry weight compared to other media. Lower callus fresh (560 mg) and dry weight (33 mg) were recorded using MS medium supplemented with 2.0 mg/l 2,4-D (Fig. 1).Callus was induced after one month using 4.0 mg/ NAA increased callus production (Fig. 2). After two months, maximum callus induction (about 33.1 mm) was obtained on MS medium supplemented with 2.0 mg/l NAA (Fig. 2). NAA enhanced callus growth after one and 3 months, callus production increased significantly (Fig. 2). Maximum callus fresh weight was obtained at 4.0 mg/l NAA, where the callus fresh weight was 1305 and 294 mg dry weight. However, 0.3 mg/l NAA formed the lowest callus weight where the callus fresh weight was 338 mg and the callus dry weight 21 mg (Fig. 2). MS medium supplemented with 2.0 mg/l NAA produced soft and creamy friable calli. Of the various concentrations tested, maximum calli was observed on MS medium supplemented with 2.0 mg/l NAA. Hormone free medium did not show any callus formation (Fig. 2).

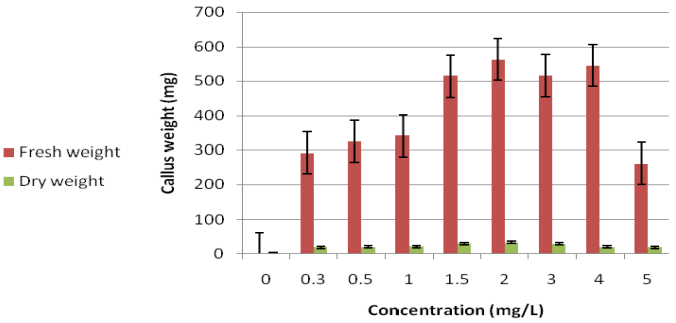

Figure 1. Effect of different 2,4-Dichlorophenoxyacetic acid (2,4-D) concentrations on fresh and dry weight of *Ruta graveolens* after four weeks of callus induction period. Error bars represent standard error (± SE).

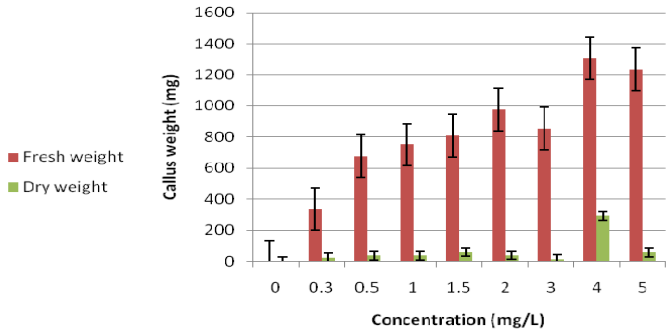

Figure 2. Effect of different naphthaleneacetic acid (NAA) concentrations on fresh and dry weight of *Ruta graveolens* after four weeks of callus induction period. Error bars represent standard error (± SE).

Callus growth and development: MS medium supplemented with 1.0 mg/l 2,4-D produced maximum fresh weight compared with other 2,4-D treatments, an overall mean callus fresh weight was 836 mg (Fig. 3).

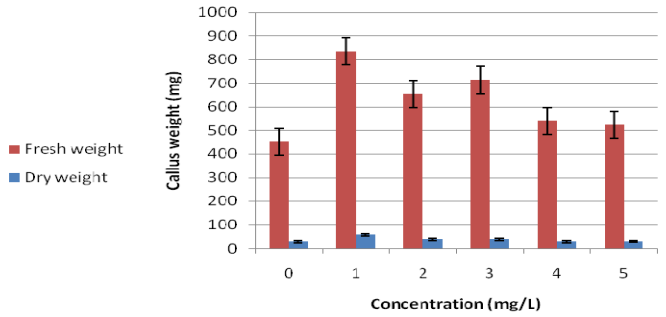

Figure 3. Effect of different 2,4-Dichlorophenoxyacetic acid (2,4-D) concentrations on callus fresh and dry weight of *Ruta graveolens* after four weeks of growth period. Error bars represent standard error (± SE).

MS medium supplemented with 1.0 mg/l NAA produced maximum callus growth compared with other treatments. Mean callus fresh weight was 794 mg when using MS medium containing 1.0 mg/l NAA compared to the four other media of NAA growth regulator. Calli developed from 1.0 mg/l NAA had the maximum fresh weight (794 mg) compared to that of all other NAA treatments (Fig. 4).

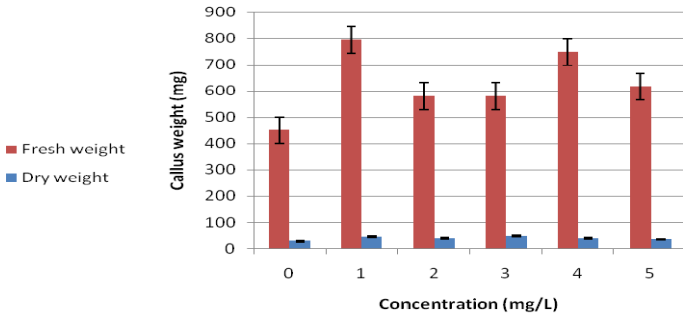

Figure 4. Effect of different naphthaleneacetic acid (NAA) concentrations on callus fresh and dry weight of *Ruta graveolens* after four weeks of growth period. Error bars represented standard error (± SE).

High performance liquid chromatography (HPLC): Plant samples were analyzed using HPLC chromatogram method. All plant samples were injected twice and the results obtained besides the HPLC chromatogram for the five standard (rutin (16.40), psoralen (22.93), xanthotoxin (23.50), bergapten (26.50) and imperatorin (31.00)) are given in Table 4. Among the different samples of *R. graveolens*, the highest rutin content was obtained in tissue culture plants extracted by using ethanol or methanol solvent where it was 3.42% and 2.16 % (w/w), respectively. The lowest concentration was in callus cultures where it was 0.22% (w/w) in methanol and 0.25% (w/w) in ethanol (Table 4). Field plants contained 0.99% (w/w) rutin in methanol extract and 1.20% (w/w) in ethanol extract.

Psoralen concentrations were always greater in field grown plants compared to tissue cultured plants when evaluated with the two solvents (methanol 0.33% w/w or ethanol 0.59% w/w) (Table 4). The solvent system had an effect on psoralen content in various cultures where the ethanol solvent showed higher

Table 4. Metabolite content of *Ruta graveolens* callus, *in vitro* (tissue culture plant) and *ex vitro* (field plant) culture using ethanolic and methanolic extraction methods.

Compound	Culture type	% w/w standard concentration	
		Methanol	Ethanol
Rutin	*Ex vitro* (field plant)	0.99	1.20
	In vitro (tissue culture plant)	2.16	3.42
	Callus	0.22	0.25
Psoralen	*Ex vitro* (field plant)	0.33	0.59
	In vitro (tissue culture plant)	0.04	0.12
	Callus	0.02	0.20
Xanthotoxin	*Ex vitro* (field plant)	0.12	0.14
	In vitro (tissue culture plant)	0.06	0.14
	Callus	0.002	0.01
Bergapten	*Ex vitro* (field plant)	0.24	0.14
	In vitro (tissue culture plant)	0.06	0.23
	Callus	0.02	0.03
Imperatorin	*Ex vitro*(field plant)	0.00	0.01
	In vitro (tissue culture plant)	0.02	0.07
	Callus	0.03	0.06

content than methanol solvent. In case of *in vitro* plants, it was 0.04 % and 0.12% (w/w) for methanol and ethanol, respectively, and 0.02% and 0.20 % (w/w) for methanol and ethanol, respectively, in case of callus culture. Callus had very negligible concentrations of xanthotoxin, approximately 0.002% in methanol and 0.01 % in ethanol solvent.

The maximum bergapten content was observed in field plants extracted by methanol (0.24% (w/w)) compared to 0.14% (w/w) in ethanol extract. In tissue culture plants, ethanol extracted bergapten was 0.23% (w/w) and it was 0.06% (w/w) when extracted by methanol. The callus culture contained lower bergapten content in both solvents; it was 0.02% in methanol and 0.03% in ethanol (Table 4). The maximum imperatorin content was 0.07% (w/w) in tissue culture ethanol extracted and was very low 0.01% (w/w) in field plants (Table 4).

Discussion

Effect of different cytokinins on shoot proliferation: Efficient methods for rapid micropropagation are needed as a preliminary step for *in vitro* propagation. The supplemented TDZ, 2-iP or zeatin increased the formation of new shoots/explant and shoot length in *R. graveolens* after six weeks growth period. Our results showed that hormone free medium did not result in a high number of new shoots formed (Tables 1-3).

The addition of TDZ enhanced shoot proliferation in *R. graveolens* (Table 1). Average number of shoots/explants at the end of the culture period varied significantly with TDZ concentration. Increasing the TDZ concentration from 0.0 (control) to 1.0 mg/l increased the mean number of proliferated shoots, and maximum shoot length was also obtained on 0.4 mg/l TDZ. Increasing TDZ concentrations increased shoot length significantly as did increasing fresh and dry weight (Table 1).

TDZ is a diphenyl urea based cytokinin growth regulator, which is non degradable by cytokinin-oxidase enzymes[19]. Furthermore, TDZ has long residual effects, increases the biosynthesis of endogenous adenine-based cytokinins and enhances nutrient uptake and assimilation in plants [19]. TDZ is considered to be one of the most active cytokinins for shoot induction in plant tissue culture [20]. This may be due to the fact that TDZ stimulates endogenous biosynthesis of cytokinins, which brings about an increase in the level of naturally occurring cytokinins [21](Table 1).

In this study, the addition of different 2-iP significantly increased the number of new shoots (Table 2). *R. graveolens in vitro* culture supplemented with 0.2 mg/l 2-iP showed greater responses (Table 2). Jahan and Anis [22] reported that 2-iP was more effective for shoot proliferation than BA and kinetin and this is similar to our results where zeatin at 0.5 mg/l was effective in the formation of new shoots (Table 3).

This is because zeatin is considered an important cytokinin that is commonly used in promoting plant regeneration [23]. Ostrolucka *et al*. [24] reported that higher multiplication rate of *Vaccinium* spp. was achieved on MS media supplemented with zeatin. An efficient shoot regeneration system of *Artemisia herba-alba* using different zeatin concentrations was achieved [25]. Therefore, this study showed that the zeatin plays an important role in microshoot development, which might be due to the higher multiplication rate.

Callus induction and growth: Calli were initiated from leaves on MS media supplemented with different concentration of 2,4-D or NAA (Figs 1 and 2). Maximum fresh weight of induced callus was achieved on MS medium enriched with 4 mg/l NAA (Fig. 2). In this study maximum callus fresh weight was obtained on MS medium supplemented with 2.0 mg/l 2,4-D and the callus was friable, light yellowish. This result similar to previous finding by Wang *et al*. [26] on *Capparis spinosa* L. in which 2,4-D produced the maximum fresh weight after four weeks. Faisal *et al*. [27] reported light yellow friable callus induction in *R. graveolens* using MS medium supplemented with 10 µM 2,4,5-trichlorophenoxyaceticacid (2,4,5-T). This result is similar to previous finding on *Oryza sativa* [28]. Our results indicated that there were significant differences among NAA treatments in regard to callus growth.

Secondary metabolites and HPLC analysis: Several types of plant material were evaluated for secondary metabolites in *R. graveolens. R. graveolens* tissue cultures are a potential source of furanocoumarins (Table 4). Higher concentrations of secondary metabolites were found in tissue culture of shoots, whereas, undifferentiated cells showed the lowest concentrations. Furanocoumarins of *R. graveolens* are present in the epidermal layer of both stems and leaves and in the mesophyll directly below the epidermis [29]. Xanthotoxin (136.8 mg/100 g dry wt.) and bergapten (210.4 mg/100 g dry wt.) were reported on microshoots. These results are in agreement with Ekiert *et al.* [30] and Ekiert and Gomolka [16] and Beier and Oertli [4] who showed that xanthotoxin was most abundant in *ex vitro* plants. Imperatorin amounts were markedly lower in all types of culture in both solvent systems compared to other metabolites. On the other hand, bergapten and xanthotoxin have also been reported in the surface wax of leaves of wild carrot (*Daucus carota* L.), a plant containing only trace levels of furanocoumarins [31]. Ekiert *et al.* [30] reported that bergapten and xanthotoxin were the dominating metabolites in *R. graveolens* shoot culture in terms of their contents. [14]. This is the first study to document the presence of all these metabolites (rutin, psoralen, xanthotoxin, bergapten and imperatorin) in *R. graveolens* at *in vitro, ex vitro* and callus at different concentrations.

Conclusions

MS medium containing 0.5 mg/l zeatin was the appropriate medium for microshoot production. Callus was successfully induced from leaf segments on MS media supplemented with 4.0 mg/l NAA under dark condition. Rutin, psoralen, xanthotoxin, bergapten and imperatorin were identified in three cultures of *ex vitro, in vitro* and callus, by performing HPLC analysis. *In vitro* shoot culture produced more secondary metabolites when compared with callus and field plants.

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