

II. AGROECOLOGY

AN EFFICIENT REGENERATION SYSTEM AND ANTIMICROBIAL ACTIVITY OF *RUTA GRAVEOLENS* L. PLANTS

N. Matvieieva, K. Drobot, L. Pasichnyk, N. Zhytkevych

Abstract. An efficient protocol of *Ruta graveolens* L. shoot regeneration has been developed. MS media supplemented with 3% sucrose, Kinetin, 6-Benzylaminopurine (BA) and α -Naphthylacetic acid (NAA) growth regulators were used to find out the regeneration ability of intermodal, root, petiole and leaf explants. Addition of BA in concentration 0.5 mg L^{-1} promoted regeneration from petioles, roots and internodes with 100% frequency. The high frequency of shoot regeneration was also observed when the explants were cultivated on the MS basal medium with BA ($0.5\text{-}1.0 \text{ mg L}^{-1}$) and NAA ($0.05\text{-}0.5 \text{ mg L}^{-1}$). Extracts of *R. graveolens* regenerated plants exhibited antibacterial activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* B918, *A. rhizogenes* A4 bacteria to the same extent as the extracts of initial plants. The extracts were not active against *Escherichia coli* and phytopathogenic bacteria. The method developed is simple and effective. It can be used for multiplication of plant material for the purpose of biologically active substances production and promote native ecosystems protection.

Keywords: *Ruta graveolens* L, shoot regeneration, growth regulators, antimicrobial activity.

1. INTRODUCTION

Development of rapid micropropagation methods, such as *in vitro* plant regeneration techniques enable a cultivation of different valuable plants in aseptic culture and allow to receive a large number of regenerated plants in a short time. The applying of those methods can be useful for further manipulations in field of plants biotechnology. Plants are a source of biologically active compounds including substances with antimicrobial and insecticide activity. However using of plants growing in nature as a source of valuable compounds is not preferable because of depending of raw material quality on several factors (weather conditions, soil toxic compounds contamination) and a potential damage of natural plant populations. Using of biotechnological approaches including micropropagational methods for valuable plants production in stable conditions are ecologically safe and may be used as an alternative approach to *in vivo* harvested plants. It should be noted that using of biotechnological methods allows to save natural plant populations and at the same time to use plant material for production of high quality plant-derived compounds for medical and agricultural purposes. Some plants that can be used to obtain compounds with insecticidal properties attract special attention. Such substances of plant origin are safe for environment, promote native ecosystems protection and have preferences in comparison with chemical insecticides using.

Recently, there has been an increased interest in regeneration methods for improvement of plants with valuable medical and commercial properties. *Ruta graveolens* L. commonly known as garden rue

belongs to such plant species. It is herbaceous perennial plant of the *Rutaceae* family grown in Mediterranean region. The simultaneously presence of alkaloids and essential oils is the peculiarity of garden rue due to rare occurrence of such combination of chemical compounds in a plant material [1]. Because of this biologically active compounds synthesis garden rue reveal the numerous medical properties. *R. graveolens* extracts can be used as antimicrobial and insecticide agent [2-6]. Active compounds from rue also possess fungicidal properties and can be used in medicine for mycosis treatment or in agriculture as alternative natural fungicide [7-9]. Flavonoids synthesized in *R. graveolens* plants have been reported to reveal antibacterial activity. Rutin is characterized with a great antioxidant activity and can be successfully used for reducing the triglyceride level in human blood [10]. Rues chemical compounds possesses also an anti-inflammatory, anticonvulsant and sedative properties [11, 12].

It is known, that *in vitro* callogenesis and regeneration effectiveness depends from the plant genotype, type of explants and also from culture medium content (the presence and concentration of growth regulators). The *Ruta graveolens* rapid shoot induction was noted from nodal segments [13-15]. Proliferation of shoots on this type of explant was obtained with high frequency – from 22% (on the medium supplemented with 0.5 mM kinetin) up to 98% on the media supplemented with 10 mM 6-Benzylaminopurine (BA) and 2.5 mM α -Naphthylacetic acid (NAA) or 3-Indoleacetic acid

(IAA). The frequency of shoot formation from leaves was lower (up to 45%) [16].

The conditions for an efficient callus formation from leaves, stems and roots were studied in [17]. The application of basal media supplemented with kinetin in combination with 2,4-Dichlorophenoxyacetic acid (2,4-D) lead to callogenesis with callus index formation 0.651 – 34.140. Stems cultivation on the medium supplemented with 1 mg L⁻¹ kinetin and 1 mg L⁻¹ 2,4-D was found as the optimal “explant type/medium content” combination for calli induction. So, there are studies on shoot regeneration from *Ruta graveolens* nodal and leaves explants. However, rue plants have phenotypical peculiarities in aseptically culture, particularly they form a small size leaves but petioles are significantly elongated, up to 40 mm long. In compare, the leaf diameter is about 2-3 mm, the intermodal segments size is less than 10 mm. So it is of interest to study the effectiveness of elongated petioles for the plant regeneration.

Study of growth regulators effect on *R. graveolens* shoot regeneration and antimicrobial activity of plant extracts was the aim of this work.

2. MATERIALS AND METHODS

2.1 Plant material and regeneration procedure

Surface sterilized *Ruta graveolens* seeds were used to introduce the plants in an aseptically culture. The plants were cultivated on hormone-free MS [18] basal medium. MS basal solidified media supplemented with 3% sucrose (w/v) and growth regulators Kinetin (Kin), 6-Benzylaminopurine (BA) and α -Naphthalene acetic acid (NAA) were used to find out the regeneration ability of four types of *R. graveolens* explants. Cutted slightly intermodal, root, petiole segments (10 mm length) and leaves were used for shoot regeneration. Growth regulators in different concentrations (Table 1) were used in the media to evaluate their effect on shoot induction ability. Regeneration frequency as the percentage of plant regeneration response was calculated dividing the number of shoot produced explants by the total number of explants and multiplied by 100.

2.2 Testing of antibacterial activity

The regenerated and initial plants crude extracts were tested for antibacterial activity using *Bacillus subtilis* ATCC 6633, *Escherichia coli* B906, *Staphylococcus aureus* B918 human pathogenic

bacteria and also using phytopathogenic bacteria *Pectobacterium carotovorum* subsp. *carotovorum* 8982, *Erwinia rhapsodici* Я7, *Xanthomonas campestris* pv. *campestris* 8003b, *Xanthomonas oryzae* pv. *oryzae* 122, *Agrobacterium tumefaciens* 8628, *Clavibacter michiganensis* subsp. *michiganensis* 102, *Pseudomonas syringae* pv. *syringae* 8511, *Pseudomonas syringae* pv. *atrofaciens* 9400, *Pseudomonas syringae* pv. *coronafaciens* 9030, *Pseudomonas fluorescens* 8573, *Agrobacterium rhizogenes* A4. The bacteria were obtained from the Institute of Microbiology and Virology NAS of Ukraine collection. The inhibitory effect to *B. subtilis*, *E. coli*, *St. aureus* and *A. rhizogenes* was determined according to the Kirby and Bauer disk diffusion method (National Committee for Clinical Laboratory Standards, 1993). Water, DMSO, ethanolic and acetone extracts (0.03 ml) were added to filter paper disks (6 mm in diameter), allowing the solvent to evaporate. Disks with pure solvents (0.03 ml) were used as negative control. Disks with antibiotics gentamicin, erythromycin, ciprofloxacin were used as positive control substances. The bacteria were incubated in Petri dishes for 24 h at 28°C (*B. subtilis*, *A. rhizogenes*) or 37°C (*E. coli* and *S. aureus*). The inhibition zones for bacteria were measured after 24 h.

Determination of the sensitivity of some phytopathogenic bacteria to plant extracts was carried out by limiting dilution method on a potato agar (PA) solid nutrient medium. 0.1 ml of the bacterial suspension in concentration 1x10⁹ colony forming units/ml was inoculated in Petri dishes with PA medium. 0.1 ml of plant extracts in two-, five- and tenfold dilution were added in the middle of each dishes. Growth inhibition zones were measured after 24-48 h incubation of bacteria at 28°C. The experiments were repeated three times.

3. RESULTS AND DISCUSSION

Regeneration ability of internodes was the highest and shoots formation frequency varied from 30% to 100% depending on the growth regulators type and concentrations. Cultivation of the internodes on the media with BA in 0.5 - 2.5 mg L⁻¹ concentration or on the media which contained both BA (0.5 - 1.0 mg L⁻¹) and NAA (0.05 - 0.5 mg L⁻¹) made it possible to regenerate new shoots with 100% frequency after short period of time - 10 days (Table 1, Fig. 1).

Table 1. *R. graveolens* regeneration frequency on the solidified Murashige and Skoog basal medium supplemented with growth regulators (23 days growth)

Medium, No	Concentration of growth regulators (mg L ⁻¹)			Regeneration frequency of different explants types (%)			
	BA	NAA	Kin	Leaves	Roots	Petioles	Internodes
1	0.5	—	—	50	100	100	100
2	1	—	—	20	100	40	100
3	2.5	—	—	60	0	80	100
4	0.5	0.05	—	50	100	100	100
5	0.5	0.1	—	30	100	80	100
6	0.5	0.5	—	80	60	40	100
7	1	0.05	—	40	100	100	100
8	1	0.1	—	10	0	40	100
9	1	0.5	—	0	0	40	100
10	2.5	0.05	—	30	60	100	100
11	2.5	0.1	—	0	0	80	30
12	2.5	0.5	—	0	0	80	100
13	—	—	0.5	20	0	15	0
14	—	—	1	0	0	0	70
15	—	—	2.5	0	0	40	70
16	—	0.05	—	0	0	0	0
17	—	0.1	—	0	0	40	10
18	—	0.5	—	0	0	0	0

The frequency of shoot formation on the leaves was the lowest as compared to the one using roots, internodes or petioles as explants for shoot regeneration and varied within 0-80%. The most effective was cultivation of leaves on MS medium supplemented with 0.5 mg L⁻¹ BA and 0.5 mg L⁻¹ NAA. This combination of growth regulators gave the opportunity to increase the regeneration frequency up to 80%.

Cultivation of root explants on the medium containing only BA (0.5-1.0 mg L⁻¹ concentration) or on the medium containing both BA and NAA at the concentration combinations 0.5 + 0.05 mg L⁻¹,

0.5 + 0.1 mg L⁻¹, 1.0 + 0.05 mg L⁻¹ allowed to regenerate new shoots at 100% frequency.

Regeneration frequency varied from 15 to 100% in case of petioles usage for plant regeneration. It was higher than frequency of shoot formation from leaves and roots and was compared to the one of used internodes for induction of shoot regeneration. The maximal 100% regeneration frequency was observed after petioles cultivation on the medium containing BA in 0.5 mg L⁻¹ concentration or after using the BA+NAA hormone combination (0.5+0.05, 1.0+0.05 and 2.5+0.05 mg L⁻¹) in basal MS medium.

It appeared that BA in high concentration (2.5 mg L⁻¹) have oppressed the regeneration and have led to production of lower number of shoots in almost all types of explants excepting internodes.

The high effectiveness of the simultaneous application of NAA and BA has been shown for many plant species cultivation, for example, *Basilicum polystachyon* [19], *Musa sapientum* [20], *Rauwolfia serpentina* [21], *Citrullus colocynthis* [22]. In our study BA and NAA simultaneous addition to the basal MS medium was the most effective for *R. graveolens* shoot regeneration using all type of explants. The addition of NAA in higher concentration (0.1-0.5 mg L⁻¹) to the media supplemented with BA (0.5-2.5 mg L⁻¹) actually have led to oppression of shoot regeneration and have caused the reduction of the number of petiole explants with shoots formed.

Using of kinetin was not effective for induction of shoot formation from all type of explants used. Regeneration frequency after using leaves, roots, petioles and internodes cultivation on the media with 0.1-2.5 mg L⁻¹ kinetin was 0 - 20%, 0%, 0-40%, 0-70% respectively. Addition of NAA without BA addition to the basal MS medium resulted in root formation in case of leaves and roots explants usage and in 40% or 10% shoot regeneration frequency in case of petioles and internodes using as the explants.

Regenerated *R. graveolens* plants did not differ phenotypically from the original plants and rooted on the basal MS medium (Fig. 1).

Earlier the antimicrobial activity (AMA) of *Ruta graveolens* plant extracts against *Escherichia coli*, *Serratia marcescens*, *Sarcina ventriculi*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and other human pathogenic bacteria was evaluated [3, 23, 24] by using *in vivo* cultivated plants. We conducted the experiments to evaluate the AMA of extracts of regenerated plants compared to the AMA of extracts of the initial *in vitro* propagated plants purposely to evaluate the possibility of using biotechnological (regenerated) plants to produce antimicrobial substances.

The results obtained in the evaluation of the activity of the different extracts against tested bacteria are shown in Table 2. Both extracts from regenerated plants and from the initial plants inhibited *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* B918 and *A. rhizogenes* A4 bacteria growth. The extracts from initial and

regenerated plant showed 8-18 and 8-20 mm diameter zone of inhibition respectively against tested *S. aureus* bacteria; 15-22 and 14-21 mm inhibition zone against *B. subtilis*; 12-22 and 12-20 mm inhibition zone against *A. rhizogenes*. Inhibition zone diameter depended on the solvent used for extraction (water, ethanol, DMSO, acetone).

The extracts did not inhibit *E. coli* and phytopathogenic *Pectobacterium carotovorum* subsp. *carotovorum* 8982, *Erwinia rhapontici* Я7, *Xanthomonas campestris* pv. *campestris* 8003b, *Xanthomonas oryzae* pv. *oryzae* 122, *Agrobacterium tumefaciens* 8628, *Clavibacter michiganensis* subsp. *michiganensis* 10₂, *Pseudomonas syringae* pv. *syringae* 8511, *Pseudomonas syringae* pv. *atropfaciens* 9400, *Pseudomonas syringae* pv. *coronafaciens* 9030, *Pseudomonas fluorescens* 8573 bacterial growth.

CONCLUSION

Regeneration frequency of *R. graveolens* plants was affected by type of explants and also by media composition. The better results were obtained when internodes were used for plants regeneration. In this case 100% regeneration frequency was noted after cultivation on the eleven media from 18 media tested. Comparison of the effect of media composition on shoot regeneration frequency from petioles and other explant types (leaves, roots, internodes) showed that cultivation on media supplemented with 0.5 mg L⁻¹ BA, 0.5 mg L⁻¹ BA+0.05 mg L⁻¹ NAA or 1.0 mg L⁻¹ BA+0.05 mg L⁻¹ NAA has an advantage for *R. graveolens* multiplication by means of shoot regeneration. Although the rate of shoot formation on the petioles was in some cases slightly lower than the one on the internodes, petioles as explant type are more preferable because of greater length compared to the length of internodes – 40 mm and 10 mm respectively. Application of one petiole enable to obtain more explants number and respectively more *R. graveolens* plants regenerated than using of one internode. So, application of petioles regeneration technology improves the efficiency of the plant material using which is important in biotechnology research. Extracts of *R. graveolens* regenerated plants exhibited antibacterial activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* B918, *A. rhizogenes* A4 bacteria to the same extent as the extracts of initial plants.

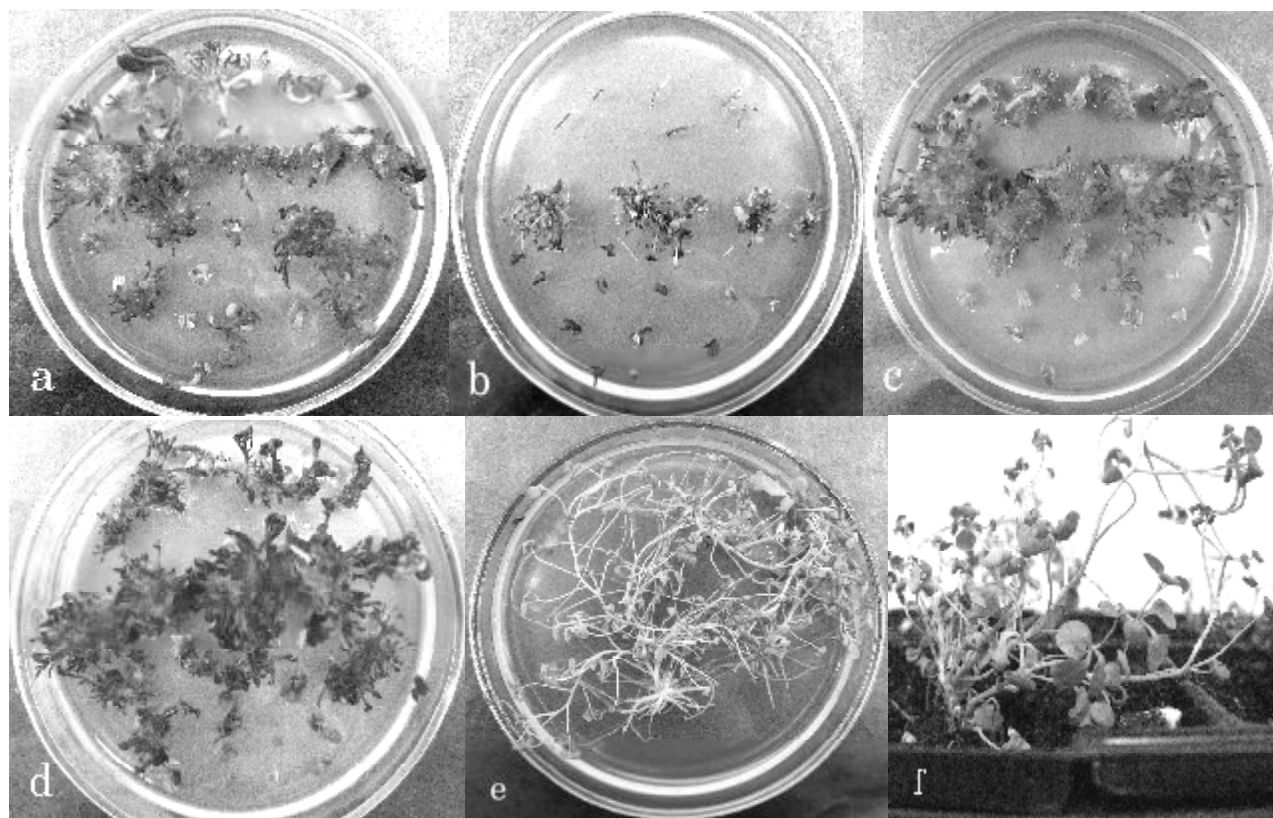


Fig. 1. *Ruta graveolens* plant regeneration on the media supplemented with 2.5 mg L⁻¹ BAP (a), 1 mg L⁻¹ kin (b), 1 mg/l BAP (c), 0.5 mg L⁻¹ BAP and 0.05 mg L⁻¹ NAA (d), *in vitro* root formation (e) and growth of plants in soil (f)

Table 2. Antibacterial activity of *R. graveolens* extracts

Bacterial strains	Inhibition zone, mm										
	<i>R. graveolens</i> extracts								Antibiotics		
	Initial <i>in vitro</i> plants				Regenerated plants						
	Water	DMSO	EtOH	Acetone	Water	DMSO	EtOH	Acetone	Gm	Em	Cf
<i>E. coli</i> B906	0	0	0	0	0	0	0	0	22	7	31
<i>B.subtilis</i> ATCC 6633	15	20	22	21	14	21	21	21	29	34	43
<i>S.aureus</i> B918	8	16	18	17	8	15	20	15	24	29	27
<i>A. rhizogenes</i> A4	12	20	22	19	12	20	20	20	22	22	40

Gm – Gentamicin, Em – Erythromycin, Cf - Ciprofloxacin

But the extracts were no active against phytopathogenic bacteria tested - *Pectobacterium carotovorum* subsp. *carotovorum* 8982, *Erwinia rhapontici* Я7, *Xanthomonas campestris* pv. *campestris* 8003b, *Xanthomonas oryzae* pv. *oryzae* 122, *Agrobacterium tumefaciens* 8628, *Clavibacter*

michiganensis subsp. *michiganensis* 102, *Pseudomonas syringae* pv. *syringae* 8511, *Pseudomonas syringae* pv. *atropfaciens* 9400, *Pseudomonas syringae* pv. *coronafaciens* 9030, *Pseudomonas fluorescens* 8573 and also were not active against *Escherichia coli* B906.

So, the method developed for rapid *R. graveolens* plants multiplication is simple, reproducible and allows shoot regeneration process from the different types of explants with up to 100% frequency. It can be used for multiplication of plant material for the purpose of biologically active substances production and for construction of transgenic plants.

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ЕФЕКТИВНА СИСТЕМА ЗА РЕГЕНЕРИРАНЕ И АНТИМИКРОБНА АКТИВНОСТ НА *RUTA GRAVEOLENS* L.

Матвеева Н., К. Дробот, Л. Пасичник, Н. Житкевич

Резюме. Разработен е ефективен метод основан на регенериращата способност на растителни израстъци на *Ruta graveolens* L. За провеждане регенерацията на междувъзлия, корени, листни дръжки и листни експланти беше използвана среда MS с добавени растителни регулатори, 3% захароза, кинетин, 6-бензиламинопурин (БАП) и α -нафтилоцетна кеселина (НОК). Добавянето на БАП в концентрации 0,5 mg.dm⁻³ допринася за 100 % регенерация на листните дръжки, корените и междувъзлията. Висока честота на регенерация се наблюдава и когато експлантите са култивирани на основната среда MS с добавка на ПАБ (0,5-1,0 mg.dm⁻³) и НОК (0,05-0,5 mg.dm⁻³). Екстракти от такива регенерирани растения показаха антибактериална активност срещу *Bacillus subtilis* ATCC 6633, но не и срещу *Escherichia coli* и фитопатогенни бактерии. Разработеният метод е прост и ефективен. Може да се използва за възпроизвеждане на растителен материал за получаване на биологично активни вещества и заедно с това съхраняващ естествените екосистеми.

Ключови думи: *Ruta graveolens* L, регенерация, растителни израстъци, растежни регулатори, антимикробна активност.

Matvieieva N.A., PhD

Head of the Laboratory of Adaptational Biotechnology,
Institute of Cell Biology and Genetic Engineering NAS of Ukraine,
148 Zabolotnogo str., 03143, Kyiv, Ukraine,
For corresponding: Tel. +38 044 5267104; Email: joyna56@gmail.com

Drobot K.O., PhD student

Laboratory of Adaptational Biotechnology,
Institute of Cell Biology and Genetic Engineering NAS of Ukraine,
148 Zabolotnogo str., 03143, Kyiv, Ukraine

Pasichnyk L.A., Dr Sci,

Senior scientist, Phytopathogenic Bacteria Department,
Institute of Microbiology and Virology NAS of Ukraine,
154 Zabolotnogo str., 03143, Kyiv, Ukraine

Zhytkevych N.V., engineer

Phytopathogenic Bacteria Department,
Institute of Microbiology and Virology NAS of Ukraine,
154 Zabolotnogo str., 03143, Kyiv, Ukraine