Regeneration of *Ruta graveolens* Transgenic Plants

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**ABSTRACT**

Here we report the construction of transgenic *Ruta graveolens* L. plants. The plants were obtained via direct shoot regeneration from leaves and petioles on Murashige and Skoog (MS) solidified medium supplemented with 6-benzylaminopurine (1.0 mg L⁻¹) and α-naphthylacetic acid (0.05 mg L⁻¹) in case of *A. tumefaciens*-mediated transformation as well as via direct shoot regeneration from rue “hairy” root culture on hormone free half-strength MS medium in case of *A. rhizogenes*-mediated transformation. Frequency of “hairy” root formation was up to 30%. All transgenic root lines were able to form shoots. Using of *A. tumefaciens* allowed to obtain transgenic rue plants with high frequency up to 100%. Indolil butyric acid addition to the medium in concentration 0.5 mg L⁻¹ enabled transgenic plant rooting. The shoots regenerated from rue “hairy” root culture were more sensitive to the growth regulator addition and rooted in 2 weeks. Rooting of the shoots obtained after *A. tumefaciens*-mediated transformation was observed only in 6 weeks under these cultivation conditions.

**Keywords:** *Ruta graveolens* L., *A. tumefaciens*, *A. rhizogenes*, genetic transformation, plant regeneration, “hairy” root culture.

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**Introduction**

*Ruta graveolens* L. is a medical plant of Rutaceae family. Numerous compounds of medical properties are known to be synthesized in rue plants. Rutin, alkaloids, essential oils and other chemicals were found in its aerial parts and roots (Orlita et al., 2008; Ekiert et al., 2005; França Orlanda et al., 2015). Rue extracts are characterized by antimicrobial (Ivanova et al., 2005; França Orlanda et al., 2015), antifungal (Meepagala et al., 2005), analgesic (Cunha et al., 2015) activity. The antioxidant and anti-inflammatory properties of *Ruta graveolens* L. were analysed earlier (Raghav et al., 2006). The study of its anti-tumor activity demonstrated that rue extracts were cytotoxic for lymphoma and carcinoma cells (Preethi et al., 2006). Ethanolic rue extracts were not toxic but caused the death of skin melanoma cells (Ghosh et al., 2015). So, *R. graveolens* plants could be used as a natural source for medical compounds production and may be used in pharmaceutical industry.

Biotechnological methods of plant improvement include construction of the transgenic plants of numerous species characterized by high level accumulation of valuable natural compounds (Sharafi et al., 2013; Zhang et al., 2014; Thiruvengadam et al., 2014; Majumdar et al., 2012). At the same time transgenic plants are able to synthesize the recombinant proteins of medical properties. So the transgenic plants are the promising source both of natural and recombinant biological active substances.

Transgenic plants construction was started at the end of 20th century. *Agrobacterium tumefaciens* and *A. rhizogenes* bacterial strains were used for this purpose. Plant transformation using *A. tumefaciens* strain resulted in direct (from plantlets) or indirect (from primarily formed callus tissue) shoot formation. Transgenic plants can also be obtained via shoot regeneration from “hairy” root culture initiated after *A. rhizogenes* – mediated transformation of plants. So, the shoot regeneration methods must be developed...
both in case of A. rhizogenes or A. tumefaciens – mediated transformation. Different types of explants (leaves, roots, hypocotyls, petioles, stems) can be used for shoot regeneration. Methods based on using of growth regulators allow to obtain the regenerated shoots (Diwan et al., 2008; Ahmad et al., 2010).

A. tumefaciens-mediated transformation method was optimized for R. graveolens transgenic plants obtained via direct plant regeneration from hypocotyls (Lièvre et al., 2009). Transformation efficiency reached up to 22% in case of using for the transformation pTDE4 plasmid harboring genes encoding neomycin phosphotransferase and β-glucuronidase. Hypocotyls, callus and shoots of R. graveolens were used for genetic transformation experiments but “hairy” root culture establishment appeared successful only after inoculation of hypocotyls with A. rhizogenes wild strain (Sidwa-Gorycka et al., 2009).

The aim of the present study was to obtain transgenic R. graveolens plants. We used A. tumefaciens GV3101 strain carrying plasmid pCB124 (Luchakivskaya et al., 2011) harboring target ifn-a2b gene coding human interferon-α2b synthesis as well the selective neomycin phosphotransferase II gene. We also used A. rhizogenes A4 strain for establishment of “hairy” root culture and obtaining of the regenerated transgenic rue plants.

Material and Methods

Plant material

Leaves and petioles of aseptic Ruta graveolens plants cultivated on half-strength Murashige and Skoog medium (1/2MS) (Murashige and Skoog 1962) were used for agrobacterial infection.

Determination of selective concentration of antibiotic kanamycin

To find out the optimal concentration of kanamycin for transformant selection we placed the shoots of in vitro rue plants on solidified ½MS medium with different concentrations of kanamycin (0–200 mg/l) (“Kievmedpreparat”, Ukraine) and cultivated them in Petri dishes for 4 weeks. The medium with minimal concentration of kanamycin which inhibited growth of green plants was chosen as selective one.

Bacterial strains and plasmid vectors

A. tumefaciens GV3101 nopaline strain with the binary pCB124 vector was used for genetic transformation. The T-DNA of the vector contained the neomycin phosphotransferase selective nptII gene under the control of nopaline synthase promoter. The used vector carried the target ifn-a2b human interferon gene (pCB124 vector) driven by cauliflower mosaic virus 35S promoter. We also used A. rhizogenes agropine A4 strain for rue genetic transformation and “hairy” root culture initiation.

The bacteria were cultivated overnight at 28°C in liquid LB medium (10 g/l of casein hydrolyzate, 5 g/l yeast extract, 10 g/l NaCl, pH 7.2, 100 mg/l carbenicillin and 50 mg/L rifampicin) on rotary shaker (200 rpm). After overnight cultivation the bacterial cultures were resuspended in liquid MS medium (OD_600 0.5–0.6).

Genetic transformation, selection and regeneration of transformants

The petiole and leaf segments were inoculated by the bacterial suspension for 30 min and then transferred onto solidified MS medium supplemented with 1.0 mg/L 6-benzylaminopurine (BA), 0.05 mg/L α-naphthylacetic acid (NAA), 30 g/L sucrose, 0.7 % agar for direct shoot regeneration in case of A. tumefaciens-mediated transformation. The infected plantlets were transferred on the mentioned medium for 1-4 days period and then were cultivated on the same medium with 600 mg/L cefotaxime (Darnitsa, Ukraine) for bacteria elimination. The plantlets were put on MS medium supplemented with 1.0 mg/L BA, 0.05 mg/L NAA, 600 mg/L cefotaxime and 25 mg/L kanamycin after A. tumefaciens-mediated transformation for selection of transgenic plants. Regenerated shoots of 1.5–2 cm long were transferred onto 1/2MS medium supplemented with 25 mg/L kanamycin and 600 mg/L cefotaxime. The obtained shoots were cultivated at 24°C under 16-hour photoperiod.

In case of A. rhizogenes-mediated transformation the explants were cultivated on 1/2MS solidified medium for “hairy” root induction. The roots formed on the plantlets were transferred to hormone-free 1/2MS medium with 600 mg/L cefotaxime in 3-8 days. The same medium with the antibiotic in mentioned concentration was used for stimulation of transgenic shoot regeneration on “hairy” root culture.

The rooted plants were transferred into soil and grown in the greenhouse.

Molecular analysis of transgenic plants

Total plant DNA was extracted from leaves of the selected transformed and wild-type plants and also from the “hairy” root culture samples by CTAB-method (hexadecyl trimethyl ammonium bromide). The total agrobacterial DNA was extracted according to Draper et al., (1988). We used primers 5’-cctgaatgaactccaggacgaggca-3’ and
5’-cttagatccccagctccgcacag-3’ for amplification of 622 bp *nptII* gene fragment; primers 5’-ttgatgcttctgcaag-3’ and 5’-tttctgcttctcagcaagtcttctagagcag-3’ for amplification of 396 bp *ifn-α2b* gene fragment; 5’-atgttagcttctgcaaga-3’ and 5’-ttagctttcttgagctttactgcag-3’ for amplification of 780 bp (*rolB*) using PCR method.

The amplification of the gene fragments was carried on under following conditions: *nptII* gene fragment amplification - 3 min at 94 °C - 33 cycles (30 s at 94 °C; 30 s at 65 °C; 30 s at 72 °C) - 3 min at 72 °C; *ifn-α2b* - 3 min at 94 °C - 30 cycles (30 s at 94 °C; 30 s at 60 °C; 30 s at 72 °C) - 3 min at 72 °C; *rolB* - 3 min at 94 °C - 33 cycles (30 s at 94 °C; 30 s at 56 °C; 30 s at 72 °C) - 3 min at 72 °C. All samples were fractionated in 1% agarose gel in TBE buffer.

**Results**

**Determination of kanamycin selective concentration**

Green shoots were formed if the medium contained less than 25 mg/l kanamycin. Whitening of shoots was observed in case of higher concentrations of antibiotic. Basing these data, we consider 25 mg/l kanamycin to be the best for selection of rue transformants.

**Transgenic shoot regeneration after A. tumefaciens-mediated transformation**

Earlier we demonstrated the possibility of high efficient shoot regeneration from leaves, roots, petioles and stems of *in vitro* cultivated *R. graveolens* plants (Matvieieva et al., 2015). For transgenic shoot regeneration after *A. tumefaciens*-mediated transformation we used the medium optimized for effective plant regeneration. This medium contained 1.0 mg L⁻¹ BA+0.05 mg L⁻¹ NAA growth regulators. The regeneration frequency of *R. graveolens* plants was found to be affected not only by media composition but also by type of explants (Matvieieva et al., 2015). Cultivation of rue petioles and leaves on the medium mentioned above resulted in shoot regeneration of 100% frequency. We consider petioles to be more preferable explants because of their greater length comparing to the length of internodes of *in vitro* cultivated rue plants (Fig. 1 a). So the petiole and leaf explants were chosen for *A. tumefaciens* inoculation because of their high regenerative capability.

We reported the possibility of direct shoot regeneration after plantlet cocultivation with *A. tumefaciens* bacterial culture. Period of plantlet cultivation on the medium without cefotaxime addition was shown to be an important factor for successful shoot regeneration. At the same time the prolongation of the cultivation period on the medium without antibiotic adding from one to four days resulted in increase of frequency of shoot formation from 61.1±1.9 to 100%. The direct shoot regeneration was observed in 10-14 days after transformation (Fig. 1 b). The presence of *nptII* gene in the constructs enabled the selection of transformants so that green plants were formed on the medium containing 25 mg/l kanamycin. The frequency of transformation (number of explants with regenerated shoots) depended on the type of explants (leaves or petioles respectively).

The shoots 10-15 mm long were transferred onto 1/2MS medium for further growth and rooting. Root formation was observed only after 8 weeks of shoot cultivation on hormone free medium (Fig. 1 c). The rooted plants were further cultivated under greenhouse conditions (Fig. 1 d).

**“Hairy” root culture induction and shoot regeneration**

We analyzed the dependence of root formation frequency on the time of plantlets cultivation on the medium without cefotaxime addition (3-8 days) because rue extract was found to inhibit *A. rhizogenes* growth. No root formation was observed in case of cefotaxime addition to 1/2MS medium in 3-5 days after transformation. Increasing of duration of plantlet cultivation on the medium without antibiotic up to 8 days enabled the agrobacterial gene transfer and “hairy” root culture initiation. Root formation on the plantlets started in 14-16 days after their cocultivation with bacterial suspension (Fig. 2 a, b). The frequency of root formation was up to 30%. The induced root culture was characterized by typical “hairy” root phenotype which is associated with the transfer of *T*₅ fragment with *rol* genes from *A. rhizogenes* T-DNA of the agropine type Ri plasmid (Tepfer 1990) and did not require any external auxines addition to the medium for their growth.

The obtained roots were subcultured every two weeks on the medium supplemented with cefotaxime (Fig. 2 c). Cultivation of *R. graveolens* “hairy” roots on hormone-free 1/2MS medium resulted in direct shoot regeneration in 6 months after the transformation (Fig. 2 d). The shoots were taken from the root culture and then grown on the same medium for rooting. Roots formation was observed in 3 weeks of their cultivation on hormone-free medium. The period required for plant rooting was considerably smaller than the one necessary for root formation in case of *A. tumefaciens*-mediated transformation. We consider this fact as an effect of *A. rhizogenes* genes transfer to the plants.
The PCR analysis proved the presence of \textit{nptII} and \textit{ifn-α2b} genes for 100% of studied plants regenerated after \textit{A. tumefaciens}–mediated transformation and also the presence of \textit{rolB} gene in case of study of DNA of the plants regenerated on the “hairy” roots (Fig. 3).

As we observed the time differences in rooting of the transgenic plants regenerated after \textit{A. rhizogenes}– and \textit{A. tumefaciens}–mediated transformation we studied the effect of Indolilbutiric acid (IBA) growth regulator in order to find out the way to make the period of root formation shorter. Using of 0.5 mg L\(^{-1}\) IBA resulted in initiation of root formation in 6 or 2-week period on the shoots regenerated after \textit{A. tumefaciens}-mediated transformation and the ones regenerated on the “hairy” roots respectively. So this regulator can be used to advance the period of \textit{R. graveolens} rooting.

There is very limited information about \textit{R. graveolens} genetic transformation though these plants are known to be susceptible to agrobacterial infection (Porter 1991). \textit{R. graveolens} “hairy” root cultures were established after inoculation of hypocotyls with wild \textit{A. rhizogenes} strain LBA 9402 (Sidwa-Gorycka et al., 2009) in order to study the level of pinnarin, rutacultin, bergapten and other compounds accumulation. It should be noted that transgenic plants were not obtained from the “hairy” root in these experiments. \textit{A. tumefaciens} bacteria were used earlier for \textit{R. graveolens} transformation by Lièvre et al., (2009). Authors reported obtaining of transgenic plants after hypocotyl co-cultivation with \textit{A. tumefaciens} strain C58C1Rif containing plasmid pTDE4 harbouring neomycin phosphotransferase and beta-glucuronidase encoding genes using acetylsyringone as virulence inducer. Routine transformation efficiency of \textit{R. graveolens} was 11% in these experiments.

Here we firstly demonstrated the possibility of \textit{R. graveolens} plants initiation after \textit{A. rhizogenes}-mediated transformation. The obtained shoots were characterized with more fast rooting compared with the shoots regenerated after \textit{A. tumefaciens}-mediated transformation.

We also demonstrated an opportunity for direct regeneration of transgenic rue plants carrying human interferon-α2b gene. Optimization of transformational conditions in particular the prolongation of the cultivation period on the medium without antibiotic cefotaxime adding from one to four days resulted in increase of frequency of shoot formation up to 100%. We would like to note that acetylsyringone as \textit{Agrobacterium} virulence inducer were not used in out experiments. Lièvre et al., (2009) noted a significant increase in the number of kanamycin-resistant plants by using acetylsyringone. However, our research has shown also the possibility of obtaining of transgenic \textit{R. graveolens} plants with high frequency without use of this compound.

**Conclusions**

Thus the transgenic \textit{R. graveolens} plants can be obtained via direct shoot regeneration from leaves and petioles in case of \textit{A. tumefaciens}-mediated transformation as well as after shoot regeneration from rue “hairy” roots in case of using \textit{A. rhizogenes} for transformation. Using of \textit{A. tumefaciens} allows the obtaining of transgenic rue plants with higher frequency up to 100%. Frequency of “hairy” root formation was up to 30% because of antibacterial activity of rue plants against \textit{A. rhizogenes}. Time of explant co-cultivation on the medium without selective antibiotic had an effect on the successful \textit{A. rhizogenes}-mediated transformation and “hairy” root formation on the plantlets. The ability to root of the shoots of rue “hairy” root origin exceeded the same ability of the plants obtained via \textit{A. tumefaciens}-mediated transformation. IBA addition to 1/2MS medium in concentration of 0.5 mg/L resulted in root growth stimulation.

The described transgenic technique could be applied to improve plant characteristics and transfer of foreign genes to rue plants. Moreover, such approach enables the obtaining of transgenic \textit{R. graveolens} plants carrying gene coding synthesis of recombinant medicinal compounds.

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Figure 1. Regeneration of rue shoots after A. tumefaciens-mediated transformation: in vitro cultivated plants used for transformation (a); green shoot regeneration on the selective medium (b); transgenic plants under in vitro conditions (c) and in greenhouse (d)

Figure 2. Initiation of Ruta graveolens “hairy” root culture and shoot regeneration after A. rhizogenes-mediated transformation: root formation on rue petiole (a) and leaf explants (b); “hairy” root culture (c); direct shoot regeneration on hormone free 1/2MS medium (d)

Figure 3. PCR analysis of the rue plants: 1, 2, 4, 5 - DNA of the plants regenerated after A. tumefaciens-mediated transformation; 7, 8 - DNA of the plants regenerated on the “hairy” root clones; 3, 6, 9 -negative control (untransformed plant DNA); npt, ifn, rolB - nptII (622 bp), ifn-α2b (396 bp), and rolB (780 bp) genes fragments; M - DNA marker ladder, Fermentas.

References

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