Plant Regeneration and Thiophene Production in Hairy Root Cultures of *Rudbeckia hirta* L. Used as an Antagonistic Plant to Nematodes*

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**Abstract:** In *Rudbeckia hirta* L., an antagonistic plant to nematodes, hairy roots were induced by infection with a wild strain (A-5) of *Agrobacterium rhizogenes*. Hairy roots cultured in half-strength MS agar medium without phytohormones showed vigorous growth and extensive lateral branching. Mikimopine (opine) was detected in the extract of hairy root by paper electrophoresis. Adventitious shoots were induced on the surface of hairy roots after 30 to 50 days of transfer to half-strength MS agar medium supplemented with BAP at 0.5—10.0 mg/l. The highest frequency of shoot formation was obtained at 0.5 or 1.0 mg/l BAP in combination with 0.1 mg/l NAA. Plants regenerated from hairy roots showed morphological alterations such as wrinkled leaves, small size of flowers and abundant lateral branching of roots. A nematocidal compound, \(\alpha\)-terthienyl, was detected in the extract from lateral roots of the regenerant.

**Key words:** *Agrobacterium rhizogenes*, Antagonistic plant, Hairy root, Nematode, *Rudbeckia hirta*, Thiophene.

Rudbeckia hirta* L. in the family Compositae is a perennial ornamental crop that is mainly used as a garden flower and a landscape plant. It has been recently demonstrated that this plant species has a nematode control ability in its roots\(^{19}\). In an antagonistic plant to nematodes, abundant roots with extensive lateral branching would be efficient for reducing the population of nematode in soil.

Hairy roots induced by infection with soil bacterium *Agrobacterium rhizogenes*, which causes hairy root disease in dicotyledonous plants, exhibit proliferous root growth\(^{3,5,10}\). Several approaches have been made to produce secondary metabolites such as ginseng\(^{4,17}\) and tropine alkaloids\(^{16}\) in hairy root cultures of some plant species. In *Tagetes patula* L. (marigold), which is an effective antagonistic plant to nematodes, thiophene, heterocyclic sulfurous compounds with strong biocidal activity, was also produced in the hairy root cultures\(^{1,2,8,10}\). On the other hand, plants regenerated from hairy roots in several plant species exhibit various phenotypes such as short internodes, wrinkled leaves and abundant root development\(^{6,12,19}\). Especially, the morphological alteration in root system in transformant by *A. rhizogenes* may be available for improvement of nematocidal ability in antagonistic plants.

In this paper we describe plant regeneration
and production of thiophene in hairy root cultures induced by a wild species of *A. rhizogenes* in *R. hirta*.

**Materials and Methods**

1. **Plant material**
   
   Seeds of *R. hirta* cv. Highway Yellow were surface-sterilized in sodium hypochlorite solution (1% active chlorine) for 10 minutes and rinsed three times with sterilized water and then placed on agar solidified Murashige and Skoog medium\(^{13}\) with one-half strength of inorganic salts dispensed into flat-bottomed test tube (25 × 120 mm). Seedlings were cultured at 25°C under continuous light at 60 μmol • photons m\(^{-2}\)s\(^{-1}\).

2. **Bacterial strain**

   *Agrobacterium rhizogenes* A-5 strain, which was isolated from hairy roots of melon plants grown in the glasshouse\(^{22}\), was cultured in 20 ml of liquid YEB medium at 25°C in the dark for 24 hr at 80 rev. min\(^{-1}\).

3. **Establishment of hairy roots**

   Several leaves of a 10–15 mm length of main vein were excised from 10 day-old seedlings after germination and cut into 2–3 segments. These segments were soaked for 15 min in *A. rhizogenes* A-5 culture described above. For the control, YEB medium was used for soaking the leaf segments instead of bacterial culture. They were wiped with sterile filter paper and then placed on filter paper wetted by sterilized water. After three days of culture, they were transferred onto half-strength MS agar medium with 500 mg l\(^{-1}\) carbenicillin and 200 mg l\(^{-1}\) vancomycin to eliminate *Agrobacterium*. The cultures were incubated at 25°C in the dark. Three independent experiments each with 20 leaf segments were conducted.

   Adventitious roots, 2–3 cm in length, formed at the cut end of the segments were transferred onto the new half-strength MS agar medium. Axenic cultures of the roots were established after two to three successive subcultures.

4. **Plant regeneration**

   Explants, 1–2 cm in length, excised from the roots proliferated on half-strength MS agar medium were placed onto the same medium supplemented with 0.5 to 10.0 mg l\(^{-1}\) 6-benzyl aminopurine (BAP) independently or in combination with 0.1 or 0.5 mg l\(^{-1}\) 2-naphthalenacetic acid (NAA). The cultures were incubated in a growth cabinet at 25°C under continuous light at 60 μmol m\(^{-2}\)s\(^{-1}\). Two independent experiments each with 15 root segments were conducted.

   Regenerated shoots with three to four firm leaves were transferred on HYPONEX (2 ml l\(^{-1}\), N : P : K = 5 : 10 : 5, liquid type, HYPONEX Japan Licensee, Murakami Bussan Co., Inc.) medium containing 8 g l\(^{-1}\) agar and no phytohormone. Regenerated plantlets were transferred to pots filled with soil and grown under glasshouse conditions.

5. **Detection of opine**

   Mikimopine\(^7\) in extracts from induced hairy roots and regenerants from the hairy roots was analyzed by high voltage paper electrophoresis using Pauly reagent as described by Tanaka\(^{14}\).

6. **Analysis of thiophene**

   Hexane extracts of the roots of both control plants and regenerants, which were being grown under glasshouse conditions, were analyzed by high performance liquid chromatography (HPLC) for detection of a nematocidal compound, thiophene, according to the procedure of Kyo et al.\(^{8}\). The HPLC was run under the following conditions: C18 ODS column, elute: acetonitrile and water at a ratio of 4 : 1, flow rate: 0.7 ml min\(^{-1}\), detection at 330 nm. Three plants of eight regenerants grown in pot were used for analysis of thiophene.

**Results and Discussion**

Small outgrowths occurred at the cut end of segments after 20 to 30 days of infection and followed by root proliferation. Root formation from the segments was rather difficult and less than 10% of segments produced roots in triplicated experiments. Each root established on the medium without phytohormones exhibited typical hairy root phenotype such as extensive lateral branching and vigorous growth (Fig. 1). On the other hand, the roots which could not grow well on the medium without phytohormones were discarded. Non-transformed roots which rarely occurred from the control cultures could not be maintained on the medium containing phytohormones.

The production of mikimopine as an evidence of transformation\(^7\) was found in extracts of all of roots which proliferated with vigorous branching (hairy roots), but not in extracts of roots of seedling grown as control
Fig. 1. Hairy root cultures of R. hirta cv. Highway Yellow induced by A. rhizogenes strain A-5 on half-strength MS agar medium without phytohormones.

Fig. 2. Electrophoretic analysis of extracts from hairy root and the regenerated plant of R. hirta cv. Highway Yellow. Lane 1: mimosine marker from transformed tobacco plant. Lane 2: hairy roots. Lane 3: lateral roots of the regenerant. Lane 4: leaves of the regenerant. Lane 5: lateral roots of the control plant (non-transformant). Lane 6: leaves of the control plant. Lane 7: histidine marker for Pauly imidazole reaction.

(Fig. 2). Although there might be any mimosine in the roots that were induced by A. rhizogenes but did not proliferate vigorously, they were not used for inducing the shoots in this experiment.

Fig. 3. Plantlet regeneration from hairy roots of R. hirta cv. Highway Yellow; shoot formation on compact callus formed on the surface of the root on half-strength MS agar medium supplemented with BAP 1.0 mg l⁻¹ (A), roots production from shoots on HYPONEX medium without phytohormones (B), and the regenerant growing in pot (C).

After 30 to 50 days of transferring the excised roots, 1-2 cm in length, onto half-strength MS agar medium supplemented with
BAP independently or in combination with NAA, one to two shoots were produced on compact calli formed on the surface of the roots (Fig. 3A). The highest frequency of shoot formation (73% of explants formed shoots) was obtained at 0.5 mg l⁻¹ or 1 mg l⁻¹ with 0.1 mg l⁻¹ NAA (Table 1).

Spontaneous shoot formation from hairy root cultures on hormone-free medium has been reported in several plant species such as *Nicotiana tabacum*, *Antirrhinum majus* and *Eustoma grandiflorum*⁶,⁹. In the present work, no shoots were observed on hormone-free medium. The observed shoot induction on *Rudbeckia* hairy roots may be attributed to the application of BAP and its effect could be stimulated by combination with NAA.

Shoots produced roots 10 days after transfer onto HYPONEX medium without phytohormones. Plantlets with several firm roots were regenerated 30 days after transfer (Fig. 3B). Eight plants are now growing in pots filled with soil under greenhouse conditions (Fig. 3).

Table 1. Effect of BAP and NAA supplemented to half-strength MS medium on shoot formation of hairy roots in *R. hirta* L. cv. Highway Yellow.

<table>
<thead>
<tr>
<th>Phytohormone (mg l⁻¹)</th>
<th>% of explants showing shoot formation*</th>
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</thead>
<tbody>
<tr>
<td>HF</td>
<td>0</td>
</tr>
<tr>
<td>BAP (0.5)</td>
<td>33</td>
</tr>
<tr>
<td>BAP (1.0)</td>
<td>53</td>
</tr>
<tr>
<td>BAP (10)</td>
<td>53</td>
</tr>
<tr>
<td>BAP (0.5) + NAA (0.1)</td>
<td>73</td>
</tr>
<tr>
<td>BAP (0.5) + NAA (0.5)</td>
<td>47</td>
</tr>
<tr>
<td>BAP (1.0) + NAA (0.1)</td>
<td>73</td>
</tr>
<tr>
<td>BAP (1.0) + NAA (0.5)</td>
<td>67</td>
</tr>
</tbody>
</table>

* Results are based on evaluations made from two independent experiments each with 15 root segments after 60 days of culture.

Fig. 4. Morphological alterations of plant regenerated from hairy root of *R. hirta* cv. Highway Yellow; wrinkled leaf of regenerant (left) and normal leaf of the control plant (non-transformant) (right) (A), flowers of regenerant (top) and control plant (bottom) (B), and abundant lateral branching of roots of regenerant (top) and normal roots of control plant (bottom) (C).
3C). Although T-DNA analysis for insertion of rol genes have not been made in this work, production of mikimopine in extracts of both leaves and roots in these regenerants indicated that they would be certainly transformed by A. rhizogenes (Fig. 2).

Regeneration from hairy roots have previously been reported in some plant species as described above. These plants exhibited the typical hairy root syndrome such as short internodes, wrinkled leaves and abundant root development\(^6\,12\,13\). In the present work, the Rudbeckia regenerants also showed morphological alterations such as wrinkled leaves, small size of flowers and abundant lateral branching of roots (Figs. 4A, B, C). It is necessary that the characteristics of these plants are evaluated in detail comparing with non-transformant as control under the same conditions of cultivation. However, it should be mentioned that regenerants with abundant lateral branching of roots were produced. Studies on propagation and characterization of these regenerated plants are now in progress.

According to HPLC analysis, \(\alpha\)-terthiienyl, a kind of thiophenes with nematocidal activity, was detected both in the roots of regenerant from hairy root and control plant grown in pots filled with soil (Table 2). Tops of both plants, on the other hand, did not produce the compound. Since only three plants each of regenerant and control were used for analysis of \(\alpha\)-terthiienyl, difference in the amount of the compound between the regenerant and the control plant was not clarified in the present experiment. However, it was definitely found that the plant transformed by A. rhizogenes in R. hirta produced a kind of thiophene in its roots.

Kyo et al.\(^8\) have reported that hairy roots in T. patula accumulated nematocidal compounds other than \(\alpha\)-terthiienyl. The compounds had higher nematocidal activities than \(\alpha\)-terthiienyl. Moreover, Croes et al.\(^2\) have reported that thiophene levels in the highly branched hairy roots in T. patula were lower than in root systems with fewer laterals. Plant regeneration from the hairy roots and its evaluation in regard to production of the nematocidal compounds in T. patula would be expected. In the present study, \(\alpha\)-terthiienyl was detected in the extracts of roots showing extensive lateral branching in the regenerant from hairy roots of R. hirta. Further studies on the actual activities of the extracted substances from the root systems of the transformant should be conducted and the effect on suppression of the population density of nematodes in a field must be evaluated.

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**References**


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**Table 2. \(\alpha\)-Terthiienyl concentration of regenerant from hairy root and control plant grown in pot in R. hirta L. cv. Highway Yellow.**

<table>
<thead>
<tr>
<th></th>
<th>Regenerant</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Top</td>
<td>Root</td>
</tr>
<tr>
<td>(\alpha)-Terthiienyl ((\mu)g/gFW)</td>
<td>ND*</td>
<td>0.86 ± 0.79</td>
</tr>
</tbody>
</table>

Values are mean of three plants with standard deviation.

ND : not detected.

* In Japanese with English summary.
** Translated from Japanese by the present authors.
*** In Japanese.