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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, α-terthienyl, was detected in the extract from lateral roots of the regenerant.
Key words : *Agrobacterium rhizogenes*, Antagonistic plant, Hairy root, Nematode, *Rudbeckia hirta*, Thiophene.

線虫対抗植物ルドベキア（*Rudbeckia hirta* L.）における毛状根からの植物体の再分化とチオフェンの産生：大門弘幸・三位正洋**（大阪府立大学農学部・**千葉大学園芸学部）

要　旨：土壌中に生息する植物寄生性有害線虫の密度低減に効果があるとされるルドベキア（*Rudbeckia hirta* L.）において，*Agrobacterium rhizogenes* の野生菌株（A-5株）を感染させることによって毛状根を誘導した．毛状根は，無機塩濃度を1/2に減じた植物ホルモンを含まないMS寒天培地上で分岐を繰り返しながら旺盛に生育した．誘導された毛状根の磨砕液を濾紙電気泳動にかけてオパインの検出を行ったところ，供試菌株に特有のオパインであるミキモピンが検出され，これらの根が形質転換されたものであることが確認された．毛状根をBAP（0.5または1.0 mg/*l*）およびNAA（0.1 mg/*l*）を添加した無機塩濃度を1/2に減じたMS寒天培地に移植したところ，移植後30～50日目に毛状根の表面に形成されたカルス上に高頻度（47～73%）に不定芽が誘導された．誘導された不定芽は植物ホルモンを含まないHYPONEX培地上で容易に発根した．得られた再分化植物を温室内で生育させたところ，毛状根由来植物に特徴的な形態である，葉の波打ち，花の小型化，分岐根の旺盛な発達が認められた．再分化植物の根において，殺線虫物質チオフェンの一つである α-ターテニールが検出された．
キーワード：アグロバクテリウム リゾゲネス，線虫，対抗植物，チオフェン，毛状根，ルドベキア．

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[13]. 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 decease in dicotyledonous plants, exhibit proliferous root growth[3,5,9]. Several approaches have been made to produce secondary metabolites such as ginseng[4,17] and tropane 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[6,12,15]. 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

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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[11] 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[3], 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 mgl^{-1} carbenicillin and 200 mgl^{-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 mgl^{-1} 6-benzyl aminopurine (BAP) independently or in combination with 0.1 or 0.5 mgl^{-1} 2-naphthaleneacetic 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 transferrd 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 to 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

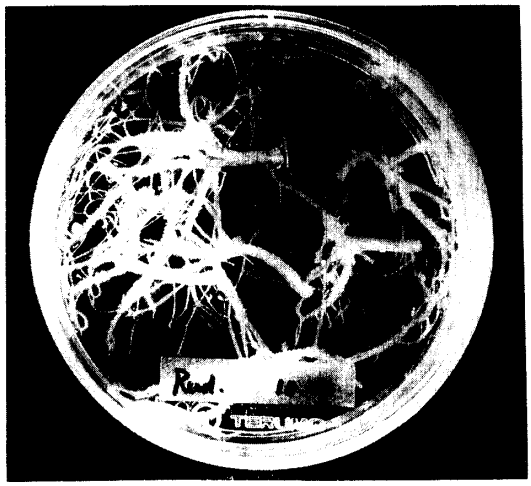


Fig. 1. Hairy root cultures of *R. hirta* cv. Highway Yellow induced by *A. rhizogenes* strain A -5 on half-strength MS agar medium without phytohormones.

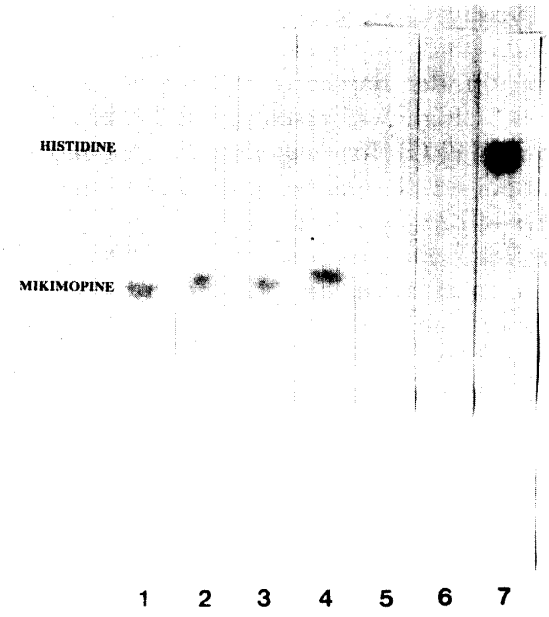


Fig. 2. Electrophoretic analysis of extracts from hairy root and the regenerated plant of *R. hirta* cv. Highway Yellow. Lane 1 : mikimopine marker from transformed tobacco plant. Lane 2 : hairy roots. Lane 3 : lateral roots of the regenerant. Lane 4 : leaves of the regenerant. Lane 5 : lateral roots 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 mikimopine 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.

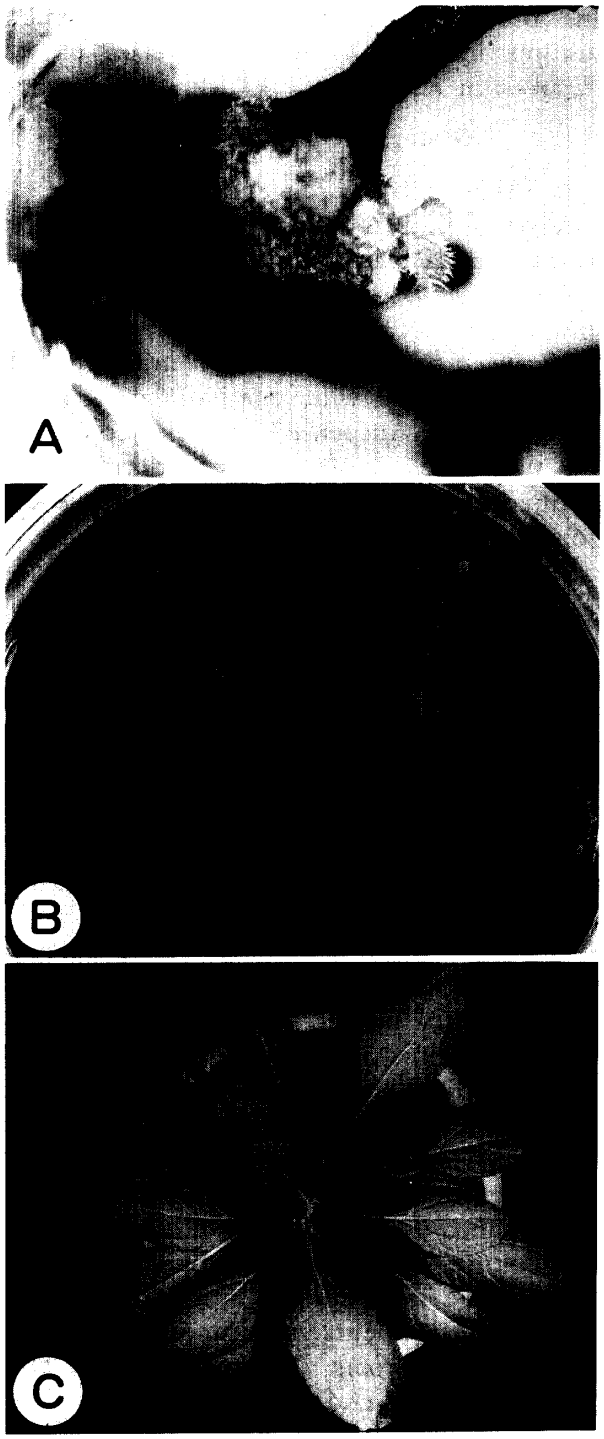
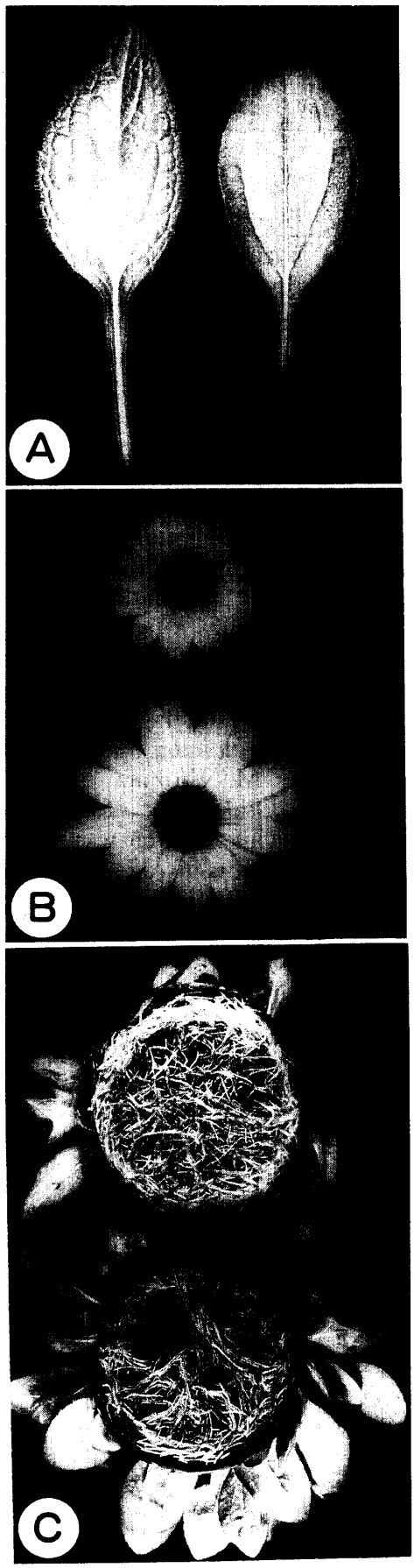


Fig. 3. Plantlet regeneration from hairy roots of *R. hirta* cv. Highway Yellow ; shoot formation on compact callus formed on the surface of the root on half-strength MS agar medium supplemented with BAP 1.0 mg l^{-1} (A), roots production from shoots on HYPONEX medium without phytohormones (B), and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

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

Phytohomone (mg l^{-1})	% of explants showing shoot formation*
HF	0
BAP(0.5)	33
BAP(1.0)	53
BAP(10)	53
BAP(0.5)+NAA(0.1)	73
BAP(0.5)+NAA(0.5)	47
BAP(1.0)+NAA(0.1)	73
BAP(1.0)+NAA(0.5)	67

* 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[6,12,15]. 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, α-terthienyl, 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 α-terthienyl, 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 α-terthienyl. The compounds had higher nematocidal activities than α-terthienyl. 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, α-terthienyl 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.

Table 2. α-Terthienyl concentration of regenerant from hairy root and control plant grown in pot in *R. hirta* L. cv. Highway Yellow.

		α-Terthienyl (μg/gFW)
Regenerant	Top	ND*
	Root	0.86±0.79
Control	Top	ND*
	Root	0.54±0.15

Values are mean of three plants with standard deviation.
ND : not detected.

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