Plant Biotechnology 29, 425–430 (2012)
DOI: 10.5511/plantbiotechnology.12.0718a

Original Paper

Plant regeneration from *Crotalaria spectabilis* hairy roots which showed inhibited growth of root-knot nematodes (*Meloidogyne hapla* and *M. incognita*) *in vitro*

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Received June 15, 2012; accepted July 18, 2012 (Edited by M. Otani)

Abstract Hairy roots were induced from leaf segments of *Crotalaria spectabilis*, which is used as a green manure crop antagonistic to nematodes, by infection with a mikimopine type wild strain of *Agrobacterium rhizogenes* A13 (MAFF02-10266). For initial proliferation of hairy roots after induction, addition of gibberellin A_3 to the medium was indispensable. These roots exhibited vigorous growth and abundant lateral branching on half-strength Murashige and Skoog (1/2MS) medium without plant growth regulators. The adventitious shoots were induced from 24% of root segments 2 months after transfer onto B5 medium containing 5 mgl^{-1} 6-benzyladenine. The axillary buds excised from these shoots produced roots 1 month after transfer onto 0.4% (wv^{-1}) gellan gum-solidified 1/2MS medium containing 30 mM fructose without plant growth regulators. Regenerated plants were successfully grown under greenhouse conditions. Infection of hairy roots of *C. spectabilis* with root-knot nematodes *Meloidogyne hapla* and *M. incognita* led to the growth inhibition of these nematodes.

Key words: *Agrobacterium rhizogenes*, *Crotalaria spectabilis*, nematode control, plant regeneration, transformation.

Agrobacterium rhizogenes, a soil-borne bacterium, is well known to induce hairy roots when Ri T-DNA is integrated into the plant genome. Plants regenerated from hairy roots in several plant species exhibit characteristic phenotypes such as shortened internodes, wrinkled leaves and abundant root mass with extensive lateral branching (Tepfer 1984). The morphological alterations of plants caused by integration of Ri T-DNA have been reported in some crops such as sweet potato (Otani et al. 1993) and ornamental plants (Otani et al. 1996; Godo et al. 1997; Hoshino and Mii 1998). These morphological changes might be of interest for breeding of some crops and especially in legumes, abundant growth with lateral branching of hairy roots is considered to be useful for improving nitrogen fixation. However, successful results on plant regeneration from hairy roots in leguminous species have been reported in some limited species such as *Lotus corniculatus* (Petit et al. 1987; Nikolic et al. 2003–2004; Fukuda et al. 2007), *Medicago sativa* L. (Spanò et al. 1987), *Robinia pseudoacacia* L. (Han et al. 1993), *Lotus japonicus* (Stiller et al. 1997) and *Astragalus sinicus* (Cho and Widholm 2002). Previously, we have reported on the regeneration of plants from hairy roots of *Crotalaria juncea* L. (sunn hemp), which is a leguminous species (Ohara et al. 2000).

Species of the genus *Crotalaria* are mainly native to Africa and Indian subcontinent and have already spread to tropical and subtropical regions as a fiber and green manure crop (Polhill 1982). Some species like *C. juncea*, *C. spectabilis* and *C. breviflora* have been introduced to temperate regions as useful crops to eliminate nematodes, because of its nematocidal root activity. Especially *C. spectabilis* is effective for various nematodes such as *Meloidogyne incognita*, *M. javanica*, *M. arenaria*, *M. hapla*, *Pratylenchus coffeae* and *Heterodera glycines* etc. (McSorley 1999; Yuhara 1969; Torigoe 1996; Kushida et al. 2003). It is also expected to introduce into paddy-upland rotated fields and ill-drained clayish fields for improvement of the physical and chemical properties of the soils (Daimon et al. 2006). For use as a green manure

Abbreviations: BA, 6-benzyladenine; GA_3, gibberellin A_3; MS, Murashige and Skoog; YEP, yeast extract peptone.

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This article can be found at http://www.jspcmb.jp/
Published online December 10, 2012

crop and as an antagonistic plant to nematodes, further improvement of the root system would be required in these species. In the present study, we report the production of transgenic plants of *C. spectabilis* obtained from hairy roots induced by infection with a domestic wild strain of *A. rhizogenes* and inoculation of nematodes to the hairy roots.

Materials and methods

Plant materials and bacterial strain

Seeds of *Crotalaria spectabilis* were surface-sterilized with sodium hypochlorite solution (1% active chlorine) containing a drop of Tween 20 for 10 min and rinsed three times with sterile deionized water. The seeds were placed on 0.8% (wv^{-1}) agar (Wako, Japan)-solidified 1/2MS medium, in which inorganic salts were reduced to the half strength of MS medium (Murashige and Skoog 1962), containing 10 $g\,l^{-1}$ sucrose. They were incubated at 25°C under continuous light condition with photosynthetic photon flux density (PPF) of 60 μmol photons $m^{-2}\,s^{-1}$ for germination. Cotyledons and hypocotyls of the seedlings thus obtained were used as explants. A mikimopine producing (Isogai et al. 1988) wild-type strain of *Agrobacterium rhizogenes*, A13 (MAFF-02-10266; Daimon et al. 1990), was used for the infection. The bacterial culture was prepared by culturing loopful bacteria in 30 ml of liquid YEP (Chilton et al. 1974) medium for 12 h at 160 revs min^{-1} at 28°C in the dark.

Establishment of hairy roots

Cotyledons and hypocotyls were excised from 5 day-old seedlings and cut into pieces of about 1 cm^2 and 1 cm long, respectively. The segments were soaked in the bacterial suspension for 10 min, blotted dry on sterilized filter paper, and placed on wet filter paper for co-cultivation at 25°C in the dark. As a control, YEP liquid medium containing no bacteria was used to soak the explants. After 3 days of co-cultivation, the explants were transferred onto 0.8% (wv^{-1}) agar-solidified 1/2MS medium containing 30 $g\,l^{-1}$ sucrose and 500 $mg\,l^{-1}$ cefotaxime (Hoechst Marion Roussel, Japan), and subcultured at 14 day-intervals to eliminate the bacteria. The cultures were incubated at 25°C in the dark. The adventitious roots were induced from the explants after several subcultures on this medium. The adventitious roots were then excised and transferred onto 0.8% (wv^{-1}) agar-solidified 1/2 MS medium containing 30 $g\,l^{-1}$ sucrose and 1 $mg\,l^{-1}$ GA_3 for the root growth without application of cefotaxime. They were further maintained independently on 0.3% (wv^{-1}) gellan gum (Gelrite, Wako, Japan)-solidified 1/2MS medium containing 30 $g\,l^{-1}$ sucrose without GA_3 at 25°C in the dark.

Opine analysis

About 100 mg fresh weight of adventitious roots induced from the explants were ground in a microtube containing some quartz sand and a drop of 1 N HCl. After centrifugation at 10,000 revs min^{-1} for 5 min, 10 μl of supernatant was spotted on a filter paper (51B Advantec, Toyo Roshi Kaisya, Japan) and subjected to high-voltage paper electrophoresis at 450 V for 2 h. The running buffer (pH 1.8) consisted of formic acid, acetic acid and distilled water (5 : 15 : 80, by vol). The detection of mikimopine was performed with Pauly reagent as described by Petit et al. (1986).

Plant regeneration from hairy roots

Hairy root segments of about 5 mm long without root tip were placed on 0.8% (wv^{-1}) agar or 0.3% (wv^{-1}) gellan gum-solidified B5 medium (Gamborg et al. 1968) containing 20 $g\,l^{-1}$ sucrose and various concentrations of BA (0, 1, 5 and 10 $mg\,l^{-1}$). The cultures were incubated at 25°C under continuous light condition with PPF of 60 μmol $m^{-2}\,s^{-1}$. Regenerated shoots were transferred onto BA-free 0.4% (wv^{-1}) gellan gum-solidified 1/2MS medium with 30 mM sucrose or fructose in test tubes (25×120 mm) each containing 10 ml medium for their growth. The elongated shoots were excised from the original hairy root segments and transferred onto the same 50 ml medium in 225 ml mayonnaise glass bottle for root formation. Each tube or bottle containing one shoot was covered with a plastic cap having a hole of 10 mm diameter, which was equipped with a disk of membrane filter (18 mm diameter) with 0.5 μm pores (Milliseal, Nihon Millipore, Japan) for acclimatization. Plantlets were transferred to pots filled with vermiculite wetted by water and grown in a greenhouse.

DNA extraction and PCR analysis

Transformation of regenerated plants from hairy roots induced by *A. rhizogenes* was confirmed by PCR analysis. Template DNA was extracted from leaves of regenerated plants by a cetyl trimethyl ammonium bromide (CTAB) method according to Murray and Thompson (1980). For the detection of *rol* genes, which are core T-DNA genes, PCR was performed using a pair of oligonucleotide primers, 1724C–1724D, which were expected to produce 1105-bp product in the *rol* genes (Kiyokawa et al. 1992). PCR amplification was carried out using the following conditions: 30 cycles of 30 s at 94°C, 30 s at 57°C and 1 min at 72°C. Amplified DNAs were detected by ethidium bromide staining after 0.8% (wv^{-1}) agarose gel electrophoresis.

Inoculation of nematodes on hairy roots

Two species of root-knot nematodes (*Meloidogyne hapla*, from Chiba Prefectural Agriculture and Forestry Research Center and *M. incognita*, from Lion Corp. Japan), which had been maintained on hairy roots of tomato (*Lycopersicon esculentum* Mill. 'Farstmore'), were used for the inoculation. Inoculation of nematodes was carried out as previously reported by Adachi et al. (1993) on hairy roots of *C. spectabilis* and tomato ('Farstmore'), which was selected as a suitable control host plant. Hairy roots of both species were maintained on 0.3% (wv^{-1}) gellan gum-solidified 1/2MS medium containing 30 $g\,l^{-1}$ sucrose at 25°C in the dark.

Before inoculation, egg masses collected from tomato

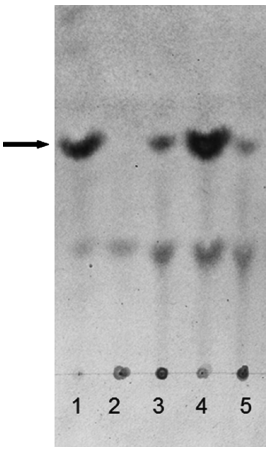

Figure 1. Paper electrophoretogram showing the presence of mikimopine in hairy root. 1: Mikimopine standard extracted from hairy roots of tobacco infected with *A. rhizogenes* A13 (MAFF02-10266) strain as a positive control. 2: Roots derived from a non-inoculated explant as a negative control. 3, 4, 5: Root strains derived from explants inoculated with *A. rizogenes* A13 (MAFF02-10266). An arrow indicates the position of mikimopine.

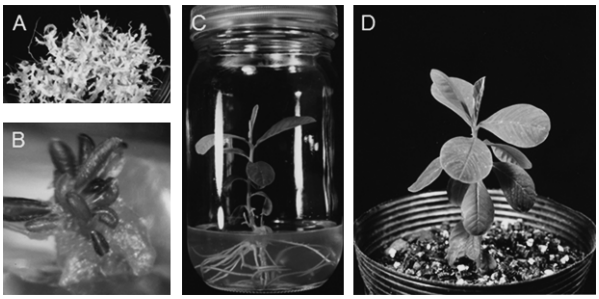

Figure 2. Plant regeneration from *C. spectabilis* hairy roots. (A) Hairy roots growth in medium without plant growth regulators. (B) Adventitious shoots induction on BA-containing medium. (C, D) Plantlet derived from hairy roots.

hairy roots incubated in sterilized water at 25°C and second-stage juveniles hatched within 48 h were used. The day before inoculation, hairy roots (approximately 5 cm) were transferred to 0.2% (wv^{-1}) gellan gum-solidified M medium (Bécard and Fortin 1988). One-hundred juveniles were inoculated to the hairy roots on M medium with 200 μl sterilized water and incubated at 25°C in the dark. For observation of nematode infection, hairy roots were bleached in sodium hypochlorite solution (1.5% active chlorine) for 15 min, washed with water for 2 min, soaked in water for 20 min, boiled with water containing acid fuchsin (70 mg l^{-1} acid fuchsin in 0.5% acetic acid) for 30 min, heated in acid glycerin, and viewed with a light microscope.

Results and discussion

Fourteen days after inoculation with *A. rhizogenes*, adventitious roots were observed at the cut surface of the cotyledon and hypocotyl explants. The frequencies of explants with adventitious root formation 28 days after inoculation were 20 and 28%, respectively. Isolated adventitious roots did not grow on 1/2MS basal medium but started to grow on the same medium supplemented with 1 mg l^{-1} GA_3. This result confirms the previous finding of the stimulation effect of GA_3 on the growth of hairy root in *Datura innoxia* (Ohkawa et al. 1989), in which GA_3 treatment showed enhanced elongation and lateral branching of hairy roots during the subcultures. In the present study, however, GA_3 did not enhance the growth but caused hyperhydricity in hairy roots of *C. spectabilis* during the subsequent subcultures. Although the reason for the temporary beneficial effect of GA_3 is still unclear, the roots proliferated on medium with GA_3 for 14 to 28 days were transferred to the medium without GA_3. About 3 months after infection with bacteria, transformation of these roots was confirmed by detection of mikimopine (Figure 1). Among eight root clones examined, seven were confirmed to be transformants, in which six were from cotyledon and one from hypocotyl explants. The transformed roots were successfully subcultured on gellan gum-solidified 1/2MS medium without addition of GA_3 and no *Agrobacterium* growth was detected in the roots without application of cefotaxime (Figure 2A). These roots showed typical hairy root character of vigorous growth with extensive branching on a medium without any plant growth regulators.

In the present study, no shoots were formed from hairy roots of *C. spectabilis* during subcultures on medium without plant growth regulators. However, adventitious shoots were induced from green spots produced on the calli derived from hairy roots after 2 months of culture on BA-containing medium (Figure 2B). In our previous study on *C. juncea*, BA was also needed for inducing adventitious shoots (Ohara et al. 2000). It has been suggested that leguminous species are mostly recalcitrant for inducing shoot regeneration from hairy roots in *in vitro* culture, and highly species-specific methods are required to overcome the difficulty (Atkins and Smith 1997) except for *Lotus corniculatus*, in which hairy root cultures regenerated adventitious shoots spontaneously without applying plant growth regulators within 30 days of culture under the continuous light condition (Petit et al. 1987). In *C. spectabilis* hairy roots, the highest frequency of adventitious shoot formation was 24% which was obtained on medium with BA at 5 mg l^{-1} (Table 1). At the higher BA concentration (10 mg l^{-1}), no shoots were formed. A large difference was found in the percentage of shoot formation among the hairy root clones, but they almost showed a similar response to BA concentrations (Table 1). The difference in the shoot regeneration ability among the hairy root clones might be caused by the difference in the insertion site of T-DNA in a chromosome as reported previously (Nagy et al. 1985).

Table 1. Effect of 6-benzyladenine (BA) concentration on induction of adventitious shoots from hairy roots of *Crotalaria spectabilis* after 60 days of culture.

Line of hairy root	% of hairy roots showing shoots formation			
	Concentraion of BA ($mg\,l^{-1}$)			
	0	1	5	10
A	0.0	3.3	40.0	0.0
B	0.0	0.0	20.0	0.0
C	0.0	0.0	0.0	0.0
D	0.0	0.0	3.3	0.0
E	0.0	20.0	26.7	0.0
F	0.0	0.0	46.7	0.0
G	0.0	10.0	30.0	0.0
Average	0.0[b]	4.8[b]	23.8[a]	0.0[b]

Values represent percentages for three replications ($n=10$ explants). Values followed by the same letter are not significantly different at 5% level by Tukey test.

Table 2. Effect of a kind of sugar in media on shoot growth and callus formation at the base from hairy root-derived shoots of *Crotalaria spectabilis* after 20 days of culture.

Sugar	No. of shoots transplanted	Callus formation (%)*		Shoots showing growth (%)
		Large	Small	
Sucrose	35	85.7	11.4	57.1
Fructose	39	7.6	38.5	82.1

* Large callus >5 mm, Small callus=2 to 5 mm.

Although shoots could be induced by application of BA, they mostly failed to grow normally due to severe hyperhydricity and callus formation at the base. To avoid the hyperhydricity, it has been recommended to apply some treatments to reduce the water potential such as an elevated concentration of gelling agents (Singha et al. 1990; Nugent et al. 1991) and forced air ventilation (Wardle et al. 1983). Also hyperhydricity was avoided in *C. juncea* by using medium containing high concentration of gelling agents and using of Milliseal on the plastic cap, which was effective for air ventilation (Ohara et al. 2000). However, shoots did not grow well in *C. spectabilis* when these methods were applied. There have been several reports which showed effectiveness of an appropriate selection of sugars to prevent the hyperhydricity in some plant species (Rugini et al. 1987; Druart 1998). Therefore, we replaced sucrose by equimolar concentration of fructose in the medium, which resulted in the reduced callus formation and hyperhydricity as well as induced shoot growth (Table 2). Although these shoots showed leaf yellowing or dead after transfer to 225 ml bottles, it was effectively prevented by removing lower part of leaves at the transplanting and keeping under dim light condition in a few days. By repeating subcultures of nodal segments with axillary bud of these shoots several times, they developed into normal shoots with firm leaflets with trichomes and finally rooted (Figure 2C). Then these hairy root-derived plantlets were successfully transplanted to the soil after acclimatization for 10 days at 25°C under 12 h lighting (Figure 2D). Transformation with Ri T-DNA of the regenerated plants from hairy roots was confirmed by PCR analysis (Figure 3). Amplified DNA of these plantlets derived from hairy roots showed 1.1 kb fragment of the *rol* gene.

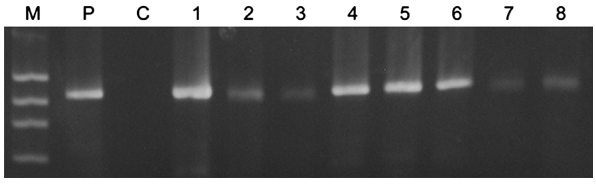

Figure 3. Confirmation of integrated Ri T-DNA by PCR analysis. M: ϕX174/*Hae*III digest. P: positive control (plasmid). C: DNA of non-transformed plant as a negative control. 1 to 8: DNA of transgenic plants.

In order to confirm the nematocidal activity in hairy roots of *C. spectabilis*, they were cultured *in vitro* with two kinds of root-knot nematodes (*M. hapla* and *M. incognita*). The tomato hairy roots used as a control were infested with nematodes one day after inoculation. Three days after inoculation, nematodes were in central cylinder of the roots (Figure 4A), and the infested parts of roots were swelled. Then enlarged nematodes were observed 10 days after inoculation (Figure 4B) and the roots followed by the formation of root knots. Finally, mature females appeared (Figure 4C) and began to lay eggs on the surface of the roots 25 days after inoculation. Both species of nematodes showed almost the same developmental process. In the case of *M. hapla*,

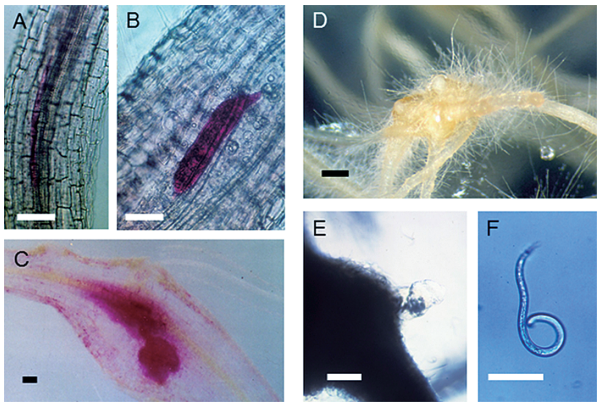

Figure 4. Nematode growth in tomato ('Farstmore') hairy roots. (A) Nematode (*M. incognita*) in the hairy roots 3 days after inoculation. (B) Nematode (*M. incognita*) in the hairy roots 10 days after inoculation. (C) Nematode (*M. incognita*) in the hairy roots 22 days after inoculation. (D) Egg masses on the hairy roots and abnormal number of lateral roots 25 days after inoculation (*M. hapla*). (E, F) Second-stage juveniles were out from the hairy roots 38 days after inoculation (*M. incognita*). Scale bar= 0.1 mm (A, B, C, F), 1 mm (D, E).

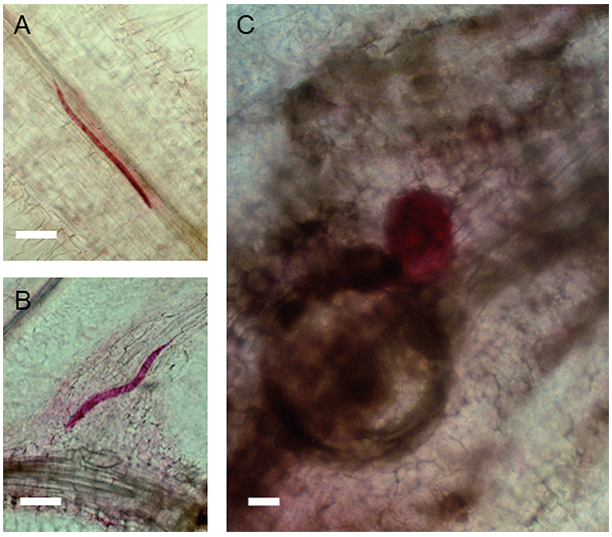

Figure 5. Inhibition of nematode growth in *C. spectabilis* hairy roots. (A) Nematode (*M. hapla*) in the hairy roots 3 days after inoculation. (B) Nematode (*M. hapla*) in the hairy roots 10 days after inoculation. (C) Nematode (*M. hapla*) in the hairy roots 40 days after inoculation. Scale bar=0.1 mm.

abnormal number of lateral roots was produced from the root knots (Figure 4D), which was characteristic to this nematode species. In both nematodes, second-stage juveniles were hatched from egg masses on tomato hairy roots 40 days after inoculation (Figure 4E, F). In *C. spectabilis*, nematodes also infested and fixed in the roots (Figure 5A, B) and formed root knots 10 days after inoculation. As in tomato, lateral roots were formed on root knots of *C. spectabilis* infested by *M. hapla*. The nematodes developed in the roots, but they were smaller than those in tomato roots, and necrosis was sometimes observed around nematodes (Figure 5C). The nematodes finally died without producing next generation. Both species of nematodes showed same incomplete developmental process, which was similar to the previous report on *M. incognita* inoculated to the roots of non-transgenic *C. spectabilis* (Sano and Nakasono 1986). These results suggest that hairy root of *C. spectabilis* has also the ability to inhibit the growth or development of nematodes like as hairy root of *C. juncea*, which was reported previously (Akasaka et al. 2003), and that the transgenic plants will be utilized more effectively to reduce the population of nematodes in the soil than the wild plants because of the increased amount of root system.

In the present study, we succeeded in producing transgenic plants from hairy roots in *C. spectabilis* and showed that the hairy roots had nematocidal activity. Detailed studies on the characterization and evaluation of the phenotypes in the transformants, such as shoot and root morphology, nodulation on hairy roots, and nitrogenase activity, are now in progress.

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