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Full Length Research Paper

Assessment of hairy roots induction in *Solenostemon scutellarioides* leaves by different strains of *Agrobacterium rhizogenes*

Saleh, N. M.* and Thuc, L. V.

Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia, 43400 Serdang Selangor Darul Ehsan, Malaysia.

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Hairy roots of *Solenostemon scutellarioides* were induced by inoculation of leaf explants with *Agrobacterium rhizogenes* strains TR 105, LBA 9402, 8196 and ATCC 15834. These strains showed different abilities to induce hairy root formation on the leaf explants. Assessment of the plant's susceptibility to the different strains of *A. rhizogenes* showed that strains ATCC 15834, TR 105, LBA 9402, and 8196 produced 56.3, 25.5, 21.5 and 13.8% transformation efficiencies, respectively. Acetosyringone was found to be useful for the enhancement of hairy roots formation in *S. scutellarioides.*

Key words: *Solenostemon scutellarioides*, hairy roots, acetosyringone, *Agrobacterium rhizogenes*, Labiatae.

INTRODUCTION

Solenostemon scutellarioides (syn. *Coleus blumei*) is a popular ornamental plant which belongs to the Labiatae family (Garcia and O'Neil, 2000). Cell suspension and transformed callus cultures of this plant are known to produce rosmarinic acid (Razzaque and Ellis, 1977; De-Eknamkul and Ellis, 1987; Petersen, 1991, Bauer et al 2004), which possesses antibacteria, antioxidation and anti-inflammatory properties (Petersen and Simmonds, 2003). Rosmarinic acid can also be used to prevent bronchial asthma, apasmogenic disorders, hepatotoxicity, ischaemic heart disease, and cancer (Al-Sereiti et al., 1999; Park et al., 2008) and has been reported to be a potent active substance against human immunodeficiency virus types1 (HIV-1) (Mazunder et al., 1997). However, cell suspension culture is not genetically and biochemically stable due to its high rate of disorganized cell division (Phillips et al., 1995; Oksman-Caldentey and Hiltunen, 1996). In contrast, organ cultures such as hairy roots are genetically stable and contain differentiated cells.

*Corresponding author. E-mail: norihanms@biotech.upm.edu.my. Tel.: 603-89468357. Fax: 603 89467510.

Hairy root is a pathological syndrome of dicotyledonous plants following wounding and infection with *Agrobacterium rhizogenes* (Draper et al., 1988). *A. rhizogenes* strains are classified by their opine types with the commonly used strains being of the agropine and mannopine types (Hamill and Lidgett, 1997). In several plant species, the presence of higher amounts of secondary metabolites has been observed in hairy root cultures when compared to the mother plants (Chae et al., 2000). For example, a 13.3-fold increase of ajmalicine production was observed in the hairy root culture of *Catharanthus roseus* (Bhadra et al., 1993) and a 7-fold increase of saponins production in the hairy root of *Pinax ginseng* (Misawa, 1994).

Currently, no record of rosmarinic acid production in hairy root culture of *S. scutellarioides* has been documented. Thus, as an initial step towards the production of rosmarinic acid in a genetically stable organ culture, we were able to induce hairy root formation in *S. scutellarioides* using different strains of *A. rhizogenes.*

MATERIALS AND METHODS

Plant Materials

Shoot tips of *S. scutellarioides* were obtained from field grown plants and surface sterilized. The shoots were then cultured onto

Table 1. Characteristic of selected *A. rhizogenes* strains.

Strain	Plasmid[a]	Opine type	Reference
8196	pRi8196	Mannopine	Dommisse et al. (1990)
ATCC 15834	pRi15834	Agropine	Dommisse et al. (1990)
TR 105	UCRi	Agropine	Dommisse et al. (1990)
LBA 9402	pRi1855	Agropine	Nguyen et al. (1992) and Ionkova et al. (1997)

[a]UCRi: uncharacterized Ri plasmid.

MS basal medium. Aseptic leaves (approximately 1-2 cm^2) were obtained from two-week old axenic plants and were used for hairy root induction.

Bacterial strains

Four bacterial strains were used in this study, namely TR 105, LBA 9402, ATCC 15834 and 8196. The first three strains were of the agropine type and the last strain was of the mannopine type (Table 1).

Activation of bacterial strains

Stocks of *A. rhizogenes* strains (TR 105, LBA 9402, 8196 and ATCC 15834) in dimethyl sulfoxide (DMSO) stored at -80°C were streaked onto separate fresh yeast extract nutrient broth (YEB) plates (13.3 g/l nutrient broth, 1 g/l yeast extract, 5 g/l sucrose, 0.24 g/l $MgSO_4$, 15 g/l gelrite agar, and pH 7.5). The plates were incubated at 30°C in the dark for 2 days. A single bacterial colony from each strain was picked and inoculated into 5 ml YEB liquid medium in 15 ml Falcon tube, and incubated with shaking at 28°C for 2 days in the dark at 150 rpm. Prior to infection and co-cultivation with plant tissues, the bacterial cultures were centrifuged for 15 min at 3000 rpm and resuspended in 5 ml hormone-free MS liquid medium (OD_{600} = 0.6) in the absence or presence of 50 µM acetosyringone.

Hairy root induction

Induction of hairy roots were performed by placing cut axenic leaf explants into a Petri dish containing 10 ml of *A. rhizogenes* culture in the presence or absence of acetosyringone (Sigma Chemical Co.). In order to test the effect of acetosyringone on the transformation efficiency of *S. scutellarioides*, 50 µM acetosyringone was added to *A. rhizogenes* and leaf explants co-cultivation medium and incubated in the dark for 12 h, and agitated at 40 rpm in the dark. The explants were then cultured onto hormone-free MS basal solid medium for 2 days in the dark at 25 ± 2°C. Two days later, the infected leaf explants were rinsed with sterile distilled water supplemented with 100 mg/l of carbenicillin (Sigma Chemical Co.), blotted dry onto sterile filter papers and subcultured onto fresh hormone-free MS basal solid medium containing 500 mg/l carbenicillin in order to eliminate *A. rhizogenes*. Stepwise elimination of *A. rhizogenes* was performed gradually by subculturing the infected explants with reduced concentration of carbenicillin (250 and 100 mg/l) and washed with sterile distilled water before each subculture. Control experiments were performed by culturing the explants into 10 ml of bacteria-free MS basal liquid medium with or without acetosyringone. Hairy roots appeared at the cut edge and midrib of the leaf explants. The roots (about 15 to 20 mm in length) were cut and transferred onto hormone-free MS basal solid medium containing 30 g/l sucrose, and 100 mg/l carbenicillin. Root tips were clear of bacterial growth after one or two cycles of weekly subcultures. The subsequent subcultures were done on the same medium without antibiotics.

Statistical analysis

One-way analysis of variance (ANOVA) was done using the MSTAT-C program (Gomez and Gomez, 1984). The analysis was performed with Duncan's Multiple Range Test at a probability of 95%.

RESULTS AND DISCUSSION

Effect of different *A. rhizogenes* strains on hairy root induction

Four strains of *A. rhizogenes* (TR 105, LBA 9402, 8196 and ATCC 15834) were tested for their infection capabilities on *S. scutellarioides* explants. The virulence of each of the strains differs as shown in Table 2. In the absence of acetosyringone, the induction of hairy root occurred after 18 ± 1.8 days with strain ATCC 15834 and 21 ± 1.0 day with strain TR 105. However, the other strains (LBA 9402 and 8196) did not induce any hairy root in the absence of acetosyringone. In the presence of 50 µM acetosyringone, hairy roots occurred in 12 ± 3.1 days with strain TR 105, 11 ± 2.7 days with strain LBA 9402, 12 ± 3.4 days with strain 8196 and 11 ± 2.2 days with strain ATCC 15834. A similar result in hairy root induction was also reported in *Allium cepa* (Dommisse et al., 1990). This indicated that the presence of acetosyringone induced hairy roots formation in shorter time with strain ATCC 15834 and TR 105. Similar observations have also been documented in other plant species where acetosyringone was able to induce and enhance the expression of *A. rhizogenes* virulence genes (Stachel et al., 1985; Bolton et al., 1986; Draper et al., 1988; Hu and Alfermann, 1993; Królicka et al., 2001, Kumar et al. 2006). No induction of hairy root was observed in the control experiments (Table 2). These leaf explants gradually became necrotic and died.

The results in Table 2 show that different strains of *A. rhizogenes* displayed various abilities to induce hairy roots on these leaf explants. Transformation frequencies of 13.8 ± 1.1, 21.5 ± 6.2, 25.5 ± 5.5 and 56.3 ± 4.5% were achieved for *A. rhizogenes* strain 8196, LBA 9402, TR

Table 2. The occurrence of hairy root induction (number of days in culture and its frequencies) in *S. scutellarioides* leaf explants in the presence or absence of acetosyringone induced by different strains of *A. rhizogenes*.

Bacterium strain	Hairy root appearance (days)		Hairy root transformation (%)*	
	with AC	without AC	with AC	without AC
Control	NI	NI	NI	NI
TR 105	12 ± 3.1	21 ± 1.0	25.5 ± 5.5[b]	8.5 ± 1.0
LBA 9402	11 ± 2.7	NI	21.5 ± 6.2[b]	NI
8196	12 ± 3.4	NI	13.8 ± 1.1[c]	NI
ATCC 15834	11 ± 2.2	18 ± 1.8	56.3 ± 4.5[a]	24.1 ± 3.4

AC: Acetosyringone.
NI: no induction of hairy root.
*Mean ± SE followed the same letter is not significant different 5% level by Duncan's test. Each value is the result of 3 replications from a single experiment.

105 and ATCC 15834, respectively when they were cultured in the presence of acetosyringone. Only 8.5 ± 1.0 and 24.1 ± 3.4% transformation efficiencies were obtained when strains TR 105 and ATCC 15834 were used in the absence of acetosyringone, respectively. Without acetosyringone, no hairy root induction was observed when strains 8196 and LBA 9402 were used. These results showed that among the four different strains of *A. rhizogenes* used, *A. rhizogenes* strain ATCC 15834 was most efficient in inducing hairy roots in *S. scutellarioides*. The enhancement of hairy root formation by acetosyringone was similarly reported in other plant species such as *Arabidopsis thaliana* (Sheikholeslam and Weeks, 1987), *Glycine max* (Owens and Smigocki, 1988), *Salvia miltiorrhiza* (Hu and Alfermann, 1993), *Brassica oleracea* (Henzi et al., 2000), *Alhagi pseudoalhagi* (Mei et al., 2001) and tobacco (Kumar et al., 2006). These results also indicated that acetosyringone was a key factor that contributed to the high rates of transformation efficiencies of these plants. Consequently, acetosyringone treatment of *A. rhizogenes* may be useful in improving the transformation rates of other plant species which are recalcitrant. The low rate of hairy root induction in *S. scutellarioides* in the absence of acetosyringone may be due to two reasons. Firstly, the natural exudates of the wounded tissues may not be sufficient to elicit the activities of the *vir* gene in the *A rhizogenes* strain. Secondly, the exudates release may have an inhibitory effect on the *A. rhizogenes* strains used. For strains 8196 and LBA 9402, this inhibitory effect was successfully overcome when acetosyringone was added.

Hairy roots were induced by all strains of *A. rhizogenes* in the presence of acetosyringone. The transformation efficiencies varied significantly among the different strains used where by the percentages of hairy root formation of strains TR 105, LBA 9402 and ATCC 15834 were higher than that of strain 8196 (Table 2). The type of opine produced by the former three strains (TR 105, LBA 9402 and ATCC 15834) is agropine, whereas the later (strain 8196) produces mannopine (Table 1). Perhaps, agropine strains contain both the T_L and the T_R regions in their Ri plasmids which make them more virulent than the mannopine strains that do not contain auxin biosynthesis genes (Otani et al., 1993; Oksman-Caldentey and Hiltunen, 1996).

In some of the leaf explants, hairy roots induction occurred at the cut midrib as well as at the cut edge of the leaf explants (Figure 1A). Generally, for all of the leaf explants treated with *A. rhizogenes* in the presence or absence of acetosyringone, hairy roots appeared at the cut midrib of these explants. A similar observation on hairy root induction was documented in *B. oleracea* by Henzi et al. (2000). According to Mei et al. (2001) the diverse responses in leaf the explants' susceptibilities to *A. rhizogenes* may be due to differences in their physical conditions and to the physiological characteristics of the tissues in response to culture conditions. The phenotypes of the hairy roots induced by all strains of *A. rhizogenes* growing onto hormone-free MS basal solid medium showed that they were highly branched and not geotropic (Figure 1B). Similar phenotypes were observed for hairy roots of *Lycopersicon esculentum* (Hashimoto et al., 1999) and *Ammi majus* (Królicka et al., 2001). The hairy roots induced by strains ATCC 15834, TR 105, and LBA 9402 grew more vigorously than those by strain 8196 on hormone-free MS basal solid medium. According to Nguyen et al. (1992) and Hamill and Lidgett (1997), the plasmids of *A. rhizogenes* strains ATCC 15834, LBA 9402 and TR 105 are of the agropine type and thus are less dependent on the internal auxin levels in the explants than those of the mannopine type like *A. rhizogenes* strain 8196.

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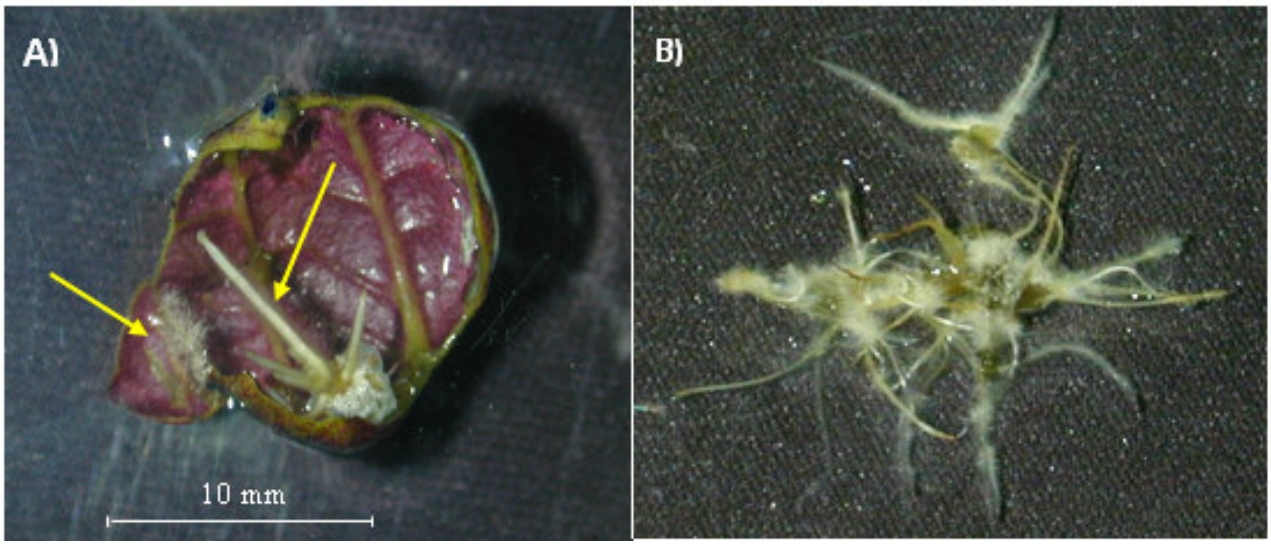

Figure 1. A) Hairy roots appeared at the cut edge of a leaf explant of *S. scutellarioides.* Arrows indicate the hairy root induced on different parts of the explant, B) Hairy roots of *S. scutellarioides* growing on hormone-free MS medium.

REFERENCES

Al-Sereiti MR, Abu-Amer KM, Sen, P (1999). Pharmacology of rosemary (*Rosmarinus officinals* Linn.) and its therapeutic potentials. Ind. J. Exptl. Biol. 37: 124-130.

Bauer N, Leljak-Levanic D, Jelaska S (2004). Rosmarinic acid synthesis in transformed callus culture of *Coleus blumei* Benth. Z. Naturforsch. 59c: 554-560.

Bhadra R, Vani S, Shanks VJ (1993). Production of indole alkaloids by selected hairyroot lines of *Catharanthus roseus*. Biotechnol. Bioeng. 41: 581-592.

Bolton GW, Nester EW, Gordon MP (1986). Plant phenolic compounds induce expression of the *A. tumefaciens* loci needed for virulence. Science, 232: 983-985.

Chae Young Am, Hee-Suk Yu, Ji-Sook S, Hee-Kyung C, Sang-Un P (2000). Indigo production in hairy root cultures of *Polygonun tinctorium* Lour. Biotechnol. Lett. 22: 1527-1530.

De-Eknamkul W, Ellis BR (1987). Tyrosine aminotransferase: the entrypoint enzyme of the tyrosine-derived pathway in rosmarinic acid biosynthesis. Phytochemistry, 26: 1941-1946.

Dommisse EM, Leung DWM, Shaw ML, Conner AJ (1990). Onion is a monocotyledonous host for *Agrobacterium.* Plant Sci. 69: 249-257.

Draper J, Scott R, Armitage P, Hamil J (1988). Transformation of dicotyledonous plant cells using Ti plasmid of *Agrobacterium tumefaciens* and the Ri plasmid of *A. rhizogenes.* In: Draper J, Scott R, Armitage P, Walden R (eds). Plant Genetic Transformation and Gene Expression-A Laboratory Manual. Black Scientific Publications, Oxford pp. 69-160

Garcia JF, O'Neil RJ (2000). Effect of Coleus size and variegation on attack rates, searching strategy, and selected life history characteristics of *Cryptolaemus montrouzieri* (Coleoptera: Coccinellidae). Biological Control, 18: 225-234.

Gomez KA, Gomez AA (1984). Statistical procedures for agriculture research, 2nd edition. An International Rice Research Institute Book. Philippines.

Hamill DJ, Lidgget JA (1997). Hairy root cultures - opportunities and key protocols for studies in metabolic engineering. In: Doran MP (ed) Hairy Roots Cultures and Application. Harwood Academic Publishers, Netherlands, pp. 1-29.

Hashimoto RY, Menck CFM, Van Sluys MA (1999). Negative selection driven by cytosine deaminase gene in *Lycopersicon esculentum* hairy roots. Plant Sci. 141: 175-181.

Henzi MX, Christey MC, McNeil DL (2000). Factors that influence *Agrobacterium rhizogenes*-mediated transformation of broccoli (*Brassica oleracea* L. var. *italica*). Plant Cell Rep. 19: 994-999.

Hu ZB, Alfermann AW (1993). Diterpenoid Production in Hairy Root Cultures of *Salvia Miltiorrhiza.* Phytochemistry, 32: 699-703.

Ionkova I, Kartinig T, Alfermenn W (1997). Cycloatane saponin production in hairy root cultures of *Astragalus mongholicus*. Phytochemistry, 45: 1597-1600.

Królicka A, Staniszewska I, Bielawski K, Malinski E, Szafranek J, Lojkowska E (2001). Establishment of hairy root cultures of *Ammi majus.* Plant Sci. 160: 259-264.

Kumar V, Sharma A, Narasimha Prasad BC, Bhaskar H (2006). *Agrobacterium rhizogenes* mediated genetic transformation resulting in hairy root formation is enhanced by ultrasonication and acetosyringone treatment. Electronic J. Biotechnol. 9: 349-357

Mazunder A, Neamati N, Sunder S (1997). Curcumin analogs with altered potencies against HIV-1 integrase as probes for biochemical mechanisms of drug action. J. Med. Chem. 40: 3057-3063.

Mei WY, Wang JB, Luo D, Jia JF (2001). Regeneration of plants from callus cultures of roots induced by *Agrobacterium rhizogenes* on *Alhagi pseudoalhagi*. Cell Res. 11: 279-284.

Misawa M (1994). Plant Tissue Culture: an alternative for production of useful metabolites. FAO Agricultural services bulletin, 108: p. 87.

Nguyen C, Bourgaud F, Forlot P, Guckert A (1992). Establishment of hairy root cultures of *Psoralea* species. Plant Cell Rep. 11: 424-427.

Oksman-Caldentey K-M, Hiltunen R (1996). Transgenic crops for improved pharmaceutical products. Field Crops Res. 45: 57-69.

Otani M, Mii M, Handa T, Kamada H, Shimada T (1993). Transformation of sweet potato (*Ipomoea batatas* (L.) Lam.) plants by *Agrobacterium rhizogenes*. Plant Sci. 94: 151-159.

Owens LD, Smigocki AC (1988). Transformation of soybean cells using mixed strains of *Agrobacterium tumefaciens* and phenolic compounds. Plant Physiol. 88: 570-573.

Park SU, Uddin MR, Xu H, Kim YK, Lee SY (2008). Biotechnological applications for rosmarinic acid production in plant. Afr. J. Biotech. 7: 4959-4965.

Petersen MS (1991). Characterization of rosmarinic acid synthase from cell cultures of *Coleus blumei.* Phytochemistry, 30: 2877-2881.

Petersen M, Simmonds MSJ (2003). Rosmarinic acid. Phytochemistry, 62: 121-125.

Phillips CG, Hubstenberger FJ, Hansen EE (1995). Plant regeneration by organogenesis from callus and cell suspension cultures). In: Gamborg OL, Philips GC (eds). Plant Cell, Tissue and Organ Culture Fundamental Methods. Springer-Verlag Berlin, Heidelberg, Germany, pp. 67-79.

Razzaque A, Ellis BE (1977). Rosmarinic acid production in *Coleus* cell

cultures. Planta, 137: 287-291.
Sheikholeslam SN, Weeks DP (1987). Acetosyringone promotes high efficiency transformation of *Arabidopsis thaliana* explants by *Agrobacterium tumefaciens*. Plant Mol. Biol. 8: 291-298.
Stachel SE, Messens E, Van Montagu M, Zambryski P (1985). Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature, 318: 624-629.