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# *Agrobacterium* pRi $T_L$-DNA *rol*B and $T_R$-DNA Opine Genes Transferred to the Spiny Amaranth (*Amaranthus spinosus* L.), A Nutraceutical Crop

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**Summary**

*In vitro* rhizogenesis occurred with a characteristic pattern typical of transformed roots following explant (internode/leaf) inoculation of *Amaranthus spinosus* L. with four different wild type *Agrobacterium rhizogenes* strains. The extent of rhizogenesis varied considerably with the explant type and source, and with the *Agrobacterium* strains employed; internodal segments performed better than leaves. Of the strains employed for cocultivation, *A. rhizogenes* LBA 9402 carrying pRi 1855 was the most virulent and infectious, causing hairy root induction in the maximum number of explants regardless of their type. Individual root clones (rhizoclones) were maintained on Murashige and Skoog's basal medium without growth regulators. The physical presence of the *rol*B gene in the $T_L$-DNA segment of the Ri plasmid of the infecting *Agrobacterium* in leaf tissues of plants regenerated from selected rhizoclones was confirmed by a positive PCR amplification. The ability of the genetically transformed plants to harbour and express $T_R$-DNA specific opine synthase genes (*man*2 and *ags*) was substantiated by PCR and opine assay respectively, demonstrating the production of characteristic opines. Such findings are implicated in the context of pharmaceutical exploitation of transformed root cultures of *A. spinosus* and also towards protecting this nutraceutically important crop, amaranth, against biotic stress challenges *via* transgenic manipulations.

*Key words*: *Amaranthus spinosus* L., *Agrobacterium rhizogenes*, rhizoclones, PCR amplification, opine assay

## Introduction

*Amaranthus spinosus* L. (spiny amaranth or spiny pigweed) of family Amaranthaceae, order Caryophyllales possesses a considerable dietary value as a calcium-rich leafy vegetable. The leaves and tender stalks contain (in %): moisture 84.5, protein 3, carbohydrates 8.1, crude fibre 1.3 and fat 0.5 along with other constituents such as (in mg per 100 g): calcium 560, phosphorus 65, iron 30.5 and ascorbic acid 30 and hence qualifies as an excellent feed for infants and lactating mothers especially in low-income food-deficit countries (LIFDC). The

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plant parts are also a highly nutritious meal for sheep and goats, which increases milk production in cattle, and seeds are eaten by birds. Besides its edible value, the plant species holds several properties for ethnomedicinal use. It is regarded as an emmenagogue and galactagogue and also used as a refrigerant, diuretic and purgative, as an enema for stomach troubles, haemorrhoids and against cholera. A decoction of the plant improves digestion and is used in kidney complaints as well as a mouthwash for toothache. The plant is used as an emollient and applied to bruises, abscesses and inflammations. The ash of the plant is applied to chancre. The decoction with palm nut soup is used to prevent miscarriage. The most important parts of this plant species are the roots which contain α-spinasterol, α-spinasterol octacosanoate and three saponins. Roots are particularly used against menorrhagia, gonorrhoea, eczema and colic. They are also used as expectorant (*1*).

In view of this, modern biotechnological intervention could be an effective means to exploit the plant species for its medicinal value. As most of the active principles of pharmaceutical relevance are present in the roots of this plant species, *in vitro* root cultures could be the most appropriate source from which useful secondary metabolites could be extracted. In this context, the neoplastic 'hairy root' disease naturally caused in plants due to infection by the soil-dwelling Gram-negative phytopathogenic *Agrobacterium rhizogenes* is, indeed, interesting and holds significance. The disease develops as a consequence of integration of oncogenic coding sequences into host plant genome and their overexpression, resulting in an unregulated auxin biosynthesis following the transfer of T-DNA from the large Ri plasmids of infecting *Agrobacterium via* a well-characterized signal transduction (*2*). The genetically transformed hairy roots, thus induced, can proliferate exceptionally fast in culture media without necessitating phytohormones (precisely auxins). Establishment of hairy root cultures mediated by the pRi T-DNA of *Agrobacterium rhizogenes* has been viewed as an effective source of high-value secondary metabolites (*3,4*). In this context, *Agrobacterium*-transformed hairy root cultures of *Amaranthus spinosus* could serve as an effective and sustainable source of medicinally important phytochemicals needed as raw ingredients for manufacturing selective drug types so as to target specific diseases.

On the other hand, a majority of amaranth plants are susceptible to fungal pathogens including *Fusarium* (*F. equisetti* and *F. moniliforme*), *Aspergillus* (*A. flavus*, *A. parasiticus* and *A. versicolor*) and *Penicillium* (*P. viridicatum*, *P. puberulum*, *P. crustosum*, *P. citrinum*, *P. expansum*) and *Rhizoctonia solani* (*5*). Several *Amaranthus* species including *A. spinosus* are also known to be attacked by foliar insects such as leaf miners, leaf rollers, cutworms, aphids, flea beetles and mites (*6*). Blister beetles and alfalfa webworms are the two leaf feeders that are known to cause substantial economic yield loss. Besides, the plants are susceptible to two common viruses namely *Amaranthus* leaf mottle potyvirus and *Amaranthus* mosaic potyvirus. Genetic transformation of a range of economically important crop plant species used as common leafy vegetables including broccoli, cabbages (rapid cycling cabbage, head cabbage, doubled-haploid cabbage), Chinese cabbage, lettuce, spinach and watercress has been possible by exploiting *Agrobacterium tumefaciens*-mediated gene transfer. *Bacillus thuringiensis* δ-endotoxin genes (*Bt*) of different isoforms such as *cry1Ac*, *cry1Ab3* and *cry1la3* were successfully transferred to broccoli (*Brassica oleracea* var. *italica* cv. Green Comet; *7*), head cabbage (*B. oleracea* var. *capitata*; *7,8*) and watercress (*Rorippa nasturtium-aquaticum*; *9*) in order to confer crop protection against insect pests. Besides, cowpea trypsin inhibitor gene (*CpTi*) was transferred to Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) with a view to bestow resistance against insect pest damage (*10*). *Agrobacterium*-mediated genetic transformation was also reported for endowing resistance to chickpea against weevil (*11*) and also to peas and azuki beans against one or more species of bruchid beetles by delivering α-amylase inhibitor gene from common bean (*α-AI-Pv*).

Therefore, it was imperative to develop a transformation system aimed at causing genetic improvement of this hitherto underexploited crop using suitable constructs of transgenes to confer resistance to biotic stress due to pests, viruses or fungal pathogens. Success in transgenic manipulation of amaranth plants is only limited to the grain-type *A. hypochondriacus* using *Agrobacterium tumefaciens* to study the expression of a light-harvesting chlorophyll a/b-binding protein gene promoter (*12*). More recently, efforts have also been made to transform a leafy type amaranth (*A. tricolor*) mediated by *Agrobacterium rhizogenes* (*13*). However, to date, there has been no report on genetic transformation of the spiny amaranth (*A. spinosus*).

A prerequisite to the success of using *Agrobacterium rhizogenes* for induction and establishment of hairy root cultures of *Amaranthus spinosus* followed by plant regeneration is the succinct evaluation and standardization of *Agrobacterium*×plant factors influencing genetic transformation. These would include bacterial strains, growth phase of the culture, cell density, virulence inducer, explant type, infection strategy, cocultivation period, bactericidal antibiotic, *etc.* Therefore, in the present study, efforts have been made to optimize each one of them with a view to maximize the transformation efficiency and thereby develop a reproducible protocol for *Agrobacterium*-mediated genetic transformation of this nutraceutically important crop, spiny amaranth.

## Materials and Methods

### *Agrobacterium strains and culture*

The wild type A4 strain of *Agrobacterium rhizogenes* (a kind gift from Dr D. Tepfer, Laboratory for Rhizosphere Biology, National Institute of Agronomic Research (INRA), Versailles Cedex, France) harbours an agropine-type pRi A4 (*14*), while LBA 9402 (a kind gift from Dr M. R. Davey, School of Biological Sciences, University of Nottingham, Nottingham, UK) is a rifampicin-resistant derivative of NCPPB 1855 and possesses the agropine-type Ri plasmid pRi 1855 (*15*).The agropine-type strain A4T (*16*) and mannopine-type 8196 (*17*) resulted from transconjugation of pRi A4 and pRi 8196 into a Ti cured cell line of *A. tumefaciens* C58, respectively. *A. rhizogenes* LBA 9402 strains were grown at 26–28 °C in modified

YEB medium containing (in g/L): yeast extract 1, nutrient broth 5, peptone 5, sucrose 5, agar 15; pH=7.4. The pH of the medium was adjusted prior to autoclaving and the medium cooled to 40 °C in water bath. Thereafter, 2 mL of 1 M $MgSO_4 \cdot 7H_2O$ and 50 mg/L of rifampicin from respective filter-sterilized stock solutions were added to it. *A. rhizogenes* A4 strains were grown in YMA medium containing (in g/L): yeast extract 5, casamino acids 0.5, mannitol 8, $(NH_4)_2SO_4$ 2, NaCl 5; pH=6.6. For explant infection, a loopful of bacteria from a single colony was inoculated into 10 mL of liquid medium in a 50-mL Erlenmeyer flask and incubated on a reciprocal shaker at 120 rpm and 28 °C. A 100-μL aliquot of the overnight suspension was reinoculated into fresh medium (10 mL in 50-mL Erlenmeyer flask). Prior to bacterial inoculation, acetosyringone (Sigma-Aldrich, St. Louis, MO, USA; 100 mM stock solution in dimethyl sulfoxide, DMSO) was added to the culture medium at final concentrations of 50, 75, 100, 150 and 200 μM. The cultures were grown for 16–18 h on a reciprocal shaker at 120 rpm and 28 °C.

### *In vivo germination of A. spinosus seeds*

Seeds of *Amaranthus spinosus* L. (NBPGR accession no. NIC-22579) were obtained from the National Bureau of Plant Genetic Resources, Government of India. Seeds were germinated in plastic HIKO™ trays containing garden soil and farmyard manure in a 9:1 ratio, moistened with tap water and kept in the glasshouse. Internode and leaf explants were then kept in a 50-mL beaker (Borosil, Mumbai, India) with a net cover and placed under running tap water for 30 min. Thereafter, explants were treated with 7.5 % (by volume) Lizol (Reckitt Benckiser India Ltd, Gurgaon, Haryana, India) for 30 min and rinsed in autoclaved tap water (5–6 changes). Explants were then surface-disinfected with 0.1 % (by mass per volume) solution of mercuric chloride in water for 8 min, followed by five rinses in autoclaved double distilled water.

### *Transformation of explants*

Leaf and internode explants from 3-month-old glasshouse-grown plants following surface-disinfection were used for cell transformation. Apical portions of the cut internodal surfaces and leaf midribs were inoculated with 10–30 μL of an overnight culture of *Agrobacterium* by means of a sterile hypodermic needle in two ways. The explants were either injected with bacterial suspension directly by hypodermic syringe or were wounded by manual pricking with the sterile needle and then immersed in the bacterial suspension (10–20 min). A third method was also attempted in which explants were floated in the *Agrobacterium* suspension without a previous manual pricking. *Agrobacterium*-treated explants were plunged in 300-mL screw-capped jars (Excel Glasses Ltd, Alleppey, Kerala, India) containing 0.8 % (by mass per volume) agar-solidified full-strength Murashige and Skoog's (MS) medium (*18*) without growth regulators (MS0; 20 mL per jar) and the jars were kept in plant growth chamber at (25±1) °C under diffuse light with a low photon flux density (PFD) of 10–15 μmol/($m^2$·s) provided by cool white fluorescent tubes (Philips, Mumbai, India) and at 60 % relative humidity. Following the cocultivation (2–8 days), the explants were transferred to MS0 agar medium supplemented with a bactericidal antibiotic. Three different antibiotics were tested, namely sporidex (Ranbaxy, Mumbai, India), carbenicillin (Sigma-Aldrich) and cefotaxime (Sigma-Aldrich). Control cultures, containing similar explants but wounded with the hypodermic needle without bacteria, were maintained under similar light and temperature conditions as the inoculated cultures.

### *Statistical analysis*

All explant transformation experiments were set up in a completely randomized design (CRD). Each treatment consisted of 10 replicate jars, each containing 1–2 leaf explants or 3–4 internodes. Each experiment was repeated 7 times. Data were analysed using analysis of variance (ANOVA) for a completely randomized design. Duncan's new multiple range test (DMRT) was used to separate the mean values of the significant effect (*19*).

### *Root cloning and maintenance of transformed rhizoclones*

Individual roots (1.0–1.5 cm) that were developed along the inoculated surface were excised and each transferred to 50×11 mm transparent plastic TPX® Petri dish (Tarsons Products Pvt. Ltd., Kolkata, India) containing 6–8 mL of MS0 agar medium. The medium was supplemented with bactericidal antibiotics cefotaxime, carbenicillin or sporidex (250–1000 μg/mL). Root clones were incubated at (25±1) °C and PFD of 15–20 μmol/($m^2$·s) and subcultured every 2–4 weeks onto 86×13 mm transparent plastic TPX® Petri dish (Tarsons Products) containing 20 mL of medium. The cefotaxime concentration in the medium was gradually reduced first to 250 μg/mL and then to 100 μg/mL. Subsequently, roots were maintained in MS0 medium without cefotaxime.

### *Plant regeneration from transformed roots*

Transformed root clones (rhizoclones) were selected on the basis of their auxin-independent growth coupled with opine proficiency and these were grown in MS0 medium for spontaneous shoot regeneration. In addition, they were also placed on MS medium containing different concentrations (1–3 mg/L) of cytokinins (kinetin/$^6$*N*-benzyladenine/zeatin) to promote shoot organogenesis. The light and temperature regime were: 16-hour photoperiod with 50 μmol/($m^2$·s) of PFD provided by photosynthetic (daylight) fluorescent tubes, and (25±1) °C, respectively. Regenerated plantlets were then transferred to plastic pots (7.5 cm in diameter) containing autoclaved vermicompost (Ranjan Agrotech, Bhubaneswar, India) moistened with autoclaved water. The potted plants, individually covered with polyethylene bag, were acclimatized inside a plant growth chamber set at (25±1) °C, 85–90 % relative humidity and 16-hour photoperiod with light intensity of 50 μmol/($m^2$·s) of PFD provided by photosynthetic (daylight) fluorescent tubes. After 3 weeks, the plants were transferred to large earthenware pots (18 cm in diameter) containing garden soil (soil/compost= 1:1) and kept in the glasshouse.

*Polymerase chain reactions*

Genomic DNA was isolated from leaf tissues of different putative transgenic lines according to a modified hexadecyltrimethylammonium bromide (CTAB) extraction protocol (*20*). Leaf DNA was also isolated from a non-transformed plant so as to serve as a negative control. *A. rhizogenes* LBA 9402 was used as the source of plasmid DNA (pRi 1855) to serve as a positive control. The oligonucleotide primer sequences (Bangalore Genei Pvt. Ltd., Bangalore, India) used for PCR amplification of the *rol*B gene were: 5′-ATGGATCCCAAATTGCTATTCCTTCCACGA-3′ (forward) and 5′-TTAGGCTTCTTTCTTCAGGTTTACTGCAGC-3′ (reverse). The primers for amplifying the *man*2 gene were: 5′-GCGCATCCCGAGGCGATG-3′and 5′-AGGTCTGGCGATCGCGAGGA-3′. Primers for amplification of *ags* gene were: 5′-CGGAAATTGTGGCTCGTTGTGGAC-3′ and 5′-AATCGTTCAGAGAGCGTCCGAAGTT-3′. The optimum PCR mixture (25 μL) contained 1 U of *Taq*DNA polymerase (Bangalore Genei), 10 mM Tris-HCl (pH=8), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dinucleotide triphosphate (dATP, dCTP, dGTP and dTTP), 1 μL of each forward and reverse primer (10 pM) and 50 ng of template DNA. The cyclic DNA amplification was performed using the following program of the thermal cycler (Applied Biosystems®, Life Technologies™, Carlsbad, CA, USA): initial template denaturation at 95 °C for 2.5 min, annealing at 55 °C for 1 min and extension (copy-synthesis) at 72 °C for 3 min for the first cycle, followed by 33 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 30 s and extension at 72 °C for 1.5 min. The final cycle was extended with an additional step at 72 °C for 5 min prior to hold at 4 °C. The amplified product was separated by 1 % agarose gel electrophoresis in 1×TAE buffer (0.04 M Tris-acetate, 1 mM EDTA; pH=8.0) at 80 V for 45 min. The gel was subsequently stained with ethidium bromide solution (0.5 μg/mL) for 15 min by continuous gentle shaking on a rocker, followed by destaining in double-distilled water (30 min). The gel was then photographed using a Gel Documentation unit (Bio-Rad, Hercules, CA, USA).

*Opine assay*

The method used to analyze transformed leaf tissues for the presence of agropine/manopine was based on that originally described for hairy roots by Morgan *et al.* (*21*) with modifications. Fresh leaves of transformed plants regenerated from randomly selected rhizoclones (200 mg of each) were separately homogenized in an Eppendorf tube (1.5 mL) with a few drops of 0.1 M HCl and centrifuged at 12 000 rpm for 5 min. The aliquot (10 μL) of the supernatant was then spotted on the Whatman 3MM Chr chromatography paper and air dried. Pure agropine solution (2 μL) as the reference standard was spotted at one extreme lane. The paper was carefully soaked with the buffer (formic acid/acetic acid/water= 1:3:16, by volume) and blotted dry to remove the extra buffer before placing it in the buffer tank. Electrophoresis was performed from cathode to anode with constant voltage of 300 V for 1 h (Biotech, Kerala, India). Then the paper was air dried for 1 h. For the development of electropherogram, the paper was dipped successively in three different solutions inside a dark room in the following sequence: (*i*) solution A (625 mg of $AgNO_3$ in 250 μL of water added to 50 mL of acetone; water was added dropwise until silver precipitate was dissolved) followed by air-drying for 20 min; (*ii*) solution B (10 mL of 20 % NaOH+90 mL of methanol) until the appearance of black spots; and (*iii*) solution C (5 % by mass per volume of sodium thiosulphate) for rinsing (15 min). The paper was gently washed under slow-running tap water to remove the excess stain and was then air dried. Leaves of *in vitro* seedlings without *Agrobacterium* treatment were subjected to opine assay to serve as a negative control.

## Results

*Genetic transformation and root development*

Roots emerged directly from the inoculated sites on stem internodes and leaf midribs. In some cases, localized swellings developed along the infected regions of the internodes and leaf midribs, from most of which roots also developed. These aerial roots were different from normal subterranean roots in exhibiting a characteristic lack of positive geotropism. They were ageotropic, most being plagiotropic (growing parallel to the culture medium), while some displayed negative geotropism with a tendency of growing towards light and away from the culture medium (Fig. 1a). Root clones resulting from inoculation were further characterized by their prolific growth on MS0 medium and a high incidence of lateral branching. They grew as closely interwoven masses over the surface of the culture medium and up to the sides of the culture dish (Fig. 1b). Uninfected control explants, lacking a bacterial treatment, did not produce roots at the inoculation sites. These, especially the stem internodes, developed a few roots (1–2) from the basal portion immersed inside the culture medium, but they failed to grow when excised and transferred to the MS0 medium.

*Plant regeneration from transformed roots*

Shoots developed spontaneously, albeit infrequently, from a limited number of transformed root clones capable of auxin-independent growth in MS0 medium (MS0). Shoot organogenesis was stimulated by the MS medium supplemented with cytokinins, of which zeatin was the most effective at an optimal level of 2.0 mg/L (Fig. 1c). Regenerated plants were successfully grown in the glasshouse (Fig. 1d) and some of these plants at a later stage of growth exhibited phenotypic anomalies such as extensive lateral branching, shorter internodes resulting in dwarfism and variations in leaf shape and size.

*Factors influencing transformation efficiency*

Explant type

Under optimum conditions, root development occurred in 8–10 days on internode explants, whereas 10–12 days were required for rhizogenesis on the leaf explants (Table 1). Of the two types of explants used in transformation experiments, internode and leaf, the former was better with respect to an early root emergence, percentage rhizogenesis and number of putative transformed roots per explant (Table 1).

Bacterial strain

Of the four different types of *Agrobacterium rhizogenes* strains (LBA 9402, A4T, A4, 8196) used, LBA 9402

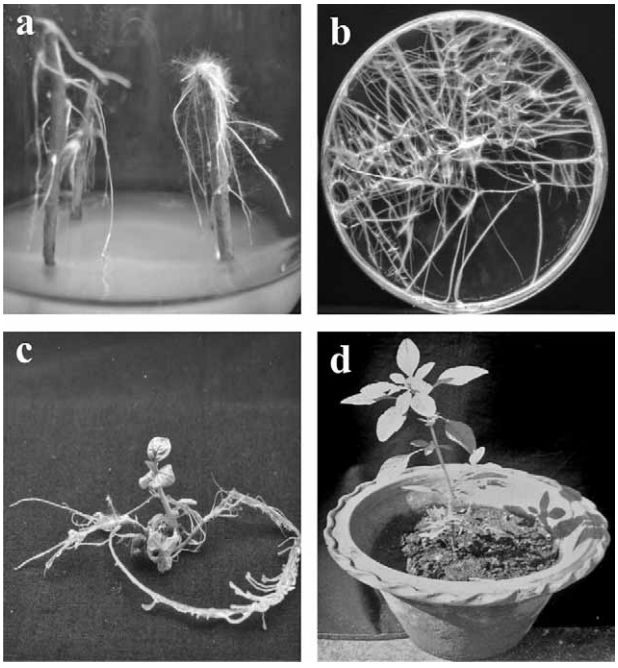


**Fig. 1.** Development of hairy roots and plant regeneration: a) emergence of hairy roots at the inoculated site of an internode of *Amaranthus spinosus* at day 20 following the infection with LBA 9402 suspension pretreated with acetosyringone, b) a proliferated hairy root clone of *A. spinosus* (AS4D9) at day 30 following transfer of a single root (a) on the growth-regulator-free medium (MS0), c) shoot regeneration from a rhizoclone (AS4D9) in MS medium with 2.0 mg/L of zeatin, and d) a 40-day-old potted plant in the glasshouse following acclimatization

was the most infectious one resulting in a markedly higher number of transformants (approx. 98.57 %) (Table 1). After 8–10 or 10–12 days of treatment with LBA 9402 strain, roots emerged from stem internode and leaf explants respectively, whereas much longer periods, *i.e.* 24–25 or 23–27 days were required, respectively, for the induction of hairy roots from internodal and leaf explants infected with the 8196 strain (Table 1).

### Bacterial growth phase and cell density

In order to determine the right stage of growth of *A. rhizogenes* for high efficiency transformation, four different growth phases were examined. At a late log phase, corresponding to the absorbance at 660 nm ($A_{660\ nm}$=0.6), maximum transformation of approx. 98.57 % occurred when using LBA 9402 strain, and of approx. 81.90 % when using A4T strain (Fig. 2a). Extensive tissue damage occurred at $A_{660\ nm}$ values greater than 1.0 because of the bacterial overgrowth. A range of bacterial cell densities (0.5 to 2.0·$10^9$ cells/mL) adjusted finally through dilution of the *Agrobacterium* suspension was evaluated for explant infection. The best results showing the highest transformation frequency (approx. 98.57 % using LBA 9402, 81.90 % using A4T, 57.62 % using A4 and 21.90 % using 8196) were obtained with a density of $10^9$ cells per mL following a 50 % (1:1) dilution of the original culture at $A_{660\ nm}$=0.6. The bacterial cell density of $10^9$ cells per mL was optimum for each *A. rhizogenes* strain; the results at other cell densities varied proportionately to the strains.

### Bacterial preculture with virulence inducer acetosyringone

A range of concentrations of acetosyringone (50–200 μM) were evaluated, and 100 μM was the most effective with respect to transformation frequency regardless of *Agrobacterium* strain employed (Fig. 2b). Acetosyringone levels below or above the threshold were less effective. Explants infected with bacterial suspension supplemented with 100 μM of acetosyringone showed an early emergence of hairy roots, *i.e.* within 8–10 days, whereas root induction required 12–15 days without acetosyringone treatment regardless of the strain and explant type.

### Infection methods

Of the three different methods used for infecting the explants with *Agrobacterium rhizogenes*, the one in which explants were pre-pricked followed by dipping in bacterial solution was the most suitable and gave the maximum number of transformants (approx. 98.57 % using LBA 9402 strain) followed by direct inoculation (approx. 75.68 % using LBA 9402 strain) (Fig. 2c). Transformation percentages were negligible in unwounded explants regardless of the explant type and *Agrobacterium* strain employed.

### Cocultivation period

With a view to determine the suitable period of cocultivation, both explant types were separately cocultivated with a different *Agrobacterium* strain for 2–8 days. The maximum transformation efficiency was achieved after 5 days of cocultivation (approx. 98.57 % using LBA

Table 1. Hairy root development in genetically transformed *Amaranthus spinosus* following explant cocultivation with various strains of *Agrobacterium rhizogenes*

| Strain | Days to root emergence | | Total no. of explants inoculated | | Total no. of explants producing roots | | Root emergence % | | Mean no. of roots per explant | |
|---|---|---|---|---|---|---|---|---|---|---|
| | I | L | I | L | I | L | I | L | I | L |
| 8196 (pRi 8196) | 24–25 | 23–27 | 210 | 140 | 46 | 15 | 21.90$^{d}$ | 10.71$^{d}$ | 4.2$^{d}$ | 2.3$^{d}$ |
| A4 (pRi A4) | 20–21 | 22–24 | 210 | 140 | 121 | 55 | 57.62$^{c}$ | 39.28$^{c}$ | 6.8$^{c}$ | 3.5$^{c}$ |
| A4T (pRi A4) | 14–17 | 15–18 | 210 | 140 | 172 | 98 | 81.90$^{b}$ | 70.00$^{b}$ | 9.6$^{b}$ | 3.8$^{b}$ |
| LBA 9402 (pRi 1855) | 8–10 | 10–12 | 210 | 140 | 207 | 133 | 98.57$^{a}$ | 95.00$^{a}$ | 15.2$^{a}$ | 5.5$^{a}$ |

Data pooled from a total of 7 cocultivation experiments each consisting of 10 replicate jars containing 1–4 explants each
I=internode, L=leaf; mean values within column with different letter in superscript are significantly different ($p \leq 0.05$; Duncan's new multiple range test)

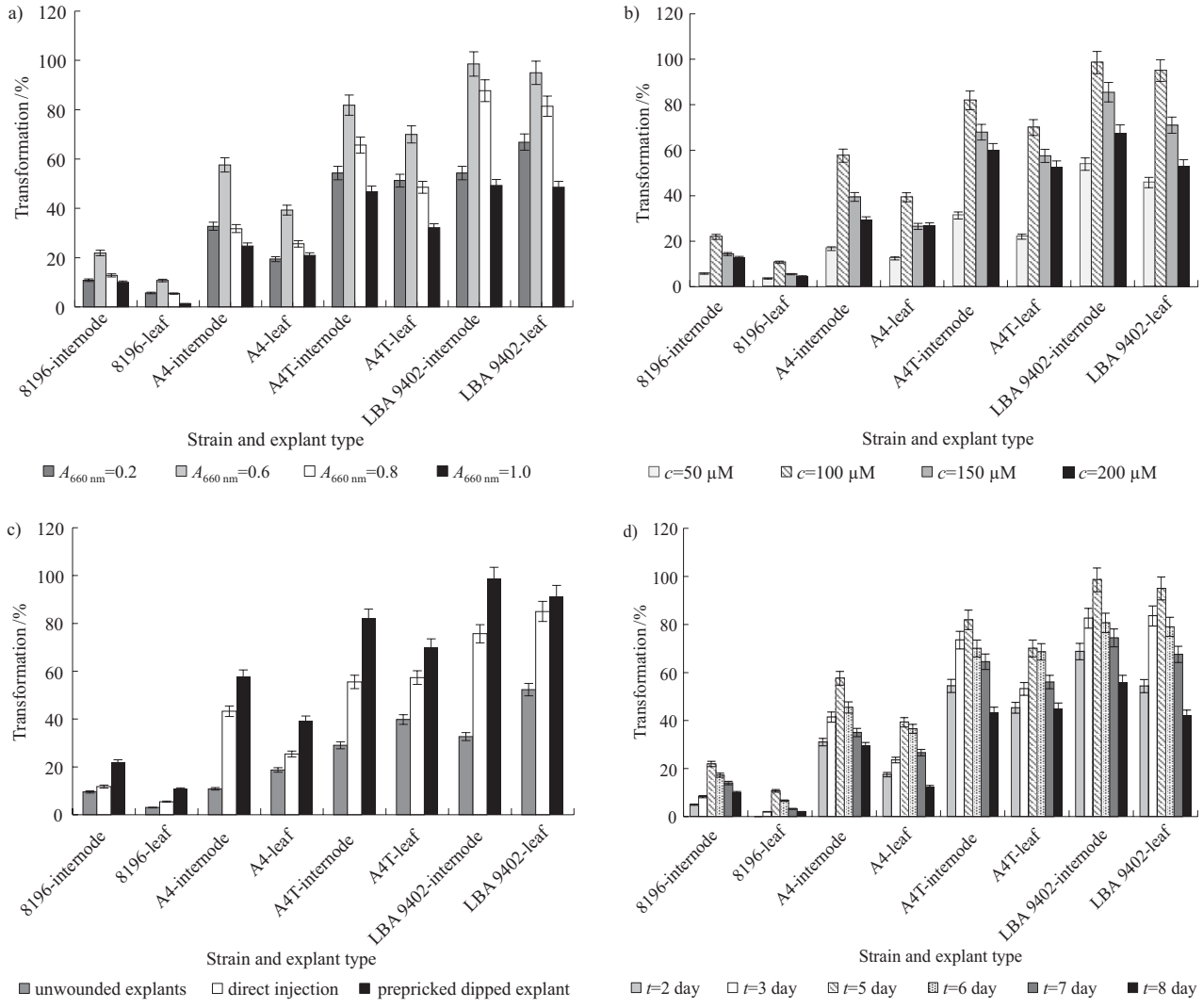


**Fig. 2.** Factors affecting transformation efficiency: a) growth phase, b) acetosyringone concentration, c) infection methods, and d) cocultivation period

9402 strain; Fig. 2d). Cocultivation periods longer than 5 days were unsuitable because of uncontrollable overgrowth of bacteria.

Bacterial overgrowth and bacteriostatic chemicals

An important consideration during infection and cocultivation was to reduce the overgrowth of the background bacteria. It was noticed that blotting the explants dry after the treatment with *Agrobacterium* affected their further development. Besides, it also drastically reduced the bacterial inoculum for subsequent cocultivation. Therefore, care was taken to blot the explants only sparingly for a very brief period (10–15 s) on preautoclaved filter paper or tissue towel (2–3 layers). This was done by inoculating the *Agrobacterium*-treated explants on two (over-layered) autoclaved filter papers, the latter being placed on the cultured medium in Petri dishes. This technique facilitated changeover to fresh media thereby reducing the bacterial overgrowth with each change.

Among different bacteriostatic antibiotics tested, cefotaxime was the most effective in eradicating background *Agrobacterium* quite successfully (data not shown), and the concentration of 500 μg/mL was the most effective. Higher concentrations were phytotoxic as most of the young roots turned necrotic and died, regardless of the *A. rhizogenes* strain employed.

## *PCR amplification for detection of rolB and opine genes in the transformed rhizoclone-derived plants*

Genomic DNA isolated from leaf tissues of plants regenerated from selected putative transformed rhizoclones, capable of showing auxin-independent growth, revealed the expected amplification product of 776 bp using *rol*B gene-specific primers (Fig. 3) as well as 512 bp or 1.6 kb using *man*2 or *ags* gene-specific primers, respectively (Figs. 4 and 5). No amplification product was detected in leaf DNA from untransformed plants when subjected to PCR amplification with the gene primers specific for the *rol*B or opine gene sequences. This indicated the presence of the *Agrobacterium rol*B and opine (*man2*/*ags*) genes as part of the pRi $T_L$–DNA and $T_R$–DNA, respectively, in the recipient plant genome *via* genetic transformation.

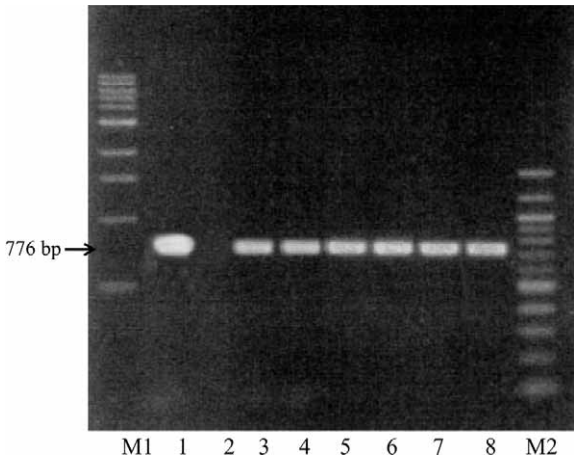


**Fig. 3.** PCR amplification of *rol*B gene in leaf tissues of plants regenerated from rhizoclones of *Amaranthus spinosus* transformed by *Agrobacterium rhizogenes* LBA 9402
M1: DNA marker (1 kb ladder), M2: DNA marker (100 bp ladder), lane 1: plasmid DNA pRi 1855 of LBA 9402 (positive control), lane 2: DNA from non-transformed plant (negative control), and lanes 3–8: DNA from putative transformed clones (AS4D2, AS4D9, AS4E7, AS4F8, AS4H2 and AS4J1, respectively)

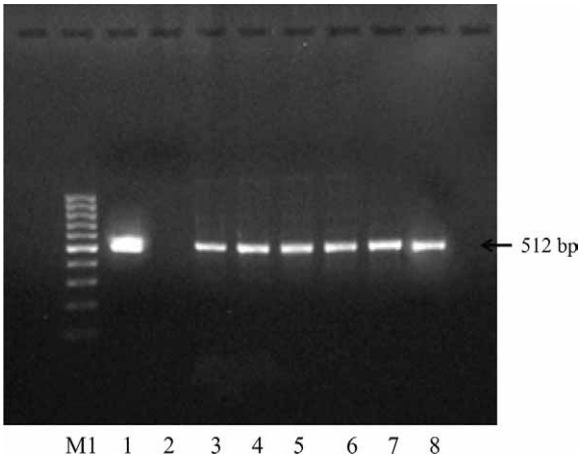


**Fig. 4.** PCR amplification of *man*2 gene in leaf tissues of plants regenerated from rhizoclones of *Amaranthus spinosus* transformed by *Agrobacterium rhizogenes* LBA 9402
M1: DNA marker (100 bp ladder), lane 1: plasmid DNA pRi 1855 of LBA 9402 (positive control), lane 2: DNA from non-transformed plant (negative control), and lanes 3–8: DNA from putative transformed clones (AS4D2, AS4D9, AS4E7, AS4F8, AS4H2 and AS4J1, respectively)

### *Opine detection*

Crude extracts from leaf tissues of putative transformed plants regenerated from selected rhizoclones were found to develop silver nitrate-positive blackish-brown spots along the electropherogram (Fig. 6). All putative transformed plants from selected rhizoclones contained considerable amounts of compounds which had the same mobility as authentic agropine and/or as additional compounds with a lower mobility representing a combination of mannopine and mannopinic acid, the opine chain precursors of agropine. Agropine was the predominant compound in all transformed clones. None of these compounds was evident in the extracts of non-transformed control plants.

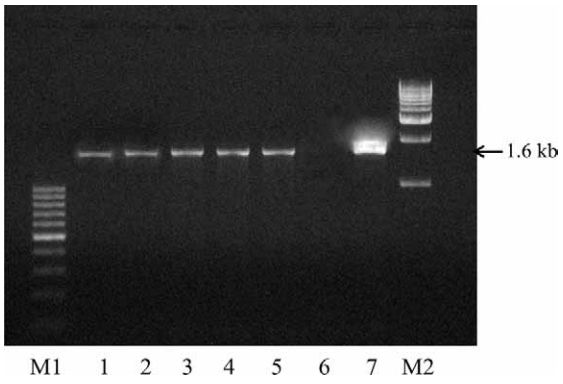


**Fig. 5.** PCR amplification of *ags* gene in leaf tissues of plants regenerated from rhizoclones of *Amaranthus spinosus* transformed by *Agrobacterium rhizogenes* LBA 9402
M1: DNA marker (100 bp ladder), M2: DNA marker (1 kb ladder), lanes 1–5: DNA from putative transformed clones (AS4D9, AS4E7, AS4F8, AS4H2 and AS4J1, respectively), lane 6: DNA from non-transformed plant (negative control), and lane 7: plasmid DNA pRi 1855 of LBA 9402 (positive control)

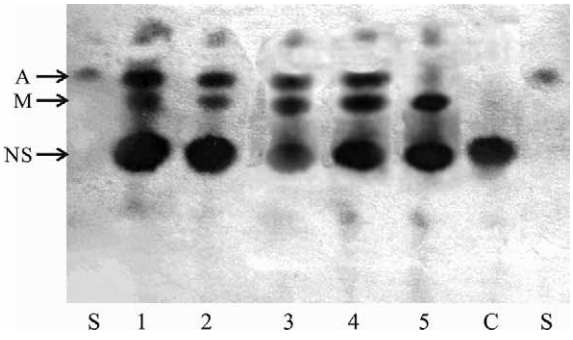


**Fig. 6.** Paper electropherogram of leaf extracts of *Amaranthus spinosus* plants regenerated from transformed rhizoclones demonstrating the presence of agropine (A) and/or mannopine (M) in addition to neutral sugar (NS)
S: agropine standard, C: untransformed control, lane 1: AS3C16 (A4T), lane 2: AS2B3 (A4), lane 3: AS2B8 (A4), lane 4: AS4D9 (LBA9402), and lane 5: AS1A7 (8196)

## Discussion

As for Ti plasmids, pRi T region apparatus of *A. rhizogenes*is is also designed in accordance with the specific types of opines produced. Different strains of *A. rhizogenes* produce different types of opines and, so far four of them have been identified, namely agropine, mannopine, cucumopine and mikimopine (*22*,*23*). Unlike the three other opine-type Ri plasmids, which share an uninterrupted T region (*24*), the agropine-type Ri plasmids harbour two T-DNA segments: $T_L$-DNA (19–20 kb) and $T_R$-DNA (10–12 kb) separated by an intervening spacer DNA fragment of 18 kb (*15*,*25*,*26*). The $T_L$-DNA region is responsible for the hairy root phenotype and the $T_R$ region for agropine as well as auxin biosynthesis (*25*,*27*). The $T_L$-DNA region carries 18 open reading frames (ORFs), a few of which are involved in the auxin-related functions and hence, its integration into plant genome is

essential for the induction of hairy roots (*28*). However, even in the absence of $T_R$ region-directed auxin biosynthesis, as in the mannopine-types, root induction does occur. The mannopine- and cucumopine-type Ri plasmids harbour T-DNA which bear strong homology to the $T_L$-DNA region of the agropine-type Ri plasmids (*29,30*). Thus, the presence of $T_R$ region is not required for the hairy root phenotype and root lines incited by agropine-type *A. rhizogenes* strains do not always synthesize agropine (*15,22*). Although $T_R$ region is not mandatory for hairy root formation, the auxin biosynthesis gene *aux1* harboured in this segment, having homology with *tms* loci (*25,31*), provides an additional source of auxin and plays a supportive role in the root differentiation (*32*). Apparently, two different mechanisms exist to account for hairy root organogenesis: one depends on auxin overproduction directed by the pRi $T_R$-DNA of certain *A. rhizogenes* strains, whilst the other is independent of the transfer and expression of genes directing the biosynthesis of auxin (*33*).

Interestingly, of the 18 open reading frames in pRi $T_L$-DNA region, ORFs 10, 11, 12 and 15 represent the 4 root-inducing loci, namely *rol*A, *rol*B, *rol*C and *rol*D respectively (*34*). These *rol* genes have been found to be essential for hairy root induction (*26*), with *rol*B playing the most important role, while the three remaining loci promote root formation synergistically (*35*). The exact cellular effects of these *rol* genes are still not clearly understood; however, they are known to enhance the sensitivity of plant cells to endogenous auxins (*36*), resulting in the expression of hairy roots (*28*). During the normal bacterial infection process, these *rol* genes are transferred and expressed in the plant cell genome (*37*); *rol*B, in particular, seems to be the most crucial in the differentiation process of transformed cells, while *rol*A and *rol*C provide accessory functions (*33*). The gene *rol*B containing a 777-bp ORF encodes a 259-amino acid protein with a molecular mass of 30 kDa, which remains localized in the plasma membrane (*38*). Plants transformed with *rol*B have tyrosine phosphatase activity, which strengthens its role in the indole-3-acetic acid (IAA) signal perception/transduction pathway (*38*). Successful plant regeneration in transformed root cultures is either spontaneous or induced by growth regulators as reported in several plants including *Crotalaria juncea* (*39*), *Catharanthus roseus* (*40*), *Taraxacum platycarpum* (*41*) and *Tylophora indica* (*42*).

Optimization of *Agrobacterium*×plant factors influencing transformation events was a prerequisite for exploiting Ri plasmid vector to its full potential. Transformation efficiency varied with bacterial strains; LBA 9402 being the best performer. Strain-specific effectiveness is documented in several plants including *Valeriana wallichii* (*43*), *Coleus forskolii* (*44*), *Picrorhiza kurroa* (*45*), *Hyoscyamus albus* and *H. muticus* (*46*). The various degrees of infectivity among strains have been ascribed to the differential expression of chromosomal virulence genes of *A. rhizogenes* (*47*). Transformation efficiency was influenced by the stage of bacterial growth and bacterial cell density. The late log phase of growth corresponding to an absorbance of 0.6 (at 660 nm) was the most ideal, while higher *A* values had adverse effect perhaps due to excessive bacteria-induced stress. The effect of growth phase and cell density of *A. tumefaciens* on transformation was reported in tea (*48*) and grass pea (*49*). Bacterial preculture with acetosyringone (AS), at an optimal level of 100 μM, caused a marked enhancement in the transformation efficiency. AS is a phenolic signal compound which is naturally secreted by wounded plant cells to the rhizosphere and acts as a virulence transducer. AS complexes with the constitutively expressed VirA membrane receptor protein of *Agrobacterium* and the AS-VirA complex triggers the signal transduction cascade leading to T-DNA transfer to the plant cell (*50*). Higher transformation frequencies have been recorded in several plants owing to AS pretreatment of infectious *Agrobacterium tumefaciens* (*8,51*) or *A. rhizogenes* (*52–55*). Among the plant-specific factors affecting the transformation by *Agrobacterium*, explant type is worth mentioning (*45,47*). In the present study, internodal explants of *Amaranthus spinosus* were more responsive than the leaves regardless of *Agrobacterium* strains tested. Interestingly, in our observation explant source was also a considerable factor; explants from garden-grown mature plants were more susceptible than those derived from *in vitro* seedling plants. Different susceptibility among explant types or plant species was ascribed to the endogenous auxin content or sucrose level (*28*). Hairy root induction in *A. spinosus* was also dependent on the mode of infection; dipping of prepricked explants in *Agrobacterium* suspension was the best, in agreement with our observation in butterfly pea (*56*). Explant wounding facilitated transformation events perhaps through secretion of phenolic inducers such as acetosyringone or α-hydroxyacetosyringone as discussed earlier.

The presence of $T_L$-DNA *rol*B gene was detected in the genomic DNA of transformed root clones of *Amaranthus spinosus* by positive PCR amplification as had been shown earlier for *Picrorhiza kurroa* (*45*), *Saussurea involucrata* (*57*) and *Gentiana macrophylla* (*47*). We have also substantiated opine gene expression as evident by synthesis of opines (agropine and mannopine) in most of the transformed rhizoclones using paper electrophoresis. Opine assay has successfully been demonstrated for several plant species including *Solanum dulcamara* (*58*) and *Picrorhiza kurroa* (*45*).

## Conclusions

The transformed hairy root cultures of *Amaranthus spinosus* resulting from the present study offer multifarious applications. These fast-proliferating renewable hairy root cultures can act as an efficient means of producing secondary metabolites that are normally biosynthesized in plant roots but in a larger quantity, or novel compounds that hold unique pharmaceutical value. Superior hairy root lines (rhizoclones) of *A. spinosus* possessing an enhanced ability of producing the expected or novel phytochemicals can be selected for long-term maintenance and utilization. The use of such cultures as a raw material for pharmaceutical industry would ensure round-the-year availability of useful bioactive compounds while reducing the dependence on natural habitats. Production of hairy root cultures can be further enhanced by various methods of chemical upgrading including elicitation, precursor feeding, cell permeabilization and trapping of the molecules released into the liquid medium.

Recent progress in the physical improvement (scaling--up) of hairy root cultures using specially designed bioreactors could render amaranth hairy root system profitably attractive for commercial exploitation. In addition, the transgenic hairy root system with new heterologous genes can serve as a suitable candidate for metabolic engineering.

Efforts should also be directed towards engineering binary or cointegrated Ri plasmid vector systems carrying suitable chimaeric gene constructs to confer protection against biotic stress agents including foliar insect pests, viruses or fungal pathogens which together pose a considerable threat to *Amaranthus spinosus*, an important leafy vegetable crop. In this context, successful plant regeneration from selected rhizoclones and demonstration of the transformed status of regenerated plants open up the avenue towards introgression of agronomically useful transgenes such as those conferring resistance to one or other biotic stress factor. Such an approach will render possible for the production of transgenic spiny amaranth plants endowed with an ability of built-in protection against major biotic challenges. This will open up opportunities for genetic improvement of this hitherto underexplored crop species of nutraceutical significance.

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