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Transformation of *Saussurea medusa* for hairy roots and jaceosidin production

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Abstract Axenically grown *Saussurea medusa* plantlets were inoculated with four *Agrobacterium rhizogenes* strains, and hairy root lines were established with *A. rhizogenes* strain R1601 in N6 medium. PCR and Southern hybridization confirmed integration of the T-DNA fragment of the Ri plasmid from *A. rhizogenes* into the genome of *S. medusa* hairy roots. In N6 medium, maximum biomass of the hairy root cultures was achieved [8 g (dry weight) per liter; growth ratio 35-fold] after 21 days of culture. The amount of jaceosidin extracted from the hairy root cultures was 46 mg/l (production ratio of 37-fold) after 27 days of culture. The maximum jaceosidin content obtained using N6 medium was higher than that obtained with Modified White, MS or B5 medium. In N6 medium, the tip segments were more efficient for hairy root growth and jaceosidin production than the middle and basal regions of the root.

Keywords *Agrobacterium rhizogenes* · Jaceosidin · *Saussurea medusa* · Hairy roots

Abbreviations *AS*: Acetosyringone · *BA*: Benzyladenine · *cef*: Cefotaxime sodium · *DW*: Dry weight · *FW*: Fresh weight · *HPLC*: High-performance liquid

chromatography · *IAA*: Indole-3-acetic acid · *km*: Kanamycin · *NAA*: α -Naphthaleneacetic acid · *SDS*: Sodium dodecyl sulfate

Introduction

Saussurea medusa Maxim is a traditional Chinese medicinal plant which has been used to treat arthritis, high-altitude diseases, and gynecological diseases. This plant contains many bioactive compounds (Fan and Yue 2003). Its flavonoids, such as 4',5,7-trihydroxy-3',6-dimethoxy flavone (jaceosidin) and 4',5,7-trihydroxy-6-methoxy flavone (hispidulin), have significant antitumor effects (Liu et al. 1985; Woerdenbag et al. 1994; Schinella et al. 1998). The demand for this plant and its flavonoids far exceeds supply. In the wild, *S. medusa* grows only in limited regions of a specific climate, and its cultivation in other climates has been unsuccessful to date. Hence, in vitro production of the bioactive compounds as an alternative supply approach has been attempted. Zhao et al. (1998) detected jaceosidin accumulation at low levels in callus and cell suspension cultures. Although cell cultures generally tend to be genetically unstable and synthesize very low levels of useful secondary metabolites (Rhodes et al. 1990), hairy root cultures transformed with *Agrobacterium rhizogenes* grow quickly and produce large amounts of secondary metabolites (Flores et al. 1999; Sevon and Oksman-Caldentey 2002).

Hairy roots result from the transfer of genes located on the root-inducing (Ri) plasmid to plant cells and their subsequent expression therein (White and Nester 1980). Two sets of *pRi* genes are involved in the root induction process: *aux* genes located in the TR region and *rol* (root loci) genes in the TL region (Jouanin 1984). The *ags* genes responsible for opine biosynthesis in the transformed tissues are also located in the TR region (Binns and Tomashow 1988). Opines are used by *A. rhizogenes* as a source of nitrogen and carbon (Moyano et al. 1999).

The aim of the investigation reported here was to obtain hairy root cultures of *S. medusa* following the

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transformation of explants of in vitro-growing plants with *A. rhizogenes*. Our results demonstrate the successful transformation of *S. medusa* cultures by *A. rhizogenes* strain R1601. The growth dynamics of hairy root cultures and jaceosidin accumulation were investigated.

Materials and methods

In vitro plant culture

Mature seeds of *Saussurea medusa* were collected from wild plants in the Qi Lian Mountain, Qing Hai Province, China. Seeds were rinsed with water for 1 h, sterilized in 70% (v/v) ethanol for 30 s, then soaked in 0.1% (w/v) mercuric chloride for 10–14 min, and finally thoroughly rinsed with sterilized distilled water. The treated seeds were then transferred to solid MS medium (Murashige and Skoog 1962) that had been gelled with 0.6% agar (w/v, Bacto agar, Sanland) and the pH adjusted to 5.8 before autoclaving. The seeds were germinated at 25±2°C under a 16/8-h (light/dark) photoperiod with light supplied by Osram 40-W fluorescent tubes at an intensity of 45 µmol/m² per second. After 10 days of culture seedlings were transferred into flasks containing MS medium supplemented with 2.0 mg/l BA, 0.1 mg/l NAA, and 0.6% agar and cultured under the same conditions for 2 weeks. The plants were maintained by sub-culturing every 3 weeks on MS solid medium containing 1.0 mg/l BA and 0.2 mg/l NAA. Three-week-old seedlings from these cultures were used as the source of explants for genetic transformation.

Transformation and the induction of hairy roots

Agrobacterium rhizogenes strains R1601, R1000, A4, and LBA9402, all stored at 4°C, were used for transformation. The bacteria were grown on YEB solid medium (Vervliet et al. 1975) overnight at 28°C, and single colonies were then inoculated into 50 ml YEB liquid medium containing 200 µl AS and cultured at 28°C on an orbital shaker (180 rpm) in the dark. The bacterial cultures were used for transformation after 12 h (approximately 10⁸ cells per milliliter).

To induce hairy roots by transformation, we tested two types of explants: leaf blades and petioles. Small cuts were made on the surface of the leaf blades before they were inoculated with the bacteria. For petioles, 20-mm sections were excised and inoculated at their cut ends. Explants were then cultured on MS solid medium supplemented with 3% sucrose and 0.6% agar at 25°C in the dark. After 2 days of co-culture the bacteria were washed from the explants with sterile distilled water and the explants transferred to N6 solid medium (Chu et al. 1975) supplemented with 3% sucrose, 0.6% agar, and 500 mg/l cef. After an initial 3 days of culture the explants were subcultured every 5–7 days in N6 solid medium containing 500 mg/l cef until adventitious roots were initiated. After approximately 1 month these (parent) roots were 8 mm in length. Hairy roots were excised from parent roots and transferred to fresh N6 solid medium with 500 mg/l cef until the residual bacteria were completely killed. Bacteria-free roots were maintained on N6 solid medium containing 50 mg/l km without growth regulators.

Liquid cultures were established with 30- to 40-mm-long tips from 20-day-old hairy roots. Ten root tips were incubated in an 100-ml Erlenmeyer flask containing 30 ml N6 medium without antibiotics. The roots were grown at 25°C on a rotary shaker (90 rpm) under a 12/12-h (light/dark) photoperiod (45 µmol/m² per second, Osram 40-W fluorescent tubes).

PCR analysis of the *rolB* gene

The plasmid DNA of strain R1601 was used as a positive control. Plasmid DNA was isolated from 12-h cultures of *A. rhizogenes*

(OD₆₀₀=0.8) using the SDS/alkaline lysis method (Sambrook and Russell 2001).

Genomic DNAs of the non-transformed leaves and hairy roots were isolated by the CTAB method (Doyle and Doyle 1990). DNA was suspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). After 50 µg/ml RNase treatment, the DNA was stored at -20°C until used.

PCR was performed to detect the *rolB* gene in both the T-DNA of the R1601 Ri plasmid and the genomic DNAs of the non-transformed leaves and hairy roots. Primers for PCR were designed according to the DNA sequence of the *rolB* gene described by Slightom et al. (1986). The primer pair used for *rolB* gene amplification of an 863-bp fragment was: 5'-TACTGCAGCAGGCTT CATGCA-3' and 5'-GCTTTCCCGACCAGAGACTG-3'. The PCR mixture consisted of approximately 200 ng plant genomic DNA (or 100 pg Ri plasmid DNA), 0.4 mM of each primer, 0.2 mM of each dNTP (Sangon, Shanghai), 1.0 U *Taq* DNA polymerase (5 U/µl, GibcoBRL, Gaithersburg, Md.), and 2.5 µl 10× buffer (100 mM Tris-HCl pH 9.0, 100 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, and 1% Triton X-100; GibcoBRL) in a final volume of 25 µl. The PCR was run under the following conditions: an initial denaturation at 94°C for 2 min; denaturation at 94°C for 1 min; primer annealing at 55°C for 1 min; elongation at 72°C for 1 min, 30 cycles; a final extension at 72°C for 5 min. The amplified PCR products were examined by electrophoresis on a 1.2% (w/v) agarose gel.

Genomic DNA Southern blot analysis

Hairy roots induced by strain R1601 were confirmed by Southern hybridization (Sambrook and Russell 2001). Genomic DNAs from over 30 passage-transformed hairy roots and non-transformed leaves were extracted by the CTAB method (Doyle and Doyle 1990). PCR analysis for the presence of the TL-DNA border sequence was carried out using the primer pair 5'-ATGGCCTC-CAAGCTCCTCCT-3' (forward primer) and 5'-ATTCAGTAGC-CAGCCAGC AG-5' (reverse primer) (Slightom et al. 1986). For PCR analysis, DNA was denatured at 94°C for 3 min followed by 30 amplification cycles (94°C for 50 s, 53°C for 50 s, 72°C for 50 s) and a final cycle of 10 min at 72°C. The expected product size was 705 bp. DNA blot analysis was used to confirm the transgenic status of hairy root lines positive for the TL-DNA border sequence by PCR analysis. A 100-ng aliquot of plasmid DNA and 15 µg of plant DNA were digested overnight at 37°C with *Hind*III, which excises a 6.7-kb fragment in pRiA4b (Jouanin 1984), fractionated by 0.8% agarose gel electrophoresis, and transferred to a positively charged nylon membrane (Roche Molecular Biochemicals, Indianapolis, Ind.). As a template for PCR, 100 pg Ri plasmid DNA were used. The probe was labeled with digoxigenin (DIG)-dUTP using the PCR DIG Probe Synthesis kit (Roche Molecular Biochemicals). Hybridizing bands corresponding to the TL-DNA border sequence were detected using the DIG Luminescent Detection kit (Roche Molecular Biochemicals), and signals were visualized by exposure to Fuji X-ray film at 37°C for 3 h.

Effect of kanamycin resistance on root growth

Roots from control and transgenic plants were cultured on N6 liquid medium containing 20–100 mg/l km to determine its inhibitory effects on growth. The km had been filter-sterilized prior to the experiment using a 0.2-µm polyethersulfone membrane. Effects on growth were evaluated during 30 days of culture.

Analysis of jaceosidin

Analysis of jaceosidin was performed according to Wang (1997). Dry hairy roots (1.0 g) were extracted with 25 ml 70% ethanol twice using a soxhlet method, each for 10 min at room temperature. The extract was filtered and reduced to about 10 ml and then di-

luted with 20 ml 95% ethanol. The solution was filtered through a 0.45- μm membrane, and a 5- μl sample was injected into a HPLC system for analysis (Waters model 244; Milford, USA). The HPLC system consisted of a M 510 pump, reverse-phase column (Zorbax RX-C18, 4.6 \times 250 mm; particle size, 5 μm), UV detector (UV 486), and a sampler (U6K). The mobile phase was methanol/water (9:1). The flow rate was 0.7 ml/min, and UV detection was at 365 nm. A standard curve was obtained by repeating the same procedure for different concentrations of the standard. Jaceosidin was detected by the UV spectra and retention time. At this wavelength, five standard jaceosidin solutions of different concentrations (0.4, 0.8, 1.2, 1.6, and 2.0 $\mu\text{g/ml}$) were used to establish the calibration curve. The amount of jaceosidin (X) was found to be proportional to its peak/area ratio (Y). The regression equation was $Y = 3X - 0.003$ ($r=0.9982$). The jaceosidin content was expressed in milligrams per liter. All data presented in the figures and tables are mean values of three independent experiments (nine shake flasks were used per experimental point).

Effect of basal media on growth dynamics and jaceosidin production

Growth and jaceosidin production in hairy roots were monitored every 3 days for 27 days under the culture conditions described above. The callus cultures were monitored for 21 days under the culture conditions described by Zhao et al. (2001). The callus has been maintained since 1997 and subcultured every 2 weeks. Callus was grown in MS (Murashige and Skoog 1962) medium supplemented with 3% sucrose, 1% glucose, 100 mg/l myo-inositol, 0.5 mg/l BA, 2 mg/l NAA, and 0.6% agar. The pH was adjusted to 5.8–6.0 with 1 M KOH solution prior to autoclaving at 121°C for 20 min. The callus was cultured at 25°C in the light (45 $\mu\text{mol/m}^2/\text{s}$, Osram 40-W fluorescent tubes). Four media were tested for this study: MS, B5 (Gamborg et al. 1968), N6, and Modified White (White 1963). All media are reported by Owen and Miller (1992). Iron was supplied in the form of FeNa_2EDTA .

Effects of cutting on root growth and jaceosidin content

Transformed roots (20 days old) were cut into three segments (base, middle, and tip; each about 30–40 mm long), and each segment was cultured in a 100-ml Erlenmeyer flask containing 40 ml N6 medium for 25 days. Five replicates (13 segments in each replicate were inoculated) were analyzed for each segment of the root. After 25 days, root dry weight and jaceosidin yield were recorded. The biomass was dried at 60°C to a constant weight, and the dry weight was determined.

Results and discussion

Establishment of hairy root cultures by means of *Agrobacterium*-mediated transformation

Hairy roots commonly emerged from wounded sites after 2–5 weeks of incubation on solid medium (Table 1). The highest transformation rates were observed in petiole and leaf-blade explants with strain R1601—100% and 83.3%, respectively; with strain A4, the rates were 3.3% and 1.7%, respectively. Root formation in the petiole and leaf-blade explants following transformation with strain R1601 was faster (14 days and 17 days, respectively) than with strain A4 (30 days and 35 days, respectively). The other two strains tested, R1000 and LBA9402, failed to induce hairy roots. Strain R1601 was more efficient than the other strains for hairy root induction in *S. medusa*, and petioles were more suitable for co-cultivation than leaf blades. Consequently, the petiole-strain R1601 combination was studied further for hairy root culture. Among the hairy root lines which were induced with strain R1601, hairy root lines HP23 from petiole explants and HB31 and HB40 from leaf-blade explants were able to grow quickly in hormone-free N6. We confirmed the integration of T-DNA into the genome of these three lines with PCR and Southern hybridization analyses. We also tested the growth dynamics and jaceosidin production of the HB40 culture and the effect of basal medium on growth and jaceosidin yield.

Pythoud et al. (1987) reported the successful induction of hairy roots in *Nicotiana tabacum* by strain R1000. Successful induction has also been reported with strain LBA9402 in *Ammi majus* (Krolicka et al. 2001). The discrepancies between our results and these reports suggest differential responses of plant species to bacterial strains.

Characterization of transgenic plants

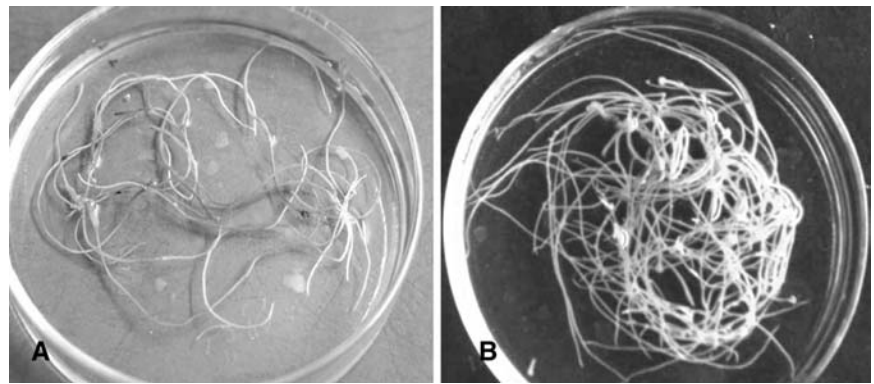
Morphology of transformed root cultures

The morphology of the transformed hairy roots (“hairy roots”) was compared with that of non-transformed roots

Table 1 Transformation efficiency of *Saussurea medusa* explants by co-cultivation with different *Agrobacterium rhizogenes* strains in MS medium supplemented with 500 mg/l cef and 0.5 mg/l gibberellic acid (N/A not applicable)

Bacterium-explant combination	Number of samples tested	Number of transformed explants	Transformed (%)	Days to root formation
R1601				
Leaf blade	60	50	83.3	17
Leaf petiole	60	60	100	14
R1000				
Leaf blade	60	0	0	N/A
Leaf petiole	60	0	0	N/A
LBA9402				
Leaf blade	60	0	0	N/A
Leaf petiole	60	0	0	N/A
A4				
Leaf blade	60	1	1.7	35
Leaf petiole	60	2	3.3	30

Fig. 1 a Non-transformed roots of *Saussurea medusa* after 20 days of growth in MS liquid medium supplemented with 0.2 mg/l BA and 2 mg/l IAA. **b** Hairy root of *S. medusa* after 20 days of growth in N6 liquid medium



(“roots”) from explants of *S. medusa* seedlings. Hairy roots (Fig. 1b) displayed a typical phenotype characterized by plagiotropic growth, a high incidence of lateral branching, and faster growth than roots (Fig. 1a). Non-transformed roots cultured on the N6 medium supplemented with 1 mg/l IAA grew without branching and sometimes formed callus-like aggregates. The most profound difference between hairy roots and roots is that hairy roots could grow in a hormone-free medium with a growth ratio (harvest weight/inoculum weight is defined as growth ratio) of 35, while roots did not grow well in this medium (growth ratio = 7). A similar phenotype has been described by several authors (David et al. 1984; Tepfer 1984; Aird et al. 1988; Wysokinska and Chmiel 1997). In our laboratory the hairy roots have been sub-cultured in liquid N6 medium with a stable phenotype for over 30 passages.

Analysis of transgenic cultures

Integration of the *A. rhizogenes* plasmid T-DNA into the *S. medusa* genome was confirmed by PCR with primers designed according to the sequence of the *rolB* gene of *A. rhizogenes*. The primers amplified the expected 863-bp DNA fragment (Fig. 2). An equivalent of this PCR product was absent from non-transformed plants. The positive results obtained in the amplification of the *rolB* gene in the transformed plants were confirmed by the stable phenotype of the cultures.

Transformation of the hairy roots with TL-DNA was further confirmed by Southern blot. Three independently derived transformed *S. medusa* lines—HB31, HB40 and HP23—were analyzed by Southern blot hybridization with a probe from the TL-DNA border sequence. Estimation of the position number of the integrated TL-DNA was made by *Hind*III digestion (Fig. 3). An expected band was observed as the 6.7-kb band in the T-DNA of strain R1601, whereas there was no hybridization band in the genomic DNA from a non-transformed plant. An approximately 8.9-kb band was observed in the hybridization fragments of three transformed lines. The band for HB31 was slightly larger than the band for HB40, indicating that they were independently transformed lines. Lines HB31 and HB40 were isolated from different leaf

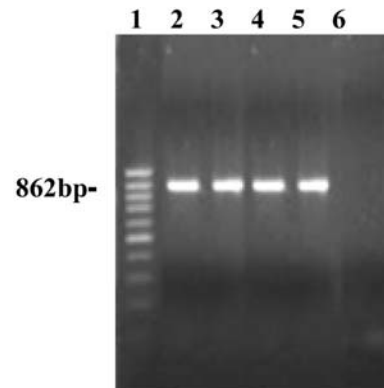


Fig. 2 Identification of the *rolB* gene fragment in transformed tissue of *S. medusa* by PCR analysis. Lanes: 1 Molecular-weight marker, 2–4 DNA isolated from the hairy roots of transformed lines HB31, HB40, and HP23, respectively, by *A. rhizogenes* R1601, 5 plasmid DNA isolated from *A. rhizogenes* R1601, 6 DNA isolated from non-transformed leaves of *S. medusa*

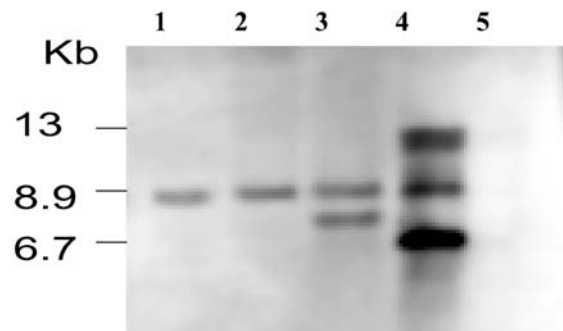


Fig. 3 Southern analysis of genomic DNAs isolated from *S. medusa*. The DNAs were digested with *Hind*III and hybridized to probe ORF 1. Lanes : 1–3 DNA isolated from the hairy roots of transformed lines HB31, HB40, HP23, respectively, 4 positive control, 5 negative control

blades. In addition, there was another band of approximately 7.3 kb in the HP23 line. These results indicated that T-DNA was integrated at different positions into the *S. medusa* genome.

Kanamycin resistance test

Km resistance (concentration: 20, 40, 60, 80, 100 mg/l, w/v) was tested to determine its effect on the growth of non-transformed roots and hairy roots. We observed that 50 mg/l kanamycin was sufficient to inhibit the growth of non-transformed roots (data not shown). Transgenic roots that contained the neomycin phosphotransferase II (*NPTII*) gene exhibited normal growth in a medium containing up to 80 mg/l kanamycin.

Growth dynamics and jaceosidin production in hairy root cultures

Growth of *S. medusa* was monitored every 3 days during the 21-day culture period for callus and 27-day culture period for hairy roots. For each sampling date, five flasks were taken for analysis of biomass (DW) and jaceosidin content. For hairy roots, the maximum biomass DW (35-fold) and jaceosidin yield (37-fold) were achieved at 21 days and 27 days, respectively (Fig. 4). For callus culture, the maximum biomass DW (6.8-fold) and jaceosidin yield (7.1-fold) were achieved at 15 days and 12 days, respectively. The maximum biomass DW and jaceosidin yield from hairy roots were much higher than those obtained from callus cultures in the exponential phase. Growth rates of hairy roots vary among plant species (Mano et al. 1986; Yoshikawa and Furuya 1987). In the present work, hairy roots of *S. medusa* grew 3.07-fold faster than those of *Panax ginseng* (Yoshikawa and Furuya 1987) but slower than those of *Atropa belladonna* (Kamada et al. 1986) and *Scopolia japonica* (Mano et al. 1986).

Quantitatively, jaceosidin production was higher in hairy roots than in callus (Fig. 4). Hairy roots displayed a maximum accumulation of secondary metabolites between the 24th and 27th day of growth (Fig. 4).

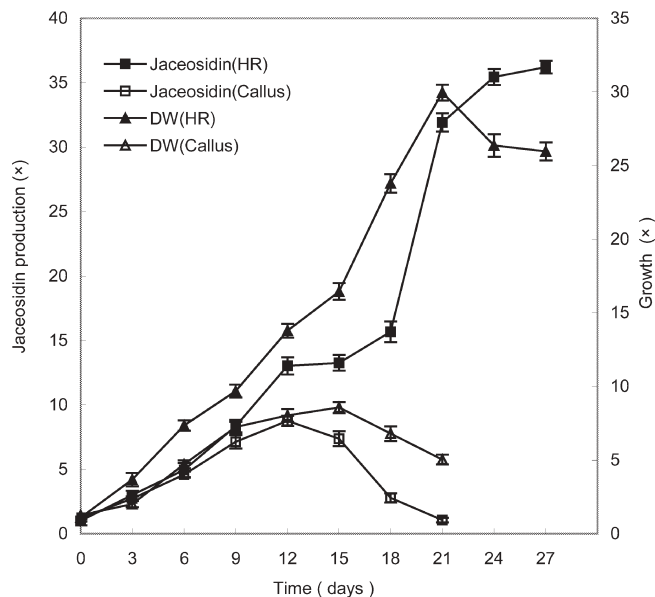
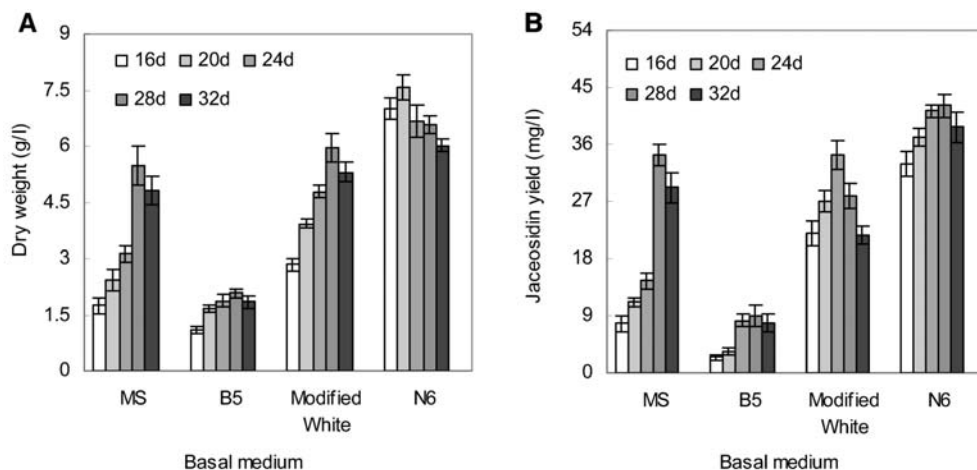


Fig. 4 Time-course of growth and jaceosidin production by hairy roots (HR) and callus culture (Callus) in N6 and MS medium. Growth ratio = harvest weight/initial inoculum weight; production ratio = amount of jaceosidin in harvest weight/amount of jaceosidin in initial inoculum weight. Bars represent standard errors

Influence of basal medium on hairy root growth and jaceosidin accumulation

S. medusa hairy roots were cultured in several growth media, each supplemented with 3% (w/v) sucrose, for 20, 24, or 28 days. N6, MS, B5, and Modified White's media were tested. Root growth largely depended on the medium formulation (Fig. 5a). The best hairy root growth (approximately 8 g/l) was achieved in N6 medium after a 20-day culture. In other media, the maximum growth was achieved after 28 days of culture (Modified White, 6.2 g/l; MS, 5.4 g/l; B5, 2.5 g/l). Different basal medium formulations affected jaceosidin content. Taking the biomass DW into account, jaceosidin yield in N6 (45 mg/l) was

Fig. 5 Effect of medium on hairy root growth (a) and jaceosidin production (b) after 16, 20, 24, 28, and 32 days. Bars represent standard errors



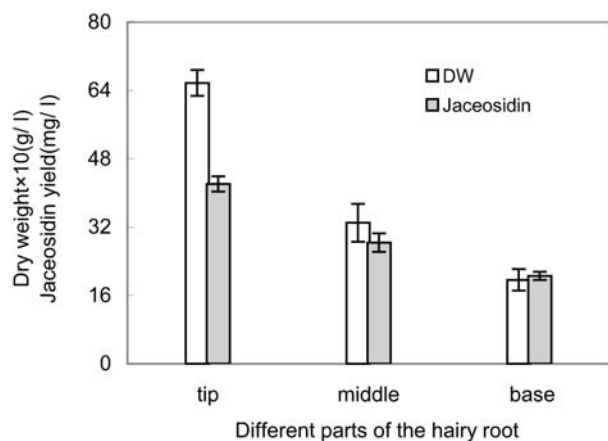


Fig. 6 Root growth and jaceosidin content found in the tip, middle, or base of the root. Bars represent standard errors

higher than in Modified White's medium (36 mg/l), MS medium (35 mg/l) and B5 medium (10 mg/l).

Different incubation times had a significant impact on both hairy root growth and jaceosidin content. For suitable incubation times, the final biomass and jaceosidin production of the hairy roots cultured in N6, Modified White, MS and B5 medium were observed after 20, 24, 28, and 28 days of culture. The results showed that for maximum hairy root growth 20 days is appropriate for N6 and 28 days for the other media tested. For maximum jaceosidin production, 24 days is appropriate for N6 and Modified White, and 28 days for MS and B5 (Fig. 5).

Jaceosidin content in various sections of the root

The tip, middle, and base of the roots showed different growth rates and jaceosidin contents in N6 medium (Fig. 6). After 25 days, the final biomass of these root segments was 6.8, 3.5 and 2.8 g/l and jaceosidin content was 68, 39 and 29 mg/l, respectively. Both the growth rate and jaceosidin content were higher in the root-tip segments than in the other segments. Lee et al. (1999) reported that tips of hairy roots of *A. belladonna* had a higher growth rate but a lower alkaloid content than the middle and base segments.

Summary

In order to obtain hairy root cultures of *S. medusa*, we tested four strains of *A. rhizogenes* and two types of explants. Hairy root cultures were established by inoculating leaves of *S. medusa* with *A. rhizogenes* R1601. The growth of the hairy roots was monitored over a period of 6 months by subculturing on hormone-free N6 medium at 3-week intervals. Hairy roots grew vigorously with lateral branches and accumulated more jaceosidin than either the original callus or ordinary roots. There is a great potential

for using hairy roots of *S. medusa* for the production of jaceosidin and other valuable secondary products.

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