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Establishment of hairy root cultures of *Ammi majus*

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Abstract

Axenically grown *Ammi majus* plantlets were inoculated with seven different *Agrobacterium rhizogenes* strains. Hairy root lines were established only after inoculation with the two agropine strains: A4 and LBA9402. The growth rate of hairy root cultures was about thirty times faster than that of callus and cell suspension cultures. Polymerase chain reaction with primers for the genes *rolB* and *rolC* confirmed the integration of the T-DNA fragment of Ri plasmid of *A. rhizogenes* to the genome of hairy roots obtained after transformation by both *Agrobacterium* strains. The furanocoumarins (psoralen, xanthotoxine, bergapten and imperatorin) usually found in seeds of *A. majus* were not detected in callus, cell suspension and hairy root cultures using Gas chromatography–mass spectrometry (GC–MS). However, umbelliferone, a precursor of furanocoumarins, was detected in callus, cell suspension and hairy root cultures. The umbelliferone content in extracts of hairy root cultures, obtained after transformation by A4, was similar to that determined in *A. majus* seeds (19 μg/g DW) and higher than those obtained for cell suspension and callus cultures (2 and 9 μg/g DW, respectively).

Keywords: *Ammi majus*; *Agrobacterium rhizogenes*; Furanocoumarins; Hairy roots; Secondary metabolites; Transformation

1. Introduction

Bishops weed — *Ammi majus* L. (*Apiaceae*) is considered to be one of the richest natural sources of linear coumarins and furanocoumarins: umbelliferone, psoralen, xanthotoxine, bergapten and imperatorin. The pharmacological activity of *A. majus* has been known since the work of Schőberg and Sina [1] when it was shown that the therapeutically effective substances of these plants are furanocoumarins. The fruit of *A. majus* has been used in the Mediterranean and bordering regions in the treatment of leucoderma, *psoriasis*, *vitiligo* and the production of suntan lotion [2,3]. In Europe the growth of *Ammi* is poor due to the cool climate (light frosts). Attempts to acclimatise *A. majus* in Central European climatic conditions were not successful: the fruits failed to ripen in moderate climatic zones and plants were highly susceptible to infection [4]. In moderate climates seed production was also limited and the germination rate poor. Great interest in therapeutic use of furanocoumarins, as well as limited natural sources, have led to a series of studies on the production of these biologically active compounds in vitro. Accumulation of furanocoumarins by *A. majus* in in vitro plant, callus and cell suspension have been investigated, but the productivity was not satisfactory [2–5].

The aim of our work was to obtain hairy root cultures of *A. majus* after transformation of explants of in vitro growing plant with *A. rhizogenes*.

Abbreviations: BAP, 6-benzylaminopurine; DW, dry weight; FW, fresh weight; GC–MS, Gas chromatography-mass spectrometry; IAA, indolyl-3-acetic acid; KIN, kinetin; MS, Murashige-Skoog's medium; NAA, naphthaleneacetic acid; PPM™, plant preservative mixture™; PCR, polymerase chain reaction.

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Upon plant transformation, T-DNA from the root-inducing Ri plasmid of a soil bacterium *A. rhizogenes* is integrated into the plant genome. Expression of the genes from the T-DNA region in plant tissue leads to the formation of adventitious roots [6]. Transformed root cultures are very useful, as they grow rapidly, are genetically stable and capable of synthesising metabolites found in the biological roots and other plant organs, in abundance [7,8].

The present study demonstrates the successful transformation of *A. majus* cultures by two different strains of *A. rhizogenes*. The growth rate, as well as coumarins and furanocoumarins concentration in seeds and in in vitro cultures (callus, cell suspension and hairy roots) have been compared.

2. Material and methods

2.1. Plant material

The plantlets of *A. majus* were grown on MS medium [9] supplemented with 2.0 mg/l NAA, 2.0 mg/l BAP, 30.0 g/l sucrose and 7.5 g/l agar, at a temperature of 20–22°C and illumination of 900 lux with photoperiod of 16-h light/8-h dark for 2–3 weeks [5].

2.2. Bacteria strains

Agrobacterium rhizogenes agropine strains: A4 [10], LBA 9402 [11], ATCC 15834 [12]; mannopine strains: ICPB TR 7 [13], NCPPB 8196 [14]; nopaline strain: ATCC 11325 [15] and cucumopine strain: ICPB TR 107 [14] were grown on MYA agar medium [16] supplemented with 200 μM acetosyringone in the dark, at 26°C. For transformation, 24 h old bacterial cultures were used.

2.3. Transformation and establishment of hairy root cultures

Explants of stalks and leaves of *A. majus* were inoculated with freshly grown *A. rhizogenes* cultures. After inoculation, the explants were transferred to the MS agar medium without growth regulators and cultured in darkness. When roots appeared, they were transferred with a fragment of tissue to fresh MS medium containing 30 g/l sucrose, 500 mg/l of claforan (Hoechst, M. Roussel) and 500 mg/l of carbenicillin (Polfa, Tarchomin) to eliminate bacteria. For more efficient and stable elimination of *A. rhizogenes* 2.0 ml/l PPM™ (Plant Cell Technology, Inc. Washington) was also used in some experiments. Axenic cultures derived from single root tips were established after 3–5 subcultures in a 250 ml Erlenmeyer flask containing 75 ml MS medium with claforan and carbenicillin, but without plant growth regulators. Bacteria-free hairy roots were then maintained on a liquid MS medium without antibiotics, in the dark at 22°C on a rotary shaker at 110 rpm. Subcultures were made every 2 or 3 weeks. Fresh weight of the cultures was measured every 10 days.

2.4. Detection of bacterial DNA in plant tissue

A. rhizogenes strains; A4 and LBA 9402 were used to isolate Ri plasmid. Plasmid DNA was isolated from 24 h cultures of *A. rhizogenes* ($OD_{600} = 0.3$) using FastDNA® Kit BIO 101. Frozen leaf tissue of untransformed *A. majus* and frozen tissue of hairy roots were homogenised with 1% polyvinylpyrrolidone and CLS–VF + PPS buffer in Fast Prep™ System FP120 (Instruments Inc. Holbrook, NY) and genomic DNA was isolated using Binding Matrix and ready-to-use buffers from FastDNA® Kit BIO 101.

Oligonucleotide primers for PCR detection of homologous sequences to *rolB* and *rolC* genes were designed on the basis of the DNA sequence of these genes described by Furner [17] (*rolB* 5′-GCTCTTGCAGTGCTAGATTT-3′ and 5′-GAAGGTGCAAGCTACCTCTC-3′; *rolC* 5′-CTCCTGACATCAAACTCGTC-3′ and 5′-TGCTTCGAGTTATGGGTACA-3′) using program BLAST.

Each PCR reaction contained: standard PCR buffer (Promega Co., Madison), 1.0 U *Taq* DNA polymerase (Promega Co., Madison), 2 mM $MgCl_2$, 0.2 mM dNTP (Gibco BRL), 8 pmol of each primer and 75–100 ng of the target DNA (final volume 50 μl). Amplification conditions: 35 cycles, initial denaturation at 94°C for 3 min, denaturation at 94°C for 1 min, primer annealing at 53.5°C for 1 min, elongation at 72°C for 1 min, followed by final elongation for 6 min. The amplified sequences were separated by electrophoresis in a 1.2% agarose gel in TBE buffer. The gel was stained with ethidium bromide and observed under UV light.

2.5. Secondary metabolites extraction

Seeds, callus, cell suspension and hairy root cultures of *A. majus* were collected, dried at 50°C and extractions of the secondary metabolites were performed in a Soxhlet apparatus. Samples (about 3 g of DW) were extracted exhaustively with petroleum ether, chloroform and methanol. Clear (free of chlorophyll) chloroform and methanol extracts were collected separately.

2.6. Gas chromatography–mass spectrometry

Identification of the components of the methanol extracts of *A. majus* seeds and in vitro growing tissue was performed by GC-MS. Chloroform extracts of plant tissue were purified on a column packed with 200-mesh silica gel (Macherey-Nagel) and eluted with 50 ml of ethyl acetate [18]. UV active fractions were evaporated to dryness in a stream of dry nitrogen and derived with a mixture (10:1) of bissilylacetamid and trimethyl silyl chloride (10 min at 100°C). Then capillary gas chromatography was applied for samples containing volatile silyl derivatives. Thus, unmodified, methoxyl-, and hydroxylated coumarins were analysed in one gas chromatography run. All fractions were subjected to GC and GC–MS.

The analyses were carried out on a GC 8000 TOP gas chromatograph, equipped with a capillary column (DB1-HT, length 30 m, I.D. 0.25 mm, 0.1 μm film thickness) with split ratio 1:30 for an injection port. Argon was used as a carrier gas. Initial oven temperature was 60°C kept for 5 min, then a temperature programme of 10°C per min. was employed to 200°C and held at 200°C for an additional 20 min [2]. Silyl derivative of heneicosanol-11 was used as an internal standard and allowed for quantitative determination of coumarins in plant tissue extracts.

Mass spectra (70 eV) were recorded on a VG TRIO-3 mass spectrometer, (Micromass UK). The samples were introduced through a Hewlett Packard 5890 gas chromatograph equipped with RTX-1 column and under the same chromatographic conditions as mentioned for gas chromatography. Helium was used as carrier gas. The ionisation chamber was kept at 230°C. A one second scan was used for mass spectra recording. For the initial 14 min no spectra were recorded to avoid solvent residues.

3. Results and discussion

Our study indicated that in vitro cultures of callus and cell suspension accumulate umbelliferone but not furanocoumarins (Table 1). The economic importance of furanocoumarins, the chemical synthesis of which is very expensive [19], has stimulated interest in transformation of *A. majus* by *A. rhizogenes*, establishing conditions for fast growing hairy root cultures and study of their ability to produce furanocoumarins.

3.1. Establishment of hairy root cultures

Hairy roots were obtained after transformation of *A. majus* with the agropine strains of *A. rhizogenes*. Hairy root formation was observed about 3

Table 1
Coumarins and furanocoumarins in *Ammi majus* seeds, callus, cell suspension and hairy root cultures[a]

Name	Secondary metabolites or their silyl derivatives		Secondary metabolites concentration (μg/g DW)			
	Characteristic ions used for detection m/z	Retention index	Seeds	Callus	Cell suspension	Hairy roots
Umbelliferone	234, 219, 163, 73	1	17 ± 2.7	8 ± 1.2	2 ± 0.3	19 ± 3.3
Bergapten	216, 201, 173	1.12	214	n.d.	n.d.	n.d.
Xanthotoxin	216, 201, 173	1.15	45	n.d.	n.d.	n.d.
Isopimpinellin	246, 231, 203	1.32	98	n.d.	n.d.	n.d.
Marmesine	259, 215, 103, 73	n.d.	n.d.	n.d.	n.d.	n.d.

[a] Callus culture-MS + 5.0 mg/l IAA + 1.0 mg/l BAP + 3.0% sucrose, 0.75% agar; cell suspension-MS + 0.5 mg/l GA_3 + 1.0 mg/l KIN + 3.0% sucrose; hairy roots-MS + 3.0% sucrose, (n.d., not detected).

Table 2
Growth of *A. majus* callus, cell suspension and hairy root cultures on MS medium[a]

Types of culture	Growth of culture (g FW)			
	0	10 days	20 days	30 days
Callus	1.7	3.1	4.1	6.9
Cell suspensions	1.3	2.7	3.8	6.4
Hairy roots	0.1	3.9	8.0	15.0

[a] Callus culture-MS + 5.0 mg/l IAA + 1.0 mg/l BAP + 3.0% sucrose, 0.75% agar; cell suspension-MS + 0.5 mg/l GA_3 + 1.0 mg/l KIN + 3.0% sucrose; hairy roots-MS + 3.0% sucrose.

weeks after inoculation of the explants by A4 and LBA9402 strains. The successful infection and root formation were obtained after inoculation of the first node and only when bacteria were grown before inoculation on MYA medium supplemented with 200 μM of acetosyringone. This phenomenon can be explained by the observation of Stachel [20] showing the activation of *Agrobacterium rhizogenes vir* genes expression by acetosyringone.

Bacterial cells were eliminated from the co-cultures by incubation of hairy root cultures on MS medium with claforan (500 mg/l) and carbenicillin (500 mg/l), [21]. After four to five passages on media with antibiotics, hairy root cultures were free of bacteria. The next few passages on MS media without antibiotics and plant growth regulators, enabled the obtaining of uniform hairy root cultures that displayed a typical phenotype characterised by plagiotropic growth and high incidence of lateral branching. These roots grew faster than normal ones. A similar phenotype was described earlier by several authors [8,22,23].

The growth rate of hairy root cultures on an MS medium without plant growth regulators was about thirty times higher than that of callus growing on an MS medium supplemented with 5.0 mg/L IAA and 1.0 mg/L BAP and cell suspension cultures growing on MS medium supplemented with 0.5 mg/l GA_3 and 1.0 mg/l KIN (Table 2). Fresh weight of hairy root cultures increased about 200-fold, from 0.08 to 15.0 g after 30 days (Table 2).

3.2. Detection of rol genes in transgenic tissue

Integration of the T-DNA into *A. majus* genome was confirmed on the molecular level by the PCR with primers constructed on the sequences of *rolB* and *rolC* genes of *A. rhizogenes*. Primers based on the sequence of *rolB* gene amplify the expected fragment of 383 bp and those specific for *rolC* gene amplify the fragment of 586 bp DNA in PCR reaction with DNA isolated from hairy root culture tissues (Fig. 1). The PCR product was absent in non-transformed tissue. Amplification of both fragments was obtained in PCR reactions performed with DNA isolated from *A. rhizogenes* cells (Fig. 1).

To be sure that *rolB* and *rolC* gene were not amplified from bacterial cells, the following procedure was performed. Hairy root cultures were maintained on a medium with antibiotics usually during the growth of four to five subcultures, then they were grown on a medium without antibiotics for several subcultures. Later on hairy root tissues were homogenised and suspensions obtained were plated on MYA and Luria Agar media. Plates were incubated for several days, but the growth of *A. rhizogenes* was never observed.

Transformation of plant tissue by *Agrobacterium* was earlier confirmed by determination of

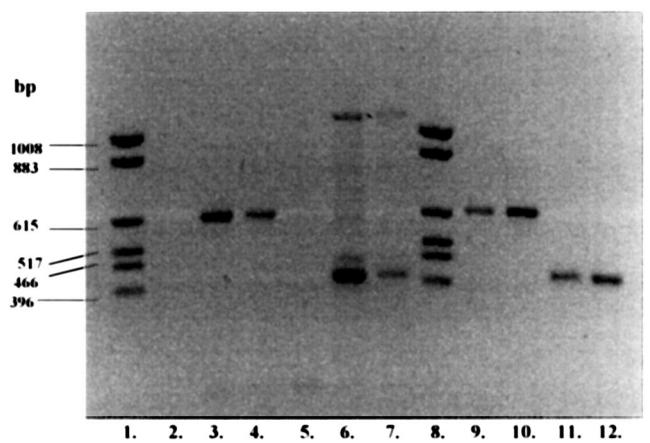

Fig. 1. Identification of *rolB* and *rolC* gene fragments in transformed tissue of *A. majus*. 1 and 8, Marker pKO3/Hinf I (Gibco BRL); PCR reactions were performed using as a target DNA isolated from: 2, Non-transformed leaves of *A. majus* + primer *rolC*; 3, hairy roots of *A. majus* transformed by *A. rhizogenes* LBA 9402 + primer *rolC*; 4, hairy roots of *A. majus* transformed by *A. rhizogenes* A4 + primer *rolC*; 5, non-transformed leaves of *A. majus* + primer *rolB*; 6, hairy roots of *A. majus* transformed by *A. rhizogenes* LBA 9402 + primer *rolB*; 7, hairy roots of *A. majus* transformed by *A. rhizogenes* A4 + primer *rolB*; 9, *A. rhizogenes* LBA 9402 + primer *rolC*; 10, *A. rhizogenes* A4 + primer *rolC*; 11, *A. rhizogenes* LBA 9402 + primer *rolB*; 12, *A. rhizogenes* A4 + primer *rolB*.

the production of opine by plant tissue [24]. Recently Henzi [25] has confirmed integration of the T-DNA to the plant genome using PCR with primers for the NPTII gene cloned earlier to the Ri plasmid of the *A. rhizogenes*. Primers for *rolB* and *rolC* genes used in this work enable molecular confirmation of the transformation with the wid type strains of *A. rhizogenes*.

3.3. Determination of coumarin contents

The analysis of methanol extracts from seeds indicated the presence of umbelliferone, bergapten, xanthotoxin, imperatorin and several other coumarins and furanocoumarins (Table 1). Furanocoumarins: psoralen, bergapten, xanthotoxin, imperatorin were absent in extracts of callus, cell suspension and hairy root cultures (Table 1). However, in vitro cultures indicated the presence of umbelliferone. Extracts of hairy roots indicated a similar level of umbelliferone (about 19 μg/g DW) to those of seeds, as well as twice the level of this compound than extracts of callus and nine times that of cell suspension (Table 1). The work of Hamerski [2] and Hamerski and Matern [26] indicated that only umbelliferone, but not furanocoumarins was present in cell suspension of *A. majus*.

Furanocoumarins biosynthesis in plants of the family *Umbelliferae* and *Rutaceae* has been studied by several groups [27–30]. It was established that psoralens are derived from umbelliferone by the prenylation [26,31]. As indicated in the work of Hamerski and Matern [26] the umbelliferone prenyltransferases, marmesin and psoralen synthase are located in the membranes of the endoplasmatic reticulum of *A. majus* cells. Activity of these enzymes was strongly dependent on induction by biotic elicitors [26,32]. Non-elicited cultures showed no activities of the enzymes involved in biosynthesis of psoralens. Fungal elicitors induce a specific set of endoplasmic reticulum enzyme: prenyltransferase, as well as marmesin and psoralen synthase in *A. majus* cells [32].

In further experiments the possibility for the induction of furanocoumarins biosynthesis in hairy root cultures of *A. majus* by abiotic and biotic elicitors will be examined.

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