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PHENOLIC ACIDS AND FLAVONOIDS IN *HYPERICUM PERFORATUM* L. HAIRY ROOTS

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ABSTRACT

Investigations have been made to develop an efficient protocol for identification and quantification of phenolic acids and flavonoids in hairy roots (HR) of *Hypericum perforatum* L. HR were induced from root segments of *in vitro* grown seedlings from *H. perforatum*, after co-cultivation with *Agrobacterium rhizogenes* A4. Transgenic status of HR was confirmed by PCR analysis using *rol*B specific primers. HR grew rapidly on hormone-free medium and had plagiotropic growth with vigorous production of lateral roots. Phenolic acids and flavonoids in control roots and HR were analyzed using HPLC/DAD/ESI-MS^n. Quinic acid was the only detectable phenolic acid in HR. Transgenic roots produced flavonol glycosides such as quercetin 6-*C*-glucoside, rutin and isorhamnetin *O*-hexoside. Chromatographic analysis of flavonoid aglycones in HR resulted in the identification of kaempferol. Transformed roots yielded higher levels of catechin and epicatechin than untransformed roots. *H. perforatum* HR represent promising experimental system for enhanced production of phenolic compounds.

KEYWORDS: ***Hypericum perforatum* L., *Agrobacterium rhizogenes* A4, hairy roots, phenolic acids, flavonols, flavan-3-ols.**

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INTRODUCTION

Hypericum perforatum L. (Saint John's wort) is a medicinal plant considered as an important natural source of secondary metabolites with a wide range of pharmacological attributes. It contains naphthodianthrones, acylphloroglucinols, flavonoids, biflavones, phenylpropanes, xanthones and an essential oil rich in sesquiterpenes[1]. Naphthodianthrones, phloroglucinols and flavonoids are distributed in the aerial parts of the plant, whereas xanthones are mainly produced in the roots[2]. The hypericins and hyperforins are the main bioactive compounds in *H. perforatum* plant exhibiting photodynamic, antidepressive and antiviral activities[3]. Flavonoids isolated from *H. perforatum* have shown anti-oxidant, anti-microbial and anti-inflammatory properties[4]. For commercial production of bioactive compounds, field-grown plant material is generally used, but the quality of these products may be greatly affected by different environmental conditions and pathogen organisms, which can result in heavy loss in yield and alter the medicinal value of plants[5]. To meet the increasing demand for plants utilized in the pharmaceutical industry, the emphasis in recent research has been focused on the development of new *in vitro* culture techniques as a useful alternative to improve the yield of bioactive metabolites in plant material.

Plant genetic transformation offers opportunity to introduce new qualities into medicinal and aromatic plants. *Agrobacterium rhizogenes*, a gram-negative phytopathogenic soil bacterium, is well known for its ability to transfer its T-DNA from the root-inducing (Ri) plasmid to the host genome thereby causing formation of hairy roots (HR). HR cultures represent an attractive experimental system for the production of high-value secondary metabolites, including pharmaceuticals and other biologically active substances of commercial importance[6,7]. Namely, HR cultures may synthesize higher levels of secondary metabolites or amounts comparable to those of the intact plant and offer a promising approach to the production of novel metabolites[8]. The first step towards the application of transformation procedures to few *Hypericum* species has been encountered. Until now, only *A. rhizogenes*-[9-11] and biolistic-mediated[12] transformation methods have been applied. Wild agropine strain *A. rhizogenes* ATCC 15834 was used in the first successful transformation of *H. perforatum*[9]. Also, an efficient transformation protocol of this species was reported with *A. rhizogenes* A4M70GUS[10]. Recently, two other *Hypericum* species (*H. tomentosum* and *H. tetrapterum*) were successfully transformed with *A. rhizogenes* ATCC 15834 and A4[11]. HR cultures of *H. perforatum* exhibited high potential for spontaneous regeneration into whole transgenic plants[9,10]. Selected *Hypericum* HR regenerated plants have been evaluated for their bioactive secondary metabolites[9,13,14]. However, no study has been published on the identification and quantification of bioactive metabolites in *H. perforatum* HR cultures.

The objectives of this study were to establish an efficient *A. rhizogenes* A4-mediated transformation system that would result in the rapid formation of HR cultures for the purposes of studying the production and accumulation of bioactive metabolites. Phenolic compounds in the control roots and transformed HR were analyzed using high-performance liquid chromatography (HPLC) coupled with diode-array detection (DAD) for routine analysis and tandem mass spectrometry (MS^n) with electrospray ionization (ESI) as a more sophisticated means for identifying phenolic acids and flavonoids. All present derivatives of phenolic acids, flavonol glycosides, flavonoid aglycones, and flavan-3-ols were identified from corresponding UV and MS spectra and quantified by HPLC-DAD.

MATERIALS AND METHODS

(i) Plant material

Seeds from *H. perforatum* were collected from wild plants growing in a natural population in the National Park Pelister at about 1394 m. Voucher specimen (number 060231) of *H. perforatum* is deposited in the Herbarium at

the Faculty of Natural Sciences and Mathematics, University "Ss. Cyril and Methodius"-Skopje, Republic of Macedonia (MKNH). As for a previous study[15], seeds were washed with 70% ethanol for 30 sec, surface sterilized with 1% NaOCl for 15 min, rinsed 3 times in sterile deionized water and cultured on MS macro and oligoelements[16], B_5 vitamin solution[17], supplemented with 3% sucrose and solidified with 0.7% agar. No growth regulator was added. The medium was adjusted to pH 5.8 before autoclaving (20 min at 120 °C). *In vitro* cultures were maintained in a growth chamber at 25±1 °C under a photoperiod of 16 hrs light, irradiance at 50 $\mu mol \cdot m^2/s$ and 50 to 60% relative humidity.

(ii) Preparation of Agrobacterium rhizogenes A4 suspension

The wild type *Agrobacterium rhizogenes* agropine strain A4[18] was used for *H. perforatum* transformation. The procedure for *A. rhizogenes* A4 culture preparation was based on the method of Di Guardo et al.,[9] with the following modifications. *A. rhizogenes* A4 was grown on nutrient agar medium (15 g/L peptone, 3 g/L beef extract, 5 g/L NaCl, 0.3 g/L KH_2PO_4 and 15 g/L agar). The suspension culture was prepared by growing a single bacterial colony in 10 mL of nutrient broth medium at 28 ºC with continuous rotary shaking (120 rpm) for 24 hrs. Subsequently, 1 mL of the bacterial suspension was transferred into 9 mL fresh nutrient broth medium and maintained under similar conditions for 12 hrs or until bacterial concentration of approximately 4.2 x 10^9 colony-forming units (CFU) per mL medium was achieved. Overnight-grown bacterial suspension was diluted 1:20 (v/v) in sterile water (0.1 absorbance at 660 nm) and used for transformation protocol.

(iii) Transformation protocol and establishment of hairy roots

A. rhizogenes A4-mediated transformation protocol was performed by Di Guardo et al.,[9] with the following modifications. Root segments (1-2 cm) without apical tip were excised from 4 week-old *in vitro* seedlings and gently wounded with a sterile lancet blade. Root explants were soaked for 15 min in bacterial suspension and blotted on sterile filter paper. Control root explants were soaked in sterile distilled water. Infected and control explants were than placed on MS/B_5 hormone-free medium in the dark at 25±1 °C. After 2 days, the explants were transferred to hormone-free medium supplemented with 200 mg/L cefotaxime. The transformation frequency was calculated in percentage ((final number of explants forming HR/initial number of infected explants) x 100) after 30 days of culture. Within 3-4 weeks, numerous HR emerged from the wounded sites. When the HR reached about 4-5 cm in length, they were excised from the explant tissue and subcultured on fresh MS/B_5 medium. After repeated transfer to fresh medium rapidly growing HR cultures were obtained. Thereafter, putative HR lines were selected by Di Guardo et al.[9] These HR lines were monthly subcultured on MS/B_5 medium and concentration of the antibiotic cefotaxime was gradually decreased (200, 100, 50 mg/L) in the next three subcultures down to the antibiotic free medium in the fourth subculture. The HR cultures were then harvested, frozen in liquid nitrogen, lyophilized and stored at -80 °C, until analysis.

(iv) Molecular analysis

Genomic DNA from transformed and non-transformed roots of *H. perforatum* was isolated using the cetyltrimethylammonium bromide (CTAB) method[19], with minor modifications. Non-transformed root DNA was used as a negative control, while plasmid DNA from *A. rhizogenes* A4 served as a positive control for polymerase chain reaction (PCR) analysis. The presence of the integrated genes in the genome of the putative transformed roots was determined by PCR amplification of *rol*B gene. The primers used for the amplification of a 348 bp DNA fragment of the *rol*B gene in the given instant were as follows:5'-AAAGTCTGCTATCATCCTCCTATG-3' and 5'-AAAGAAGGTGCAAGCTACCTCTCT-3', according to the sequence of *rol*B gene from *A. rhizogenes* A4[20]. Bacterial contamination of plant tissue was excluded by testing the amplification of a 421 bp DNA fragment of the *vir*C1 gene which is located outside the

bacterial T-DNA and is not transferred to the plant genome using the following primers: 5'-CTCGCTCAGCAGCAGTTCAATG-3' and 5'-ACGGCAAACGATTGGCTCTC-3'[21]. The PCR reactions were performed in a total 10 µM volume and contained 30-50 ng of DNA, 0.5 µM of each primer, 0.2 mM dNTP, 1 unit Taq DNA polymerase, 1xPCR buffer and 3 mM $MgCl_2$. The PCR mixture was incubated in a DNA thermal cycler (Perkin Elmer 2400, USA). PCR conditions for *rol*B and *vir*C1 fragment amplification were: 95 ºC for 5 min (initial denaturation), 35 cycles of 95 ºC for 30 sec, 64 ºC for 1 min and 72 ºC for 1 min and a final extension at 72 ºC for 7 min. PCR amplification products were analysed by electrophoretic separation on 2% (w/v) agarose gel in TE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3) and were detected by fluorescence under UV light after staining with ethidium bromide.

(v) HPLC/DAD/ESI-MS^n analysis of phenolic acids and flavonoids

Phenolic compounds extraction from freeze-dried lyophilized and powdered root cultures was performed as previously reported[22,23]. The HPLC system was equipped with an Agilent 1100 series diode array and mass detector in series (Agilent Technologies, Waldbronn, Germany). It contained a binary pump (G1312A), autosampler (G1313A), degasser (G1322A) and photo-diode array detector (G1315B), controlled by ChemStation software (Agilent, v.08.03) Chromatographic separations were carried out on 150 mm x 4.6 mm, 5 µm XDB-C18 Eclipse column (Agilent, USA). The mobile phase consisted of two solvents: water-formic acid (A; 99:1, v/v) and methanol (B) in the following gradient program: 90% A and 10% B (0-20 min), 80% A and 20% B (20-30 min), 65% A and 35% B (30-50 min), 50% A and 50% B (50-70 min), 20% A and 80%B (70-80 min) and continued with 100% B for a further 10 min. Each run was followed by an equilibration period of 10 min. The flow rate was 0.4 mL/min and the injection volume 10 µL. All separations were performed at 38 °C. Formic acid (HCOOH) and methanol (CH_3OH) were HPLC grade solvents (Sigma-Aldrich, Germany). HPLC-water was purified by a Purelab Option-Q system (Elga LabWater, UK). The commercial standards chlorogenic acid, rutin, quercetin, kaempferol, catechin and epicatechin (Sigma-Aldrich, Germany) were used as reference compounds. The reference compounds were dissolved in 80% methanol in water. The concentration of the stock standard solutions was 1 mg/mL and they were stored at -20 ºC. Spectral data from all peaks were accumulated in range 190-600 nm, and chromatograms were recorded at 260 nm. Peak areas were used for quantification at wavelengths where each group of phenolic compounds exhibited an absorption maximum. The HPLC system was connected to the Agilent G2445A ion-trap mass spectrometer equipped with electrospray ionization (ESI) system and controlled by LCMSD software (Agilent, v.6.1.). Nitrogen was used as nebulizing gas at a pressure-level of 65 psi and the flow was adjusted to 12 L/min. Both the heated capillary and the voltage were maintained at 350°C and 4 kV, respectively. MS data were acquired in the negative ionization mode. The full scan mass covered the mass range from *m/z* 100 to 1200. Collision-induced fragmentation experiments were performed in the ion trap using helium as a collision gas, with voltage ramping cycle from 0.3 up to 2 V. Maximum accumulation time of the ion trap and the number of MS repetitions to obtain the MS average spectra was set at 300 ms and 3, respectively. Identification of the component peaks was performed by the UV/Vis, MS and MS^2 spectra and retention times of the above mentioned available standards.

RESULTS

1. Establishment of hairy roots

Hairy roots of *Hypericum perforatum* L.

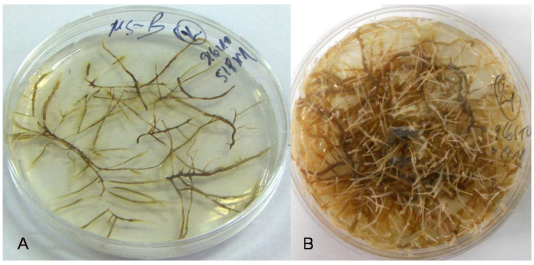

Figure 1
Control roots (A) and hairy roots (B) of H. perforatum cultivated on solid hormone-free MS/B_5 medium.

HR cultures of *H. perforatum* were initiated by inoculation of root explants with *A. rhizogenes* A4. After one week of bacterial infection, some root segments subsequently regenerated adventitious roots from wounded sites on explants. The adventitious roots elongated within the next 3 weeks reaching up to 4-5 cm in length and showing high level of lateral branching. In contrast, control root segments rarely produced adventitious roots and further elongation of these roots was very slow (Fig 1A). Transformation of HR lines was confirmed by PCR analysis and transformation frequency was recorded 1 month past the fourth subculture on antibiotic-free medium. The percentage of HR induction from infected root explants was 33%. HR cultures grew rapidly in the dark and showed characteristics of transformed roots. Namely, the HR cultures were thin and whitish in colour showing plagiotropic growth with active branching and a vigorous production of elongated lateral roots (Fig 1B). On the other hand, the non-transformed roots grew slowly without branching or displaying altered geotropism (Fig 1A). The phenotype of HR cultures was stable for over one year of maintenance on a hormone-free medium in *in vitro* conditions. There was not any variability in the morphology and growth patterns among individual HR clones, despite the fact that each HR clone arose from a separate transformation event. It was seen that the growth of HR was generally most vigorous between the 3rd and 4th weeks of the cultivation period (1 month), but their growth declined after the 5th week. For this reason, 4-week-old HR cultures were further evaluated for PCR and HPLC analysis.

2. Molecular analysis

PCR analysis

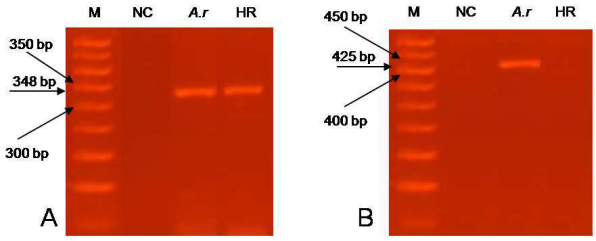

Figure 2
Gel electrophoresis of PCR products amplified from H. perforatum genomic DNA. A. PCR performed with rolB primers; the black arrow indicates the 348 bp amplification product. B. PCR performed with virC1 primers; the black arrow indicates the 421 bp amplification product. A.r: positive control (pRi A4); HR: hairy roots; M: molecular weight marker; NC: negative control (non transformed roots).

The transgenic nature of the selected HR cultures was confirmed by PCR analysis of the presence of *rol*B sequences from T_L-DNA of *A. rhizogenes* Ri plasmid. PCR analyses (Fig 2) performed on HR led to the amplification of the expected *rol*B fragments (348 bp) which were identical to those of the positive control (pRi A4). No such product was obtained from the non-transformed roots (negative control). To confirm the transformation and exclude any possibility of bacterial contamination, primers directed against a *vir*C1 gene which is not transferred into the HR were used. No product was obtained either from the non-transformed or from the tested transformed roots when using the *vir*C1 primers. The *vir*C1 amplification band (421 bp) was visualised only in *A. rhizogenes* DNA samples (Fig 2). Negative results from the attempted amplification of the *vir*C1 gene suggested that HR cultures were bacteria-free and the Ri T_L-DNA was successfully incorporated into the genome of *H. perforatum* HR cultures.

3. HPLC/DAD/ESI-MS^n analysis of phenolic acids and flavonoids

Table 1
HPLC/DAD/ESI-MS^n data of the major identified phenolic acids and flavonoids in H. perforatum control and hairy roots.[a]

Compounds		t_R (min)	UV (nm)	$[M-H]^-$ (*m/z*)	$-MS^2$ $[M-H]^-$ (*m/z*)	Control roots mg/100g DW±S.D.	Hairy roots mg/100g DW ±S.D.
Phenolic acids							
F1	quinic acid	3.9	262, 310	191	173, 127	26.26±3.19	166.77±1.20
F2	3-caffeoylquinic acid	13.7	240, 294sh, 326	353	191, 179, 135	18.24±3.01	ND
F4	3-*p*-coumaroylquinic acid	19.9	314	337	191, 163	4.39±0.09	ND
F6	3-feruloylquinic acid	25.3	314	367	193	15.54±2.17	ND
F15	rosmarinic acid	49.7	238, 294sh, 324	359	223, 197, 179, 161	7.63±1.46	ND
Flavonol glycosides							
F9	quercetin 6-*C*-glucoside	33.9	256, 356	421	331, 301	36.64±1.75	2.99±0.79
F11	kaempferol 3-*O*-rhamnoside	37.3	256, 264 352	431	285	9.03±0.53	ND
F12	isorhamnetin *O*-hexoside	38.1	254, 356	477	316, 315, 271	ND	11.80±0.94
F13	kaempferol hexoside	41.2	256, 266, 350	447	285	8.01±0.97	ND
F14	rutin (quercetin 3-*O*-rutinoside)	44.9	263, 298sh, 356	609	301	5.21±0.78	14.72±2.16
F16	kaempferol 3-*O*-rutinoside	52.2	256, 266, 350	593	285	10.20±1.32	ND
Flavan-3-ols							
F3	catechin	19.5	280	289	245, 205	ND	27.28±3.20
F7	(epi)catechin	29.9	280	289	245, 205	24.24±1.55	184.85±12.92
F5	proanthocyanidin dimer	24.5	280	577	559, 451, 425, 407, 289	151.27±5.31	146.95±9.13
F8	proanthocyanidin dimer	33.4	280	577	559, 451, 425, 407, 289	135.34±1.76	41.43±1.03
F10	proanthocyanidin dimer	36.8	280	577	559, 451, 425, 407, 289	71.15±1.30	29.24±2.41
Flavonoid aglycones							
F17	quercetin	57.1	256, 372	301	273, 229, 179, 151	5.63±0.11	ND
F18	kaempferol	59.5	256, 266, 350	285	/	ND	3.92±0.38

[a]ND not detected, DW dry weight, sh shoulder, t_r retention time. MS^2 ions in bold indicate the base peak. For information on peak numbers see Figure 3.

Chromatographic analysis

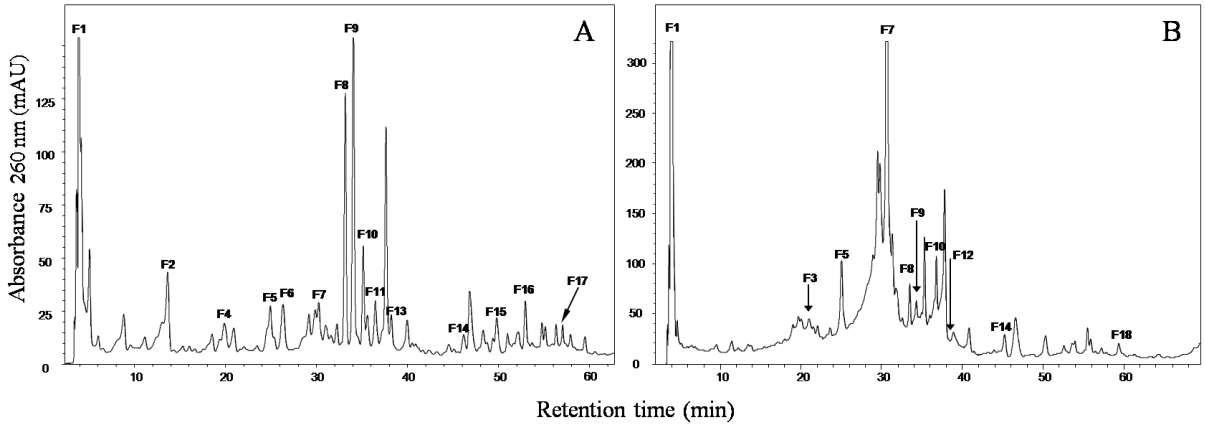

Figure 3

HPLC/DAD data of the major identified phenolic compounds in H. perforatum control roots (A) and hairy roots (B). Compound symbols correspond to those indicated in Table 1.

The HPLC/DAD/ESI-MS^n technique was used to analyse the phenolic profile of *H. perforatum* HR cultures. Four groups of phenolic compounds such as phenolic acids, flavonol glycosides, flavan-3-ols and flavonoid aglycones were recorded in HR cultures (Tab 1). Their identification was based on the typical UV/Vis spectral data and LC/MS in the negative ionization mode $[M–H]^–$ with the subsequent MS^2, MS^3 and MS^4 analysis for further identification with reference to similar data previously reported[24-29]. The HPLC analysis of secondary metabolites revealed marked differences between control roots (Fig 3A) and HR cultures (Fig 3B).

Phenolic acids. HPLC chromatograms confirmed the presence of 5 phenolic acids (F1, F2, F4, F6 and F15) in root extracts (Tab 1, Fig 3). Compound F1 occurred at a retention time of 3.9 min and exhibited a $[M–H]^–$ ion at *m/z* 191. Its MS^2 fragmentation produced a $[M–H–CO–2H_2O]^–$ ion at *m/z* 127 as a base peak. A $[M–H–H_2O]^–$ ion at *m/z* 173 was also observed. Compound F1 was identified as quinic acid, taking in account its MS^n fragmentation pattern and literature data[24]. Quinic acid (F1) was the only detectable phenolic acid in HR cultures. A 6-fold increase of quinic acid was observed in HR cultures compared to control roots. Four peaks, F2, F4, F6 and F15 were detected in samples from the control roots with identical UV spectra characterized by absorption bands at 240–246 nm and 320–325 nm and by a sharp diagnostic shoulder at 290–300 nm typical for compounds containing a caffeoyl group[25]. The chemical structure of 3-caffeoylquinic acid (F2) was confirmed by ESI-MS/MS analysis. Its full mass spectrum exhibited an intense $[M–H]^–$ ion at *m/z* 353 in the negative mode, consistent with a caffeoylquinic acid derivative. The molecular ion fragmentation yielded fragment ions corresponding to quinic acid (base peak *m/z* 191) and caffeic acid (*m/z* 179) moieties. 3-*p*-coumaroylquinic acid (F4) and 3-feruloylquinic acid (F6) were readily distinguished by their cinnamic acid-derived MS^2 base peaks at *m/z* 163 and at *m/z* 193, respectively. Compound F15 with a molecular ion $[M–H]^–$ at *m/z* 359 was identified as rosmarinic acid. In the MS^2 spectra of the $[M–H]^–$ ion of the compound F15 exhibited ions at *m/z* 179 and 161 derived from neutral loss of caffeic acid (180 amu) or 3,4-dihydroxyphenyllactic acid (198 amu).

Flavonol glycosides and flavonoid aglycones. In *H. perforatum* HR, the flavonol glycosides and flavonoid aglycones were observed to be qualitatively and quantitatively distinct from those of the corresponding control roots (Tab 1, Fig 3). A major group of identified compounds belonged to flavonols according to their characteristic UV spectra of flavonols glycosylated at C3 (257, 265sh, 355 nm). The detected compound F9 can be identified as *C*-glycoside of quercetin. The deprotonated molecular ion $[M–H]^–$ of compound F9 was

detected at *m/z* 421. It showed an MS^2 fragmentation characteristic of mono-*C*-hexosyl flavones, with losses of 90 and 120 amu[26], giving *m/z* ions characteristic for quercetin. The compound F11 had UV-spectrum and MS data consistent with those of kaempferol 3-rhamnoside. This compound gave deprotonated molecular ion $[M–H]^-$ at *m/z* 431 and its MS^2 gave a single ion at *m/z* 285. The compound F12 had molecular ion $[M–H]^-$ at *m/z* 477. MS^2 spectra of this compound showed fragmentation ions at *m/z* 315 (loss of 162 amu), suggesting presence of hexose residue. So, compound F12 was tentatively identified as isorhamnetin *O*-hexoside. The compound F13 was identified as kaempferol derivative with glycosilation in position 3 according to its UV-spectra (256, 266, 350 nm). The MS and MS^2 spectra were consistent with the presence of a hexose residue and confirmed the kaempferol aglycone. Therefore, this compound was identified as kaempferol hexoside. Compounds F14 and F16 had molecular ions $[M–H]^-$ at *m/z* 609 and 593, and their MS^2 gave a single ion at *m/z* 301 and 285, respectively, indicating quercetin and kaempferol derivatives with rutinose at C3[27]. The absence of intermediate fragmentation between the deprotonated molecular ion and the aglycone ion is indicative of an interglycosidic linkage 1→6[28]; therefore these compounds were putatively identified as quercetin 3-*O*-rutinoside (rutin) and kaempferol 3-*O*-rutinoside. Three compounds (F9, F12, and F14) could be distinguished in HR cultures that belong to the group of flavonol glycosides. A 2.8-fold increase of rutin (F14) was observed in HR compared to control roots. In contrast, quercetin 6-*C*-glucoside (F9) was in lower amounts compared with those in control roots. Isorhamnetin *O*-hexoside (F12) was *de novo* synthesized in transformed roots while kaempferol 3-rhamnoside (F11), kaempferol hexoside (F13) and kaempferol rutinoside (F16) were not detectable in HR cultures. Two compounds in the extracts were detected as flavonoid aglycones (F17, F18) but only F18 was identified in HR while F17 was observed in control samples. The peaks at *m/z* 301 and 285 correspond to quercetin (F17) and kaempferol (F18), respectively.

Flavan-3-ols. The HPLC analysis confirmed the presence of 5 flavan-3-ols (F3, F5, F7, F8 and F10) in HR extracts (Tab 1, Fig 3). The mass spectrum in full scan mode showed the deprotonated molecules $[M–H]^-$ of catechin and epicatechin at *m/z* 289 (compounds F3, F7), with characteristic MS^2 ions at *m/z* 245 and 205 and UV maximum at 278 nm. Compounds F5, F8 and F10 had $[M–H]^-$ at *m/z* 577 and main fragmentation with loss of 152 amu, characteristic fragmentation pathway by retro Diels-Alder reaction[29] and were recognized as proanthocyanidin dimers. Regarding the group of flavan-3-ols in HR cultures, catechin (F3) was *de novo* synthesized while compound epicatechin (F7) was 8-fold increased, compared to control roots. In contrast, proanthocyanidin dimers (F5, F8 and F10) were generally in lower quantities in HR cultures as compared to control roots.

DISCUSSION

1. Establishment of hairy roots

In the present study, we have successfully described a method for an *A. rhizogenes* A4 mediated transformation of *H. perforatum*. The results showed that root segments, as primary explants, displayed susceptibility to an *A. rhizogenes* infection which resulted in the development of HR cultures. Namely, HR formation with pRiA4 occurred at a transformation frequency of about 33%. Recent studies on different primary explants infected with *A. rhizogenes* reported lower HR transformation rates. Efficient transformation with *A. rhizogenes* A4M70GUS was observed in 21% of infected shoots[10]. Di Guardo et al.[9] showed that 25% of leaf explants and only 13% of root segments had been successfully transformed with *A. rhizogenes* ATCC 15834. These authors suggested that the transformation of leaf segments was more troublesome and occurred only on a medium supplemented with indole-3-acetic acid and zeatin. Phytohormones promote cell division of the host target tissue and it is reasonable that wound sites associated with actively dividing cells are capable of undergoing a successful transformation[9]. As presently established, efficient *Agrobacterium*-mediated transformation occurred when *H. perforatum*

root segments were maintained on a hormone-free medium. Therefore, it is possible to consider that root segments are promising explants and better target sites for a higher transformation rate. Present results confirmed that transformed roots of *H. perforatum* had characteristic traits of HR previously described by Tepfer[30]. Namely, *H. perforatum* HR phenotype includes a high degree of lateral branching, plagiotropism, and rapid growth on hormone-free medium. Indeed, *H. perforatum* HR lines have a homogeneous morphology and similar growth patterns among individual root clones. The uniformity of HR phenotypes obtained in this study is curious, because the HR morphological traits depend of particular expression levels of various *rol* genes within the clones, differences in length or copy number of inserted T-DNA, positional effects or by an epigenetic control[7].

2. Molecular analysis

T-DNA of agropine type of *Agrobacterium* Ri plasmid consists of T_L-DNA and T_R-DNA which is separated by 16-18 kb non-transferred DNA sequence[31]. Both T_L-DNA and T_R-DNA are transferred and integrated independently into the host plant genome, but the transfer of T_L-DNA is essential for HR formation. White et al.[31] identified the *rol* loci on T_L-DNA to be the most important virulent factors and indicated that *rol*B gene has a main role in pathogenicity. In our study, the integration of T_L-DNA region in *H. perforatum* HR genome was confirmed by showing the presence of *rol*B gene segment. In other studies, the transgenic nature of *H. perforatum* HR cultures was verified by the amplification of *rol*C gene[9], while transgenosis of *H. tetrapterum* and *H. tomentosum* was confirmed by the presence of *rol*ABCD genes[11]. Considering that the *rol* genes are essential genetic determinants, it is reasonable to assume that these gene loci have a large impact on secondary metabolism in transformed plant cells[32].

3. Production of phenolic acids and flavonoids

The main advantage of using HR lies in their differentiated nature, genetic and biochemical stability, rapid growth and capability for enhanced production of secondary metabolites[33]. So far, secondary metabolite profile of *H. perforatum* HR cultures has not been the subject of extended research. Therefore, in the present study we used HPLC/DAD/ESI-MS^n method to thoroughly analyse HR extracts for the production of phenolic compounds. The results revealed the presence of phenolic acids, flavonol glycosides, flavonoid aglycones and flavan-3-ols in root extracts. The HPLC profiles obtained in the course of this work clearly evidenced a distinct secondary metabolite production between control roots and HR cultures. As established, while HR did not exhibit a superior potential for the accumulation of various phenolic acids, it is noteworthy to mention in this study that they did exhibit the potential to accumulate quinic acid. Quinic acid is the most important component as a key intermediate in the biosynthesis of aromatic compounds. The condensation between quinic acid and caffeic acid leads to the formation of chlorogenic acid in the shikimic acid pathway. Chlorogenic acid is an important antioxidative compound recently produced by *H. perforatum* adventitious roots cultivated in bioreactor[34], shoot cultures[35] and transgenic plantlets[13].

With regard to the class of flavonol glycosides, our results showed that HR have the capability to produce quercetin derivatives such as quercetin 6-*C*-glucoside, quercetin 3-*O*-rutinoside (rutin) and isorhamnetin *O*-hexoside. However, there is no available study for the potential of *H. perforatum* root cultures to produce flavonol derivatives. Several differences can be pointed out when comparing the composition of flavonol glycosides in HR extracts with those of *H. perforatum in vitro* cultures. In our previous work[22,23], we indicated that *H. perforatum* cells, calli and shoots demonstrate a considerable potential for producing quercetin, isoquercitrin and quercitrin upon elicitation with jasmonic acid and salicylic acid. The LC-MS screening of twelve *H. perforatum* HR transgenic plant lines showed a large variability in the content of rutin, hyperoside, quercetrin and quercetin[13]. Moreover, the above mentioned flavonol glycosides had been identified in *H. perforatum* regenerated plantlets[36] and *H. undulatum* shoot cultures[37].

HPLC-MS analysis of flavonoid aglycones in HR cultures resulted in the identification of kaempferol but the aglycone quercetin was not detected. Kaempferol and quercetin are typical flavonoid aglycones in *H. perforatum* wild plants, which are considered to have strong antioxidant properties and neuroprotective action[38]. The absence of aglycone quercetin in HR extracts posses a potentially interesting finding, since it is well-known that quercetin is a biologically active flavonoid that interacts synergistically with other bioactive substances[39]. One of the main achievements in this study was the identification of flavan-3-ols (catechins) as the major flavonoid fraction in root extracts. Namely, HR cultures were better producers of both catechin and epicatechin than control roots. Furthermore, catechin and epicatechin can exert marked medicinal effects and play important role as antioxidants[40] and antibacterial agents[41]. *H. perforatum in vitro* cultures had never been reported to posses catechin derivatives. Nevertheless, catechin, epicatechin and proanthocyanidin dimers had been previously identified in shoots and calli of *H. erectum*[42] and *H. undulatum* shoot cultures[37].

CONCLUSION

In conclusion, we have developed an efficient transformation system for *H. perforatum* which leads to the formation of HR cultures producing various groups of phenolic compounds. Distinct profile of phenolic acids and flavonoids between control and HR cultures was shown as detailed for the first time. Quinic acid was the only detectable phenolic acid in HR cultures. Three flavonol glycosides identified as rutin, quercetin 6-*C*-glucoside and isorhamnetin *O*-hexoside were detected in HR. Among these, isorhamnetin *O*-hexoside was *de novo* synthesized in transformed roots. HPLC-MS analysis of flavonoid aglycones in HR cultures resulted in the identification of kaempferol but the aglycone quercetin was detected only in control roots. Chromatographic analysis of flavan-3-ols confirmed the presence of catechin, epicatechin and proanthocyanidin dimers in HR extracts. Regarding this group of flavonoids, catechin was *de novo* synthesized in HR. The present study demonstrates that *H. perforatum* HR can offer a valuable source of different groups of flavonoids widely used in pharmaceutical industry.

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