Bioactive Constituent Production in St. John's Wort in Vitro Hairy Roots. Regenerated Plant Lines[†]

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A wild strain of *Agrobacterium rhizogenes* was used to regenerate twelve in vitro plant lines from different hairy roots of *H. perforatum* (St. John's Wort). The production of the main bioactive constituents was observed even though their yields varied in the different plant lines. Two lines were selected for the hyperoside production (4.9−4.6 mg/g_{dw}) while nine were characterized by significant yields of chlorogenic acid (ranged from 0.47 to 1.09 mg/g_{dw}). Furthermore, one out of twelve lines showed a 10-fold higher hypericin content (0.25 mg/g_{dw}) than that reported for the in vitro shoots in the literature. Morphological and phytochemical features were determined in order to select *H. perforatum* genotypes enriched in valuable bioactive compounds.

INTRODUCTION

Hypericum perforatum L. (Hypericeae) is a spontaneous herbaceous perennial plant, widely distributed in the temperate zones of Europe, Asia, and North Africa. Since antiquity it has been used as a medicinal plant for its antiinflammatory, diuretic, and sedative properties. Hyperici herba (St. John's Wort, dried aerial parts) is included in many pharmacopoeias and in the ESCOP monographs (*1*).

H. perforatum contains many compounds investigated for their biological activities, and it is used as herbal medicine expecially for the treatment of mild and moderate depression (*2, 3*). In fact its clinical efficacy is comparable to that of tricyclic antidepressants. It is possible to attribute this effect not to specific compounds but to the synergic action of the large variety of its constituents (*4, 5*).

The increasing demand of *H. perforatum* extracts has promoted a biotechnological approach for the plant material production. Several typical flavonoids, hypericin and hyperforin, were found also in the extracts obtained from in vitro St. John's Wort cultures, calli, and regenerated organs (*6–11*). Recent studies (*12*) demonstrated that hypericins were accumulated in a bunch of

secondary cells located on the callus surface in their late development phase. Some researchers (*13*) used jasmonic acid as elicitor to induce the hypericin production in cell suspension cultures of *H. perforatum.* Other studies (*14*) showed the influence of several enhancers on the hypericin and hyperforin prodution in in vitro seedlings. It is well-known that the genetic transformation by wild types of *Agrobacterium rhizogenes* produced neoplastic roots (hairy roots) which were characterized by a high growth rate and genetic stability (*15*). Moreover, hairy roots of many plant species have been reported to produce large variety of phytochemicals (*16*). In certain genotypes, hairy roots spontaneously regenerated whole transgenic plants during in vitro culture (*17*). Transgenic plants, obtained from the T-DNA of *A. rhizogenes* Ri plasmid, showed peculiar phenotypic alterations, related to a particular auxin/cytokinin metabolism or sensitivity to typical morphological features (*18*). The presence of hypericin was reported in the literature only for two tranformed plant lines of *H. perforatum* obtained by hairy roots induced from in vitro root and leaf tissues with the *A. rhizogenes* ATCC 15834 strain (*19, 20*). In the present study, a wild Italian accession of *H. perforatum* was chosen according to Kornfeld et al. (*20*) who demonstrated that wild accessions were an excellent source for hypericin and hyperforin. The morphology, the biomass production, and the phytochemical differences of several hairy-root-regenerated plant lines were all taken into consideration together in order to select *H. perforatum* plant material enriched in bioactive secondary metabolites (**Figure 1**).

Bioactive Constituent Production in St. John's Wort

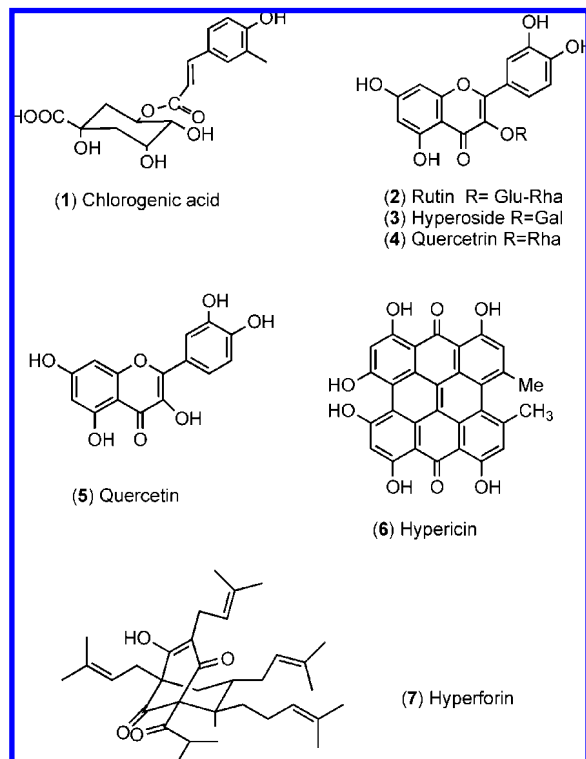*J. Agric. Food Chem.,* Vol. 56, No. 13, 2008   **5079**

Figure 1. *H. perforatum* compounds (**1–7**) analyzed in the transformed plant lines by LC-PDA-ESI-MS.

MATERIALS AND METHODS

Plant Material And Transformation Procedures. A micropropagated line of *Hypericum perforatum* was established from a wild Italian accession. Transgenic hairy roots (HR) were obtained by transformation of leaf and root fragments with a wild agropine type *A. rhizogenes* strain ATCC 15834 (*19*). Different HR lines originated from single independent transformation events were established. All the HR lines were first grown on agarized medium which contained mineral salts and vitamins, according to Murashige and Skoog (*21*). The pH parameter was adjusted at 5.7 before autoclaving at 120 °C for 20 min. The cultures were maintained in the growth chamber at 23 ± 1 °C with a 16/8-h photoperiod (cool white fluorescent lamps) and a quantum flux density of 28 μE m^{-2} s^{-1}. The twelve selected HR lines (A, B, C, D, G, H, I, L, M, N, R, and S) were grown in 300 mL glass vessels with 30 mL of liquid MS0 medium and cultured in the growth chamber with a continuous rotary shaking (78 rpm). The cultivated medium was renewed at 7-day intervals. After about 4 weeks, shoots regenerated from the HR in all the lines, providing the "hairy root-derived plant lines", which showed shoots and roots. The culture medium pH and the fresh weight of the cultures were measured at each medium renewal. The pH average and the daily growth index (DGI) (final fresh weight − initial fresh weight/days of culture) were calculated after 42 days in order to detect eventual differences in biomass development. Furthermore the productivity coefficient (PC) was then calculated by multipling the daily growth index per dry weight yield (DGI × DWY, **Table 1**). The morphological characterization was evaluated by the number of shoots, the number of leaves, the height of the shoots, and the number of black spots on the leaves (BSL). The morphological data were used to calculate the index of compactness [IC = no. of leaves per shoot/shoot length (cm)] for each line in order to describe with a single value the plant behavior due to the *A. rhizogenes* transformation.

Phytochemical Analysis. *Chemicals.* Chlorogenic acid (**1**) (Extrasyntheses, Lyon, France), rutin (**2**) (Merck, Darmstadt, Germany), hyperoside (**3**) (Extrasyntheses, Lyon, France), hypericin (**6**) (Extrasyntheses, Lyon, France), and hyperforin (**7**) (Sigma-Aldrich, Munich, Germany) were used as commercial standard compounds. Quercetrin (**4**) (STD37HH17) and quercetin (**5**) (STD10HH17) are included in a homemade database of natural compounds isolated and characterized by NMR and MS techniques in DCBB laboratory (*22*). All these compounds (**1–7**) were analyzed and

Table 1. Growth Parameters of the HR-Derived Plant Lines Grown in Liquid Culture after 42 Days of Culture at 23 ± 1 °C and at 16/8-h Photoperiod (Light Intensity at 28 μE m^{-2} s^{-1})

line	DGI[a]	DWY[b] (%)	pH ± SE	PC[c]
A	0.92	5.25	4.44 ± 0.04	0.048
B	1.44[d]	3.29	4.78 ± 0.16	0.047
C	1.08[d]	4.44	4.54 ± 0.06	0.0479
D	0.76	6.29	4.49 ± 0.1	0.048
G	0.64	7.41	4.41 ± 0.07	0.047
H	0.47	8.57	4.3 ± 0.07	0.040
I	0.91	5.22	4.34 ± 0.08	0.047
L	0.34	11.45	4.29 ± 0.07	0.038
M	1.3[d]	3.73	4.58 ± 0.08	0.038
N	0.96	5.37	4.53 ± 0.05	0.051
R	1.3[d]	3.65	4.52 ± 0.1	0.047
S	0.85	5.69	4.36 ± 0.05	0.048

[a] Daily growth index: final fresh weight − initial fresh weight/days of culture. [b] Dry weight yield: dry weight/fresh weight. [c] Productivity coefficient: DGI × DWY. [d] Fast growing lines.

Table 2. ASE Extraction and LC-PDA-ESI-MS Data for the *H. perforatum* Constituents (**1–7**) Detected in the Analyzed HR-Regenerated Plant Lines

compound	ASE recovery %	RSD[a]	μg/mL	regression equation
1 chlorogenic acid	98.4	4.7	2.8–288	$y = 0.347 + 1.199 \times 10^{-4}x$ ($r = 0.997$)
2 rutin	88.7	3.0	2.6–264	$y = -0.393 + 4.522 \times 10^{-5}x$ ($r = 0.999$)
3 hyperoside	93.7	3.3	2.7–274	$y = -1.122 + 6.110 \times 10^{-5}x$ ($r = 0.997$)
4 quercetrin	86.3	5.1	4.9–198	$y = 0.0517 + 7.194 \times 10^{-5}x$ ($r = 0.999$)
5 quercetin	102.3	4.1	2.1–206	$y = 0.999 + 9.243 \times 10^{-5}x$ ($r = 0.999$)
6 hypericin	89.7	7.1	0.04–44	$y = -6.296 \times 10^{-3} + 2.557 \times 10^{-5}x$ ($r = 0.997$)
7 hyperforin	87.6	8.3	0.12–85	$y = 0.777 + 5.944 \times 10^{-5}x$ ($r = 0.996$)

compound	UV, nm	[M − H]$^-$, m/z	fragment ions, MS/MS[b]
1 chlorogenic acid	234sh, 244, 297sh, 328	352.9	191.1, 173.1
2 rutin	259, 266sh, 299sh, 359	609.1	301.0, 178.9
3 hyperoside	257, 362, 269sh, 299sh	463.0	392.7, 301.0
4 quercetrin	256, 265sh, 350	447.0	301.0
5 quercetin	270, 371, 310sh	301.0	178.9
6 hypericin	270sh, 590	503.2	487.2, 459.1
7 hyperforin	270	535.1	466.0, 397.0

[a] Relative standard deviation (RSD); $n = 3$ replica. [b] EC = energy collision range 30−43%.

purified by HPLC-PDA-ESI-MS (>98−99%) before using them as reference material (**Figure 1**). Acetonitrile (CH$_3$CN), formic acid (HCOOH), and methanol (MeOH) were HPLC grade solvents (Baker, The Netherlands). HPLC-water was purified by a Milli-Q Plus system (Millipore, Milford, MA).

ASE Extraction and Purification. The freeze-dried samples (1.5−2.0 g) were extracted by Accelerated Solvent Extraction (ASE, Dionex 200, 33 mL). The extraction was performed by two different static steps using dichloromethane and methanol (75 °C, 140 bar, 5 min) in turn as reported in the literature for wild *H. perforatum* plant samples (*23*). The methanolic extraction recoveries of each standard compound were reported in **Table 2**.

Sample Preparation for HPLC. Three samples (20 μL) of each *H. perforatum* plant line extract were dissolved in methanol (2 mg/mL) and were filtered through a cartridge-type sample filtration unit (PTFE, 0.45 μm, 25 mm) before HPLC injections.

*Calibration Curves for the Reference Compounds (**1–7**).* The dosage of the analyzed constituents (**1–7**) was performed by the external

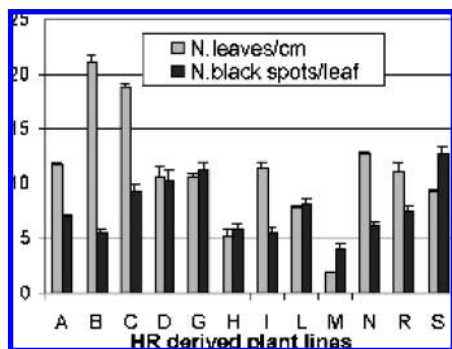**5080** *J. Agric. Food Chem.,* Vol. 56, No. 13, 2008

Bertoli et al.

Figure 2. Index of compactness [IC: number of leaves per shoot/shoot length (cm)] evaluated in the analyzed plant material and the number of black spots per leaf (BSL) on the leaves of the plants regenerated HR lines (number ± SE).

standard method, using six levels of concentration for each compound (**Table 2**). The standard solutions for the authentic samples (**1**−**5**) were prepared in methanol. Hypericin (**6**) (4.4 μg) was dissolved in pyridine (2 mL) before adding methanol (8 mL) while hyperforin (**7**) (11.7 μg) was dissolved in MeOH−ascorbic acid 0.1% (10 mL). Hypericin and hyperforin solutions were prepared and kept protected from light. An aliquot (20 μL) of each standard compound was analyzed in triplicate under the same HPLC-PDA-ESI-MS conditions used for the extract samples.

HPLC-PDA-ESI-MS Analyses. The HPLC system consisted of a Surveyor ThermoFinnigan liquid chromatograph pump equipped with an analytical Lichrosorb RP-18 column (250 × 4.6 mm i.d., 5 μm, Merck), a Thermofinnigan photodiode array detector, and an LCQ Advantage mass detector. The analyses were carried out by a linear gradient using water with 0.1% HCOOH (solvent A), CH₃CN (solvent B), and MeOH (solvent C) at flow rate of 1.0 mL/min under the following conditions: from 5:95 v/v (B-A) to 15:85 (B-A) in 10 min and then to 40:50:10 v/v (B-A-C) (20 min), to 75:10:15 (B-A-C) (10 min), to 80:5:15 (B-A-C) (15 min), and then conditioning to the initial condition (5:95 v/v B-A) for 10 min. (total run time was 65 min).

An aliquot (20 μL) of each sample was analyzed in triplicate. Chromatograms were detected at 270 nm for all analyzed compounds except for hypericin (590 nm).

Both full and MS/MS scan mode chromatograms (mass range *m/z* 200 to 700 amu, negative ion mode) were registered. (**Table 2**; **Figure 3**). ESI-MS spectra were performed by the following parameters: sheath gas flow-rate 72 arb units, auxilary gas flow 10 arb units, capillary voltage −16 V, and capillary temperature 280 °C.

Statistical Analysis. The analytical data were subjected to one-way ANOVA, and the significance of treatment differences was established by *F*-tests at the 95% confidence level.

RESULTS AND DISCUSSION

(1) Plant Material and Transformation Procedures. The selected liquid medium was very effective for in vitro fast growth of *H. perforatum* plant tissues. Transgenic hairy roots, cultivated under light conditions on a medium without plant growth regulators showed a fast and good increase in fresh weight. Furthermore, they spontaneously regenerated shoots, providing a biomass suitable for the extraction of the typical active constituents of *H. perforatum* (**Figure 1**). The pH averages measured during the growing phase are showed in **Table 1**. All these values were slightly acid and ranged between 4.2 and 4.6 pH values. The B line was characterized by the highest pH at 4.78 ± 0.16. The productivity of each HR-derived plant line was related to the daily growth index (DGI) mean value and to the biomass yield in dry weight (DWY) (**Table 1**). It was possible to select two different kinds of plant material: the fast growing-lines with a DGI higher than 1 (lines B, C, M, and R) and the slow ones with the index lower than 0.5 (**Table**

1). The calculated productivity coefficients (PC) were very similar between the two groups even if some lines had a fast development in their fresh weight. Furthermore, the fastest growing lines had a very high tissue hydration, and this feature could be induced by the growth in the liquid culture immersion. Other in vitro culture systems could be explored in order to avoid this excessive water uptake, for instance in a temporary immersion system (TIS), characterized by short flooding that could increase plant biomass (*24*). The morphologic data were used also to define the Index of Compactness (IC) for each line. The B and C lines showed the highest IC values (**Figure 2**) and this fact was visually confirmed by the plant shape too. In fact they had severe compact shape and miniaturized architecture, typical of HR derived-plants, which was also maintained after their transfer to the greenhouse. The black spots, typically developed on the *H. perforatum* leaves grown in the open-field, were showed also by the leaflets of the in vitro growing plants. These structures were counted and each line was classified by the number of black spots per leaf (BSL). The G and S lines showed the highest BSL values (**Figure 2**).

(2) Phytochemical Analysis. ASE procedure was performed for the extraction of the HR-regenerated plants using dichloromethane and methanol in turn. The yields and percentage recoveries for each analyte (**1**−**7**) are shown in **Table 2**.

The ASE extracts were analyzed by LC-PDA-ESI-MS (**Table 2**; **Figure 3**) and the extract content of the reference compounds (**1**−**7**) was estimated by using a multilevel external standard procedure in the linear range of the calibration. The linear regression equations and their correlation coefficients for each analyte are shown in **Table 2**. An overview of the amounts (mg/g$_{dw}$) of the selected compounds (**1**−**7**) in the *H. perforatum* extracts is given in **Table 3**. The production of some typical St. John's Wort constituents was observed in the selected HR-regenerated plant lines. Chlorogenic acid (**1**) was one of the most representative secondary metabolite of the analyzed plant material. It was synthesized in significant amounts by several transformed lines (nine out of twelve) apart from G, H, and S lines. Its concentration range was 0.47−1.09 mg/g$_{dw}$, and the highest production was obtained by the fast growing B, C, M, and R lines (**Table 3**). The C line produced chlorogenic acid (**1**) together with a significant amount of hypericin (**6**) and hyperforin (**7**).

Originating from the shikimic acid pathway, chlorogenic acid rises from the condensation between quinic acid and caffeic acid (*25*). It is well-known that an increase of this compound influences the levels of other polyphenols in plants. Furthermore chlorogenic acid is an important bioactive compound recently produced by engineered plants for its antioxidant activity against degenerative diseases (*25*). The in vitro procedure performed in the present study guaranteed the selection of B, C, M, and R lines, which were the most productive in chlorogenic acid (**1**). The LC-MS screening of the methanolic extracts showed a large variability also in the content of other typical flavonoids such as rutin (**2**), hyperoside (**3**), quercetrin (**4**), and quercetin (**5**) (**Table 3**). They ranged from the N line, characterized by the presence of all selected flavonoid markers, to the C line, in which only chlorogenic acid, hypericin, and hyperforin were detected. Hyperoside (**3**) was produced in seven out of twelve transformed lines, and the I and L lines seemed particularly efficient in its biosynthesis (4.91 and 4.60 mg/g$_{dw}$, respectively) (**Table 3**). Hyperoside (**3**) is a typical constituent of *H. perforatum* wild plants, which is considered for its strong antioxidant properties and its protective action against cytotoxicity (*26*).
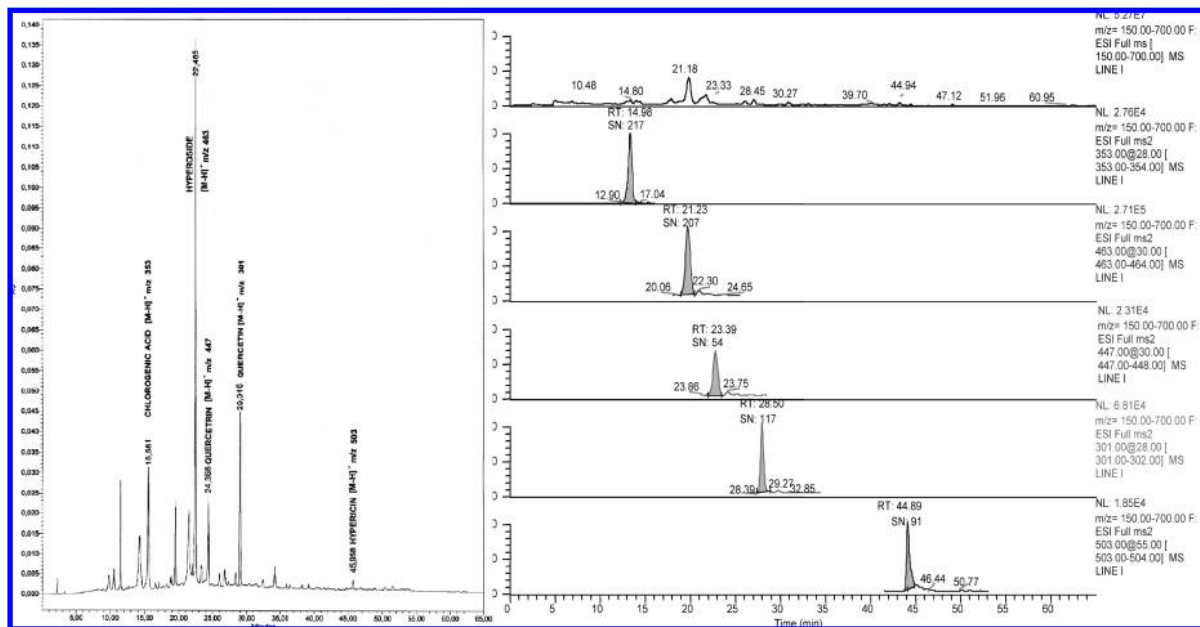
Bioactive Constituent Production in St. John's Wort

J. Agric. Food Chem., Vol. 56, No. 13, 2008   **5081**

Figure 3. LC-PDA-ESI-MS profiles of the *H. perforatum* transformed plant line I (270 nm, full scan and MS/MS mode).

Table 3. Production of the Marker Compounds (1−7) (mg/g $_{dw}$) in the *H. perforatum* Hairy Root Regenerated Plant Lines [a]

	chlorogenic acid 1	RSD	rutin 2	RSD	hyperoside 3	RSD	quercetrin 4	RSD	quercetin 5	RSD	hypericin 6	RSD	hyperforin 7	RSD
line A	0.47	5.01	-		0.38	6.61	0.38	10.0	0.45	4.77	0.10	8.76	0.05	1.63
line B	1.02	1.14	0.56	3.78	-		0.30	7.21	0.40	7.47	0.07	2.47	0.06	1.64
line C	1.04	1.61	-		-		-		-		0.01	5.1	0.06	4.11
line D	0.77	1.31	0.21	2.17	-		0.31	2.43	0.49	10.44	-		0.08	16.5
line G	-		0.11	2.17	-		-		0.16	1.18	0.01	3.38	0.09	2.61
line H	-		-		0.10	2.18	-		-		0.03	5.89	0.05	5.73
line I	0.54	2.06	-		4.98	0.26	0.30	2.46	0.26	2.74	0.01	26.46	-	
line L	0.54	2.48	-		4.60	0.21	0.46	2.85	0.28	3.06	0.02	1.75	0.01	1.91
line M	0.96	0.78	-		0.45	1.65	0.35	1.71	0.17	2.88	0.01	4.6	0.01	4.43
line N	0.84	7.04	0.24	5.68	0.62	7.21	0.35	9.54	0.58	6.56	0.25	9.12	-	
line R	0.98	4.36	0.11	4.97	-		0.23	4.50	0.41	3.52	0.05	3.48	0.07	2.72
line S	-		0.14	10.5	0.27	5.55	0.89	2.55	-		0.05	2.88	0.09	10.1

[a] Means of triplicate analyses are expressed with their relative standard deviation (RSD)

Line B showed the highest rutin content (0.56 mg/g$_{dw}$), while six out of twelve lines did not produce this secondary metabolite (**Table 3**). It is important to point out that wild-Italian *Hypericum* rutin-free chemotypes were reported by Martorfi (*27*) and by Avato and Guglielmi (*28*). Some analyzed HR lines showed a very low variety of the selected flavonoids (1−7). In fact the G line was characterized only by the presence of two flavonoids, rutin (**2**) and quercetin (**5**) while the H line showed only hyperoside (**3**) production. Hypericin (**6**) and hyperforin (**7**) were quite ubiquitarious in the analyzed plant material. Hypericin content ranged from 0.01 to 0.25 mg/g$_{dw}$, and the N line showed the highest production (0.25 mg/g$_{dw}$). The same line was selected also for the best productivity coefficient PC (**Table 1**). It is important to point out that the N line, grown without exogenous plant growth regulators, showed a hypericin content up to 10-fold higher than the best reported from Gadzovska (*9*) with in vitro micropropagated plants using 6-benzyladenine (BA). Moreover, the L line showed a significant amount of hypericin (**6**), always higher (2.5 fold) than that obtained in callus, grown by auxin feeding (IAA and IBA) (*9*). The N line did not produce hyperforin (**7**) so it was considered a selected line for the hypericin production. However, other lines such as D, S, and G showed significant amounts of hyperforin (**7**) (0.08, 0.09, and 0.09 mg/g$_{dw}$, respectively). Briskin and Gawienowsky (*29*) proposed that hypericins were accumulated into the dark glands of *Hypericum* leaves, but the undifferentiated tissues never

showed these secretory structures even if they were grown under dark conditions (*10*). In a recent work, Kornfeld (*20*) demonstrated by SEM (scanning electron microscopy) and TEM (transmission electron microscopy) techniques that hypericins may occur in other different parts of the in vitro grown leaves instead of in the glands. In fact the peripheral cells may be related to the production of hypericins, too. In our experiments, after in vitro leaf organogenesis or leaf development under light conditions, the black spots were observed.

The analyzed plant material was produced by the whole plant biomass in which the leaves showed a huge number of dark glands. In fact the number of BSL seemed to be a target for each line, and it was much higher in the S, G, and D lines (**Figure 2**). The same lines were characterized by significant amounts of hyperforin (**7**) and a lower production of hypericin (**6**) which was absent in the D line. Therefore the present study showed that the presence of black spots should be related not only to hypericin but also to hyperforin content. Furthermore the lack of hypericin (**6**) in the D line and the absence of hyperforin (**7**) in the I and N lines could be related to a genetic modification due to the *A. rhizogenes* transformation rather than to the differentiation step. Several hypotheses may be drawn in order to understand the genetic modification among the analyzed hairy root-regenerated plant lines. In fact previous studies already suggested that the variability of secondary metabolite production may be due to the copy numbers and to the different

Bertoli et al.

insertion sites of the foreign T-DNA in the plant genome or in other cases to the silent genes involved in the secondary metabolism (*30*). As reported previously (*31*) for *Panax ginseng*, it is possible to select high-producing lines of secondary metabolites by screening a large population of independent lines after *A. rhizogenes* transformation.

In conclusion the present study demonstrated that it is possible also for *Hypericum perforatum* to screen the corrispondent stable lines originated from independent transformation events and to select hairy root-regenerated plant material with specific metabolic profiles. The transformation protocol performed in this study was able to select transgenic *Hypericum perforatum* plant lines characterized by significant amounts of its main bioactive constituents.

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