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Short communication

Influence of different elicitors on the synthesis of anthraquinone derivatives in *Rubia tinctorum* L. cell suspension cultures

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Abstract

An elicitation method increased the synthesis of anthraquinone derivatives occurring in cell suspension cultures of *Rubia tinctorum* L. High performance liquid chromatography coupled with diode array detection and mass spectrometry (HPLC–DAD–MS) was used for the detection and quantification of the two glycosides, lucidin primeveroside and ruberithric acid and eight aglycones, namely pseudopurpurin, lucidin, alizarin, purpurin, alizarin-2-methylether, lucidin-ω-ethylether, nordamnacanthal and munjistin ethyl ester. The improved elicitor treatment of the suspension cultures increased the amounts of the natural dyes; the different elicitors, such as fungal polysaccharides and endogenous signal molecules (salicylic and jasmonic acid) provided specific yields of anthraquinone derivatives. After 7 days of elicitation, the total yield of anthraquinones increased from $\approx 70\ mg\ g^{-1}$ to $\approx 262\ mg\ g^{-1}$ while the amounts of lucidin primeveroside and ruberithric acid grew threefold and the quantity of pseudopurpurin increased by a factor of 28.

Keywords: Anthraquinone analysis; Natural dyes; Dye production; *Rubia tinctorum* L; Elicitors

1. Introduction

Anthraquinones are well-known and are widely used in the dye industry, medicine and food chemistry [1,2]. The natural and synthetic derivatives of 9,10-anthracenedione, in particular, display numerous beneficial effects both in mammals and humans such as antibacterial, antitrypanosomal and antineoplastic activities; other effects such as lipid peroxidation, intestinal motility inhibition and inhibition of human telomerase have also been described. Antharcycline antibiotics, which are known to be key substances for the therapy of several cancers, are also 9,10-anthracenediones [3]. In addition to the above mentioned properties, the anthraquinones from the *Rubia* genus of the Rubiaceae family also show some important physiological effects. These substances exhibit hepatoprotective, renal calculus eliminative, immunomodulant, anti-inflammatory, calcium channel2 antagonistic, antithrombotic as well as DNA binding activities in both animals and humans [4]. Among other important molecules, the *Rubia* plants contain more than 60 anthraquinone derivatives and their glycosides.

While the rhizomes and roots of *Rubia tinctorum* L. (madder) are known as the main sources of several anthraquinone derivatives and have been used to dye textiles since ancient times, nowadays synthetic derivatives of 9,10-anthraquinone are more often used for dyeing [5–7]. Furthermore, the appropriate age of madder roots for economic anthraquinone production is 3 years and harvesting results in the destruction of the established plants. However, the continuous production of anthraquinone derivatives in the suspension cultures of *R. tinctorum* cells has been proven [8,9].

The natural dye production by suspension cultures can be influenced by different exogenous and endogenous molecules (called elicitors), which act as signals for the cells. These alarm signals generate complex defence responses via different signal transduction pathways. In addition to the main responses, plant cells elevate the production of some secondary metabolites

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[10]. Elicitation is a well-known technique and several authors demonstrated that the synthesis of anthraquinones in *R. tinctorum* cell cultures could be improved using this technique [11–18]. In addition to synthetic preparation, the biotechnological production of the dyes provides a useful tool because of its favorable environmental impact and economic importance.

The aims of this study were to increase the production of anthraquinones by applying fungal elicitors and two endogenous signal molecules (jasmonic acid and salicylic acid), to determine the optimal treatment period and obtain information on the specificity of selected elicitors on anthraquinone synthesis. To systematically identify and quantify anthraquinone production it was necessary to employ high performance liquid chromatography (HPLC) coupled with photodiode array (DAD) and mass selective (MS) detection [19–23].

The anthraquinone composition of madder roots is well documented, but less information is available concerning the anthraquinone-derivative contents of *in vitro* cultures. It has been found that cell suspension cultures contain seven anthraquinone derivatives [24] whereas callus cultures have superior levels of ruberythric acid and rubiadine-3-glucoside [25]. In hairy root cultures, alizarin and purpurin were the main constituents of hydrolyzed extracts [26]. Other authors describe the presence of alizarin, purpurin, lucidin and nordamnacanthal in transformed cell cultures [27–29] and the main component in non-hydrolyzed samples of hairy root cultures was shown to be lucidin primeveroside [30].

In order to determine the composition of madder root samples we recently developed a combination of HPLC–DAD–MS and gas chromatography GC–MS [31] to identify and quantify two glycosides and eight aglycones in control and elicited cell suspension cultures of *R. tinctorum*.

2. Materials and methods

2.1. Chemicals

If analytical grade reagents and substances were available, they were used, otherwise, technical grade or locally prepared alternative versions were utilized. Acetonitrile, ammonium acetate, ethyl alcohol were obtained from Reanal (Budapest, Hungary), trifluoroacetic acid (TFAA) from Serva (Heidelberg, Germany). Jasmonic acid and salicylic acid were from Sigma-Aldrich (Sigma-Aldrich Ltd., Budapest, Hungary). Anthraquinone standards, such as alizarin, anthraquinone were from Reanal (Budapest, Hungary), purpurin, and quinizarin from ICN (Aurora, OH, USA). Other anthraquinone derivatives were found and identified in extracts, prepared in our laboratory, such as lucidin, lucidin-ω-methylether, alizarin-2-methylether, lucidin-ω-ethylether, munjistin ethylester, and nordamnacanthal, while 1-hydroxyanthraquinone and 2-hydroxyanthraquinone were prepared according to the literature [31,32].

2.2. Plant and fungal materials

Three year old *R. tinctorum* plants were sourced from the Botanical Garden of the Research Institute for Medicinal Plants (Budakalász, Hungary). Suspension cultures were prepared from the roots and grown in Murashige–Skoog liquid medium (containing 30 g l^{-1} sucrose, 1 mg l^{-1} indoleacetic acid (IAA), 0.2 mg l^{-1} naphatleneacetic acid (NAA) and kinetin) at room temperature and under natural light (in 250 ml Erlenmeyer flasks) [33]. Before sterilization in an autoclave (1 at, 20 min) the pH of the medium was set to 5.8. Suspensions were maintained on a rotary shaker at 125 rpm and were subcultured every 14 days. Since cell division reaches a maximum between the 7th and 14th days and this period is followed by a stationary phase [11,12], the elicitations were made before the end of the exponential phase of the cell divisions (on the 14th day).

Fruit bodies of the necrotroph-parasite fungus *Coriolus versicolor* (Fr.) Quel. were collected from the Buda Hills in Hungary.

The phytopathogenous fungus *Botrytis cinerea* Pers. ex. Pers. isolates were obtained from the Department of Cell and Molecular Biology of Drogs, Comenius University (Bratislava, Slovakia) and were cultured on 3% (w/v) malt medium [11].

2.3. Preparation of the elicitors

The isolation and purification of the cell wall material of *B. cinerea* (BC), and its hydrolysis with trifluoroacetic acid (TFAA) was performed as described previously [11].

Water-soluble polysaccharides of *C. versicolor* were isolated using the methods described before [34] and were separated by means of gel filtration chromatography using Sephadex G-200 columns (Pharmacia, Uppsala Sweden) [35]. The separation resulted in three different M_r fractions of polysaccharides (CVI: $M_r > 60$ kD; CVII: 60 kD $> M_r >$ 5 kD; and CVIII: $M_r < 5$ kD) [36]. Finally, the obtained fractions were lyophilized.

2.4. Elicitation procedure

Using the methods described in recent literature, the following sterile aqueous solutions of elicitors were prepared with the use of distilled water:

(i) Increasing concentrations of jasmonic acid (JA), (3, 7, 15, and 30 μl ml^{-1} designated as JA1–JA4 in the order of increasing acid content);
(ii) Increasing concentrations of salicylic acid (SA), (13, 27, 67.5, and 100 mg ml^{-1}; designated as SA1–SA4 in the order of increasing acid content);
(iii) 80 mg ml^{-1} concentration of CVI, CVII and CVIII polysaccharides fractions derived from *C. versicolor*.

One millilitre of these elicitor solutions was then added to 100.0 ml of *R. tinctorum*'s cell suspension cultures [11,36–9]. In the case of *Botrytis* derived elicitor, 1.0 ml of BC solution was added to 100.0 ml of *R. tinctorum*'s cell suspension culture. The control suspension cultures were treated with 1 ml of autoclaved distilled water (DW).

After the different elicitors had been added to the cell cultures the amount of anthraquinones was determined at various elicitation times (after 1, 3, 5, and 7 days and in

selected cases after 10 days). The production of anthraquinones in the elicited suspension cultures was compared to the yield of the control samples.

2.5. Extract preparations from R. tinctorum's roots and suspension cultures

After collecting *R. tinctorum* cells by means of filtration at the appropriate elicitation times the cell materials and the root samples were dried (60 °C, 5 days) and pulverized. 0.20 g of each pulverized cell and root materials was extracted with 30 ml of 80% (v/v) ethyl alcohol using a reflux condenser (for 1 h). The insoluble filtrate was then extracted for the second time using the same method. The stock solutions were obtained by unifying the filtered extracts of each sample, then diluting them to 100.0 ml. Fifteen millilitres of each stock solution was evaporated to dryness using a vacuum evaporator at 30–40 °C. Finally, the dried extracts were dissolved in 1.50 ml of 80% (v/v) ethyl alcohol and analyzed by HPLC–DAD–MS.

2.6. Sample evaluations by HPLC–DAD–MS

In order to determine the composition of the samples HPLC–DAD–MS measurements were performed using an Agilent 1100 HPLC–DAD–1946A MSD system (Agilent, Waldbronn Germany) equipped with an autosampler (injection: 20 μl). Analysis was carried out at room temperature using MOS Hypersyl BDS 150 mm × 4 mm ID, 5 μm particle, RP C_{18} column, (Shandon Southern Products, Runcorn, UK) [31]. Gradient elution was carried out. In eluent A, the volume percentage of acetonitrile to 0.02 M ammonium-acetate buffer (pH = 4.0) was 15:85 v/v, and in eluent B, the volume percentage of acetonitrile to 0.02 M ammonium-acetate buffer (pH = 4.0) was 85:15 v/v [14]. A linear gradient program was performed where the ratio of eluent B was raised from 20% to 80% in 40 min at a flow rate of 1 ml min^{-1}.

DAD parameters: spectra, on-line: 200–600 nm; quantification at: $\lambda = 254$ nm.

MS parameters: ES (electrospray) ionization, positive ion mode; scan: 140–700 *m/z*; drying gas: N_2 − 8 l min^{-1}; nebulizer pressure: 40 psig; capillary voltage: 3500 V; fragmentation voltage: 80 V.

The quantification of the anthraquinone derivatives in *R. tinctorum* samples was carried out using an external standard calibration method described in Ref. [31]. However, to calculate the quantities of lucidin primeveroside and ruberithric acid the response of alizarin was used and for determining the pseudopurpurin quantity the response of purpurin was used.

3. Results and discussion

3.1. Identification of the extracts' constituents by HPLC–DAD measurements

Fig. 1A shows the chemical structure of anthraquinone derivatives (substances 1–16) in the order of their retention times. These can be identified and quantified by using HPLC–DAD–MS separation shown in Figs. 2–4. The chemical structure of SA and JA can be found in Fig. 1B and C, respectively.

The standard anthraquinone derivatives were dissolved in 80% (v/v) ethyl alcohol and their chromatogram can be seen in Fig. 2A, where the corresponding peaks appear at $\lambda = 254$ nm. The retention times and the DAD–MS spectra of these standards were the basis of the characterization of the experimental samples. Fig. 2B–D shows the chromatograms of 2B, the control suspension sample; 2C, sample with CVI elicitor; 2D, sample with JA2 elicitor.

The identification and quantification of the anthraquinone derivatives in all of the suspension cultures and root extracts of *R. tinctorum* can be clearly determined on the basis of their retention times and observing their DAD (Fig. 3A–D) and mass spectra (Fig. 4).

The experimentally determined DAD spectra of the anthraquinone compounds had been grouped according to similarities in their molecular structure (Fig. 1A). Fig. 3A demonstrates the DAD spectra of lucidin (4), lucidin-ω-ethylether (12) and nordamnacanthal (13), where the three specific maximum wavelengths are: 244, 280 and 412 nm. Fig. 3B shows maximum absorbances at wavelengths 258–260 nm and 478–492 nm, which correspond to the DAD spectra of pseudopurpurin (3) and purpurin (7). Fig. 3C presents the DAD spectra of alizarin (6) and its 2-methylether (10) with their fairly identical maximum values at 250, 280 and 428 nm, respectively. Fig. 3D shows the spectral similarity of munjistin ethylester (15) and a non-identified compound (8) which provided special maximum absorbance at 410 nm, although their structural similarity was not confirmed by their mass spectra (see Fig. 4: spectra of compounds 8 and 15).

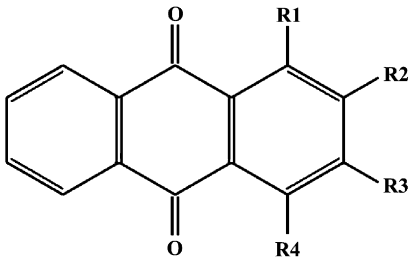
3.2. Evaluation of the mass spectra obtained by on-line HPLC–MS (ESI)

On-line HPLC–MS (ESI) measurements were performed in order to identify the anthraquinone derivatives in *R. tinctorum*'s suspension cultures and roots (Fig. 4).

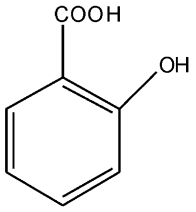
According to the mass spectra, 10 constituents provided informative protonated, ($m/z = [MH]^+$), and/or Na cationized ($m/z = [MNa]^+$) molecular ions. In the order of listing, the masses have been confirmed as follows (see Fig. 1 for molecular weights and Fig. 4 for spectra):

1+2: lucidin primeveroside ($M_w = 564$); $m/z = [MNa]^+ = 587.1$ + ruberithric acid ($M_w = 534$); $m/z = [MNa]^+ = 557.1$;
3: pseudopurpurin ($M_w = 300$); $m/z = [MNa]^+ = 301.1$;
4: lucidin ($M_w = 270$), $m/z = [MH - H_2O]^+ = 253.0$; $m/z = [MNa - H_2O]^+ = 275.0$; $m/z = [MNa]^+ = 293.0$;
6: alizarin ($M_w = 240$), $m/z = [MH]^+ = 241.0$;
7: purpurin ($M_w = 256$), $m/z = [MH]^+ = 256.9$;
8: unknown ($M_w = 418$, presumably), $m/z = [MH]^+ = 419.0$; $m/z = [MNa]^+ = 441.1$;
10: alizarin-2-methylether ($M_w = 254$), $m/z = [MH]^+ = 255.0$;
12: lucidin-ω-ethylether ($M_w = 298$), $m/z = [MH - (C_2H_5OH)]^+ = 253.0$; $m/z = [MNa - (C_2H_5OH)]^+ = 275.0$; $m/z = [MNa]^+ = 321.1$;

A



B



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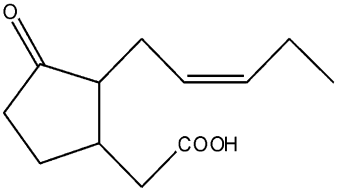

No.	Compound	R1	R2	R3	R4	Mw
1.	**Lucidin primeveroside**	OH	CH_2OH	O-Pr*	H	564
2.	**Ruberythric acid**	OH	O- Pr*	H	H	534
3.	**Pseudopurpurin**	OH	COOH	OH	OH	300
4.	**Lucidin**	OH	CH_2OH	OH	H	270
5.	2-hydroxyanthraquinone	H	OH	H	H	224
6.	**Alizarin**	OH	OH	H	H	240
7.	**Purpurin**	OH	OH	H	OH	256
9.	Lucidin methyl ether	OH	CH_2OCH_3	OH	H	284
10.	**Alizarin-2-methyl ether**	OH	OCH_3	H	H	254
11.	Antraquinone	H	H	H	H	208
12.	**Lucidin-ω-ethylether**	OH	$CH_2OCH_2CH_3$	OH	H	298
13.	**Nordamnacanthal**	OH	CHO	OH	H	268
14.	1-hydroxyanthraquinone	OH	H	H	H	224
15.	**Munjistin ethyl ester**	OH	$COOCH_2CH_3$	OH	H	312
16.	Quinizarin	OH	H	H	OH	240

Mw=molecular weight
*= primeverose (6-*O*-β-D-xylopyranosyl-β-D-glucose);
Bold printed compounds: identified and quantified in suspension cultures.

Fig. 1. Traces A–C: (A) chemical structures of anthraquinone derivatives 1–16, in the order of their retention time (corresponding to the peaks on Figs. 2–4.), (B) chemical structure of salicylic acid, (C) chemical structure of jasmonic acid.

13: nordamnacanthal ($M_w = 268$), $m/z = [MH]^+ = 269.0$; $m/z = [MNa]^+ = 291.0$;
15: munjistin ethylester ($M_w = 312$), $m/z = [MH]^+ = 313.0$; $m/z = [MH - (C_2H_5OH)]^+ = 267.0$; $m/z = [MNa - H_2O]^+ = 319.0$; $m/z = [MNa]^+ = 335.0$.

3.3. Quantification of the anthraquinone derivatives

The relative standard deviation (RSD%) from the average reproducibility of the anthraquinone's quantification was 4.46% (varying between 2.59% and 6.5%) (Table 1).

3.4. Results and discussion of the elicitation procedures

During the investigated time period (1–10 days) the anthraquinone derivatives' content of the control cultures varied within the experimental error of the measurements.

In order to have a comparable basis for the anthraquinones' production the optimum elicitation time had to be defined. For this purpose, the CVI fungal polysaccharide fraction was selected due to its promising behavior at the preliminary measurements. The effect of this elicitor on the anthraquinones' yield was measured after 1, 3, 5, 7 and 10 days and the results showed that the 7th day was the optimum elicitation time. Thereafter, in order to compare the different elicitor's effectivity, the anthraquinones' production was measured after 7 days (Table 1).

3.4.1. Effect of fungal polysaccharides on the anthraquinone production

After evaluating the total production of the anthraquinones in the experimental samples it was found that the fungal polysaccharide elicitors provided the maximum yields. The elicitor's effectiveness was found to be in the following order: CVI > CVII > CVIII > BC and the corresponding yield values of the dried suspension cultures were 262.0, 182.9, 148.5, and 107.7 mg g^{-1}. In comparison, the average yield of the distilled

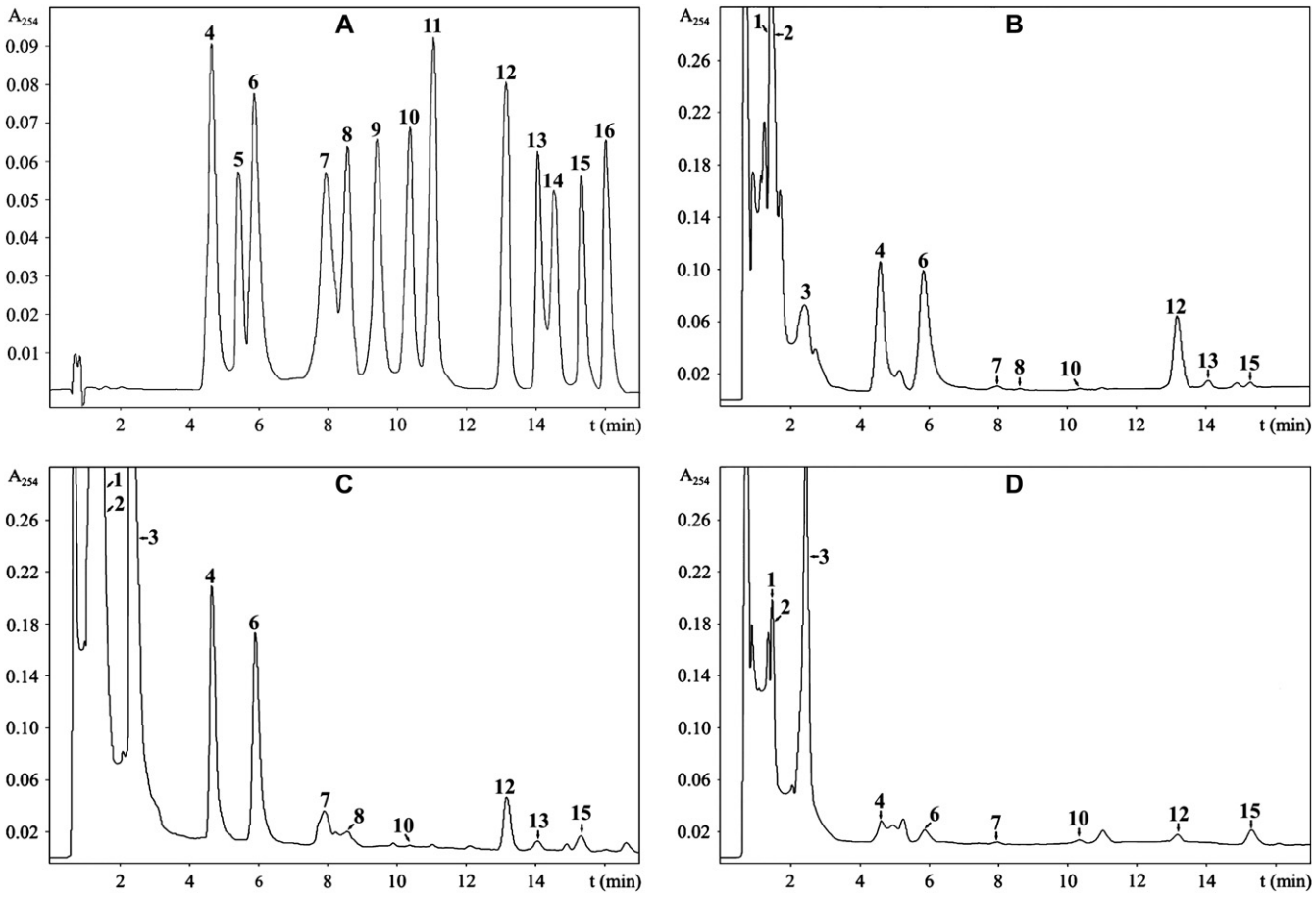

Fig. 2. Traces A−D: HPLC−DAD chromatograms obtained at $\lambda = 254$ nm. Demonstrated anthraquinones: (A) in standard solution; (B) in the control suspension culture (after 10 days); (C) in CVI elicited suspension culture (after 7 days) and (D) in JA2 treated suspension cultures (after 7 days). Peaks: lucidin primeveroside and ruberithric acid (1, 2); pseudopurpurin (3); lucidin (4); 2-hydroxyanthraquinone (5); alizarin (6); purpurin (7); unknown (8); lucidin methylether (9); alizarin-2-methylether (10); antraquinone (11); lucidin-ω-ethylether (12); nordamnacanthal (13); 1-hydroxyanthraquinone (14); munjistin ethyl ester (15) quinizarin (16).

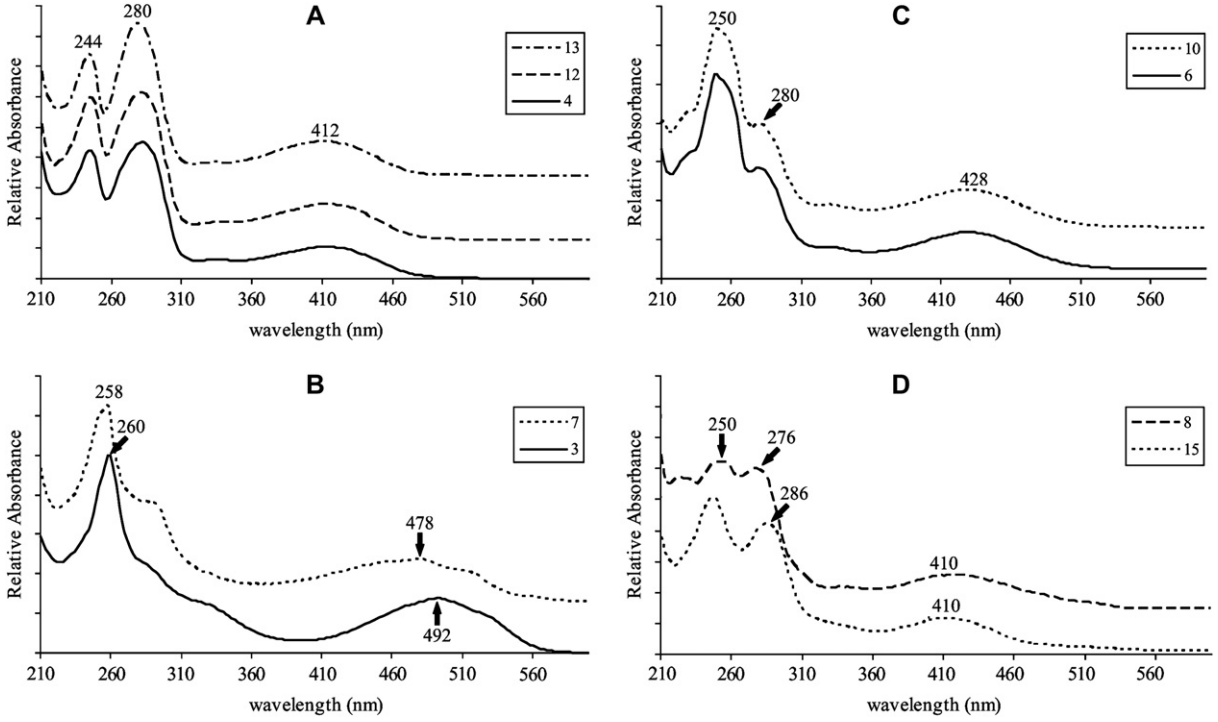

Fig. 3. Traces A−D: HPLC−DAD spectra of the identified compounds in *R. tinctorum* L. suspension cultures (numbers correspond to the peaks indicated in Fig. 2).

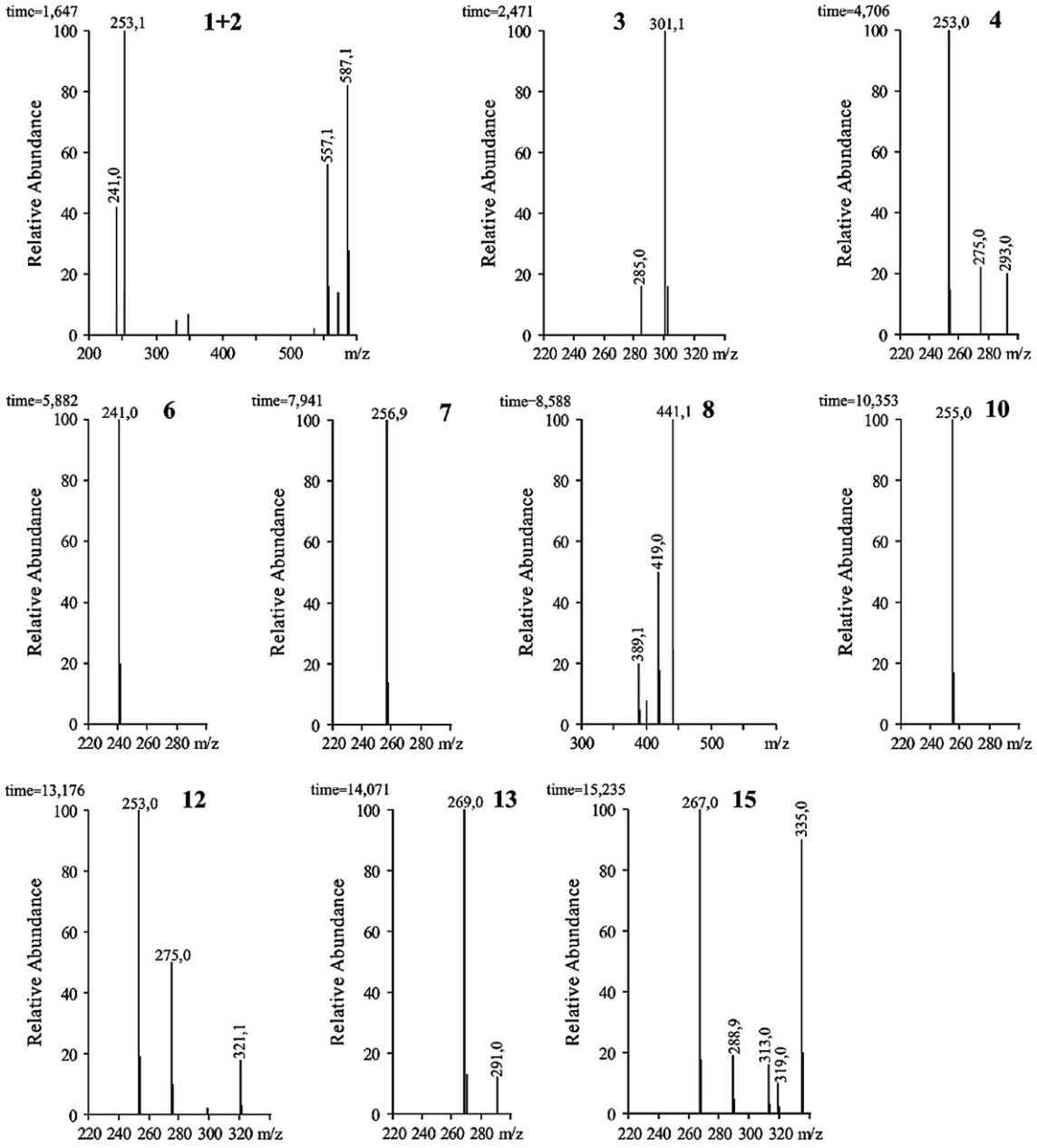

Fig. 4. HPLC–MS (ESI) spectra of the identified compounds in *R. tinctorum* L. suspension cultures (numbers correspond to the peaks indicated in Fig. 2).

water treated control samples was only 72.5 mg g^{-1}. This can be regarded as a spectacular increase in yields. It is also worth mentioning that the total anthraquinone content found in the madder root sample was only 82.8 mg g^{-1} and this amount is significantly less than the yield of the elicited cell suspension cultures (107.7–262.0 mg g^{-1}). Therefore the elicitations of cell suspension samples are clearly advantageous.

Similarly, high lucidin primveroside, ruberithic acid and pseudopurpurin production were measured in the cell suspensions elicited by fungal polysaccharides (Table 1).

Elicitors are recognized by plant receptors or R proteins localized in the plasma membrane or in the cytoplasm before initiating signaling responses, which (among other responses) lead to elevated production of secondary metabolites [10]. This recognition process is essential in response generation, and realized by pathogen associated molecular patterns (PAMP) of the elicitors [40]. Coriolus derived protein-bound polysaccharides exhibit immunomodulatory, antitumor, analgesic and hepatoprotective effects in mammals [41,42]. The compositions of this kind of polysaccharopeptides had been described by several authors and some repetitive regions has been found in the structure of the polysaccharide parts and the peptide content of the extracted molecules ranged from 6.4% to 10% [34,42–44]. Unfortunately, the PAMP of Coriolus derived protein-bound polysaccharides is not known.

Based on our findings, the CVI–CVIII elicitors had similar effects on the production of the anthraquinones (Table 1) with the difference in yields. The different molecular weights of

Table 1
Quantity of anthraquinone derivatives in dried roots and suspension cultures of *R. tinctorum L.*

Compounds	Constituents, mg g^{-1} of dried suspension cultures																			Constituents of dried roots mg g^{-1}
	Constituents of control (distilled water treated) suspension cultures			Constituents of suspension cultures after different elicitor treatments																
				Coriolus derived elicitor treaments							BC Treatment	Jasmonic acid treatments				Salicylic acid treatments				
				CVI					CVII	CVIII		JA1	JA2	JA3	JA4	SA1	SA2	SA3	SA4	
	1 day	5 days	10 days	1 day	2 days	5 days	7 days	10 days	7 days	7 days	7 days	7 days	7 days	7 days	7 days	7 days	7 days	7 days	7 days	
Lucidin primeveroside + ruberithric acid	52.5 (4.94)	55.2 (3.84)	51.0 (4.34)	55.8 (3.37)	59.6 (5.4)	63.8 (5.7)	172.4 (4.13)	165.9 (5.6)	131.4 (4.27)	110.5 (3.74)	82.6 (5.1)	37.3 (4.27)	12.2 (4.88)	10.7 (6.04)	11.8 (3.34)	44.6 (3.53)	18.5 (3.46)	17.8 (2.89)	19.2 (4.78)	57.4 (3.14)
Pseudopurpurin	2.98 (6.3)	2.76 (4.02)	3.04 (2.68)	5.6 (4.16)	9.2 (4.35)	28.0 (4.67)	60.8 (4.28)	60.2 (3.06)	25.2 (3.81)	19.2 (2.59)	10.7 (4.33)	3.12 (3.91)	48.6 (4.56)	70.3 (4.18)	84.9 (4.66)	3.79 (4.52)	10.8 (4.24)	12.6 (6.42)	17.3 (4.49)	23.3 (4.92)
Lucidin	7.2 (3.71)	7.4 (2.89)	7.1 (3.72)	7.9 (4.82)	8.8 (3.24)	12.2 (3.38)	13.1 (6.2)	12.9 (4.71)	11.9 (5.7)	7.9 (4.58)	8.0 (2.92)	6.5 (4.77)	0.36 (3.53)	0.48 (5.82)	0.5 (4.58)	7.1 (4.84)	0.76 (5.2)	0.28 (3.6)	0.26 (4.63)	0.35 (4.30)
Alizarin	6.9 (4.42)	6.7 (5.2)	6.8 (4.66)	7.6 (4.06)	8.5 (4.62)	9.9 (5.4)	10.5 (4.31)	10.1 (3.42)	9.5 (2.79)	6.4 (3.72)	2.70 (3.84)	4.22 (2.68)	0.35 (4.21)	8.3 (3.85)	10.3 (2.99)	5.8 (3.94)	0.81 (5.59)	17.1 (4.37)	16.9 (3.87)	0.92 (5.19)
Purpurin	0.23 (2.98)	0.26 (5.1)	0.23 (4.53)	0.69 (3.19)	1.20 (5.5)	1.25 (3.42)	1.30 (5.5)	1.28 (5.0)	1.20 (3.97)	1.11 (4.23)	0.23 (4.40)	0.34 (5.12)	0.20 (3.16)	0.22 (4.65)	0.28 (4.21)	0.32 (6.11)	0.23 (6.1)	0.19 (4.46)	0.24 (4.18)	0.63 (4.65)
Alizarin-2-methylether	d	d	d	d	d	d	d	d	d	d	d	d	d	d	0.12 (3.84)	d	d	0.18 (5.17)	0.2 (4.12)	d
Lucidin-ω-ethylether	2.13 (5.1)	2.09 (4.74)	2.19 (4.86)	2.04 (4.69)	2.57 (3.62)	3.50 (4.14)	3.51 (3.76)	3.87 (3.38)	3.38 (6.5)	3.05 (4.72)	2.91 (3.25)	1.98 (5.36)	0.11 (4.57)	0.14 (6.23)	0.1 (5.7)	1.87 (5.27)	0.32 (5.44)	0.12 (5.39)	0.17 (5.81)	d
Nordamnacanthal	0.17 (4.79)	0.14 (5.3)	0.15 (4.78)	0.14 (5.1)	0.16 (4.06)	0.16 (4.76)	0.18 (4.84)	0.17 (6.2)	0.14 (5.9)	0.17 (4.39)	0.15 (6.1)	nd	nd	d	d	nd	nd	d	d	d
Munjistin ethylester	d	d	d	d	d	0.18 (4.23)	0.25 (4.48)	0.23 (5.1)	0.15 (4.76)	0.13 (3.92)	d	d	0.27 (5.1)	0.25 (5.56)	0.26 (5.31)	d	0.10 (6.2)	d	d	0.2 (4.11)
Identified in total (average)	72.2	74.6	70.6	79.8	90.1	119.0	262.0	254.7	182.9	148.5	107.7	53.5	62.1	90.4	108.3	63.5	31.5	48.3	54.3	82.8

Indications: CVI–CVIII: *Coriolus versicolor* derived polysaccahride elicitors; BC: *Botrytis cinerea* derived elicitor; JA1–JA4: different concentrations of jasmonic acid elicitor; SA1–SA4: different concentrations of salicylic acid elicitor. Numbers in parentheses represent the %(RSD) values obtained from three independent measurements, d: detected, nd: not detected.

polysaccharopeptides seem to contain the same recognition sites but not in equal proportions. These proportions were found to be dependent on the molecular weight as the larger molecules generated significantly higher yields of anthraquinones in *R. tinctorum* cell suspension cultures.

After elicitor signal perception, the plant receptors activate their effectors (such as ion channels, G proteins, protein kinases, NADPH oxidases) and via second messenger molecules (such as H_2O_2, JA, SA, internal calcium release, MAP kinases etc.) the response leads to the modulation of several genes (eg. defence genes). Finally, the altered defence gene expression may manifest in elevated generation of secondary metabolites [10,13–16].

The chorismate/*o*-succinilbenzoic acid pathway leads to anthraquinone biosynthesis in *R. tinctorum* cell suspension cultures. Regulation of this pathway by different environmental (light, nutritional components, elicitors) and endogenous (metabolic and developmental) stimuli strongly influences the anthraquinone biosynthesis. Accordingly, different elicitors (like fungal polysaccharides, JA and SA) are useful and well-known tools to influence secondary metabolites in plant suspension cultures of *R. tinctorum* [9,10,13–18,27,29].

3.4.2. Effect of signal molecules on the anthraquinone production

Compared to the control cultures, the effects of the different concentrations of JA and SA elicitors on the major anthraquinone derivatives were the following (see data in Table 1):

(i) Lower levels of these two endogen signal molecules (JA1 and SA1) were not as effective to alter the production of the anthraquinone derivatives significantly.
(ii) JA and SA are specific elicitors for pseudopurpurin. The average pseudopurpurin production of the control suspension cultures was 2.93 mg g^{-1} where the use of JA4 increased this to 84.9 mg g^{-1} and SA4 to 17.3 mg g^{-1}.
(iii) Both elicitors decreased the quantity of lucidin primeveroside and ruberithric acid even at higher concentrations but not in equal proportions.
(iv) Both elicitors decreased the quantities of lucidin at higher than JA1 and SA1 concentrations (JA2–JA4; SA2–SA4).
(v) Both elicitors increased the quantities of alizarin at higher than JA2 and SA2 concentrations (JA3–JA4; SA3–SA4).
(vi) The production of alizarin was reduced at low concentrations of JA and SA (JA1–JA2; SA1–SA2).

Overall, JA and SA are two important regulating compounds affecting plant responses and their signaling crosstalk acts in an inhibiting/activating relationship [37,45–47]. Our results showed that SA and JA have characteristic patterns on the regulation of anthraquinone formation in *R. tinctorum* cell suspension cultures. Moreover, the effect of these two signaling molecules significantly differs from the fungal elicitors used by us (Table 1).

4. Conclusions

1. We determined that 10 anthraquinone derivatives could be separated, identified and quantified by applying HPLC–DAD–MS measurements in the suspension cultures and root samples of *R. tinctorum*.
2. Four fungal polysaccharides (CVI, CVII, CVIII and BC) and two endogenous signal molecules (JA, SA) were selected as possible elicitors. Results confirmed, that
   (i) The treatment with fungal polysaccharide elicitors provided outstanding total anthraquinone production, which was particularly high in the case of CVI. Using the madder root samples as a comparison, these elicitors can increase the production of the anthraquinones by more than threefold.
   (ii) JA had a specific impact, which is due to its effect on selective pseudopurpurin production.
   (iii) SA had a specific impact, which is due to its effect on elevated alizarin production.
3. Overall, based on our experiments associated with fungal polysaccharides and two endogen regulators, both types of elicitors have advantageous features. Our data supports the possibility of an effective positive influence on the synthesis of anthraquinone derivatives. In addition to the natural and synthetic routes, this method is a useful biotechnological tool to produce the desired anthraquinone compounds.

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