Stimulation of anthocyanin production by two-phase growth system, in callus of *Rudbeckia hirta*

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1. Introduction

Rudbeckia hirta L. of *Asteraceae* family grows in its natural habitat in North America, where it is used in traditional Indian medicine as a remedy against various infections [1,2]. The species arrived in Europe in the 17th century, it became naturalised and at present it is commonly grown, also in Poland [3]. Due to its traditional therapeutic applications and the fact that it is closely related to the *Echinacea* genera, *R. hirta* is an interesting subject for phytochemical research.

Extensive research of this species carried out at the Department of Pharmacognosy, Medical University of Gdańsk, proved the presence of a rich set of flavonoid compounds, phenolic acids and traces of anthocyanin compounds in the flowers of the natural plant [4-6].

As part of the research into new sources of polyphenolic compounds, a task was set to obtain callus tissue of *R. hirta*, which would be characterised by high vitality and ability to produce a selected set of secondary metabolites.

The initial phytochemical analysis (TLC, PC, HPLC) of the tissue material obtained from sections of seedlings of *R. hirta* showed clear tendencies of the propagated callus to produce anthocyanins, only minimal amounts of which were present in the natural plant. Thus, the above described, unexpected change in the metabolic paths of the callus of *R. hirta* in comparison with the natural plant, led to the formulation of a new goal of biotechnological research, which was to obtain high production of anthocyanins from material propagated *in vitro*. It has to be stressed, that anthocyanin compounds have applications in a number of areas, including medicine [7,8]. Moreover, the pigments are only obtained from natural plants, which means that the supply of the material is limited. A rich source of anthocyanins in

the form of *in vitro* cultures would allow production independent of environmental conditions (such as the season of the year and the climate).

It was decided that in order to achieve the goal, an appropriate experimental medium should be selected, which would guarantee good tissue growth of *R. hirta* and high content of anthocyanins in the propagated tissue. Moreover, the main components of the obtained set of anthocyanin compounds were to be isolated and identified.

2. Experimental

2.1. Plant material and culture methods

Seeds of *Rudbeckia hirta*, obtained in 1992 from the Medicinal Plant Garden of the Medical University of Gdańsk, were used in the development of callus cultures. (Vouchers are on deposit in the herbarium of Medicinal Plant Garden of the Medical University of Gdańsk). The biological material underwent surface sterilisation with 0.1% $HgCl_2$ (45 min), then it was rinsed 3 times with redistilled water, and placed on Petri dish, on absorbent paper soaked in a solution of giberelic acid and kinetin (10^{-4} g/dm^3). The dishes were kept in darkness 24 hours a day, at 25°C, and after a week seedlings with good vitality were obtained. These seedlings were used as explants for the callus cultures; they were cut into 2 mm sections and placed on MS medium [9] and G-5 medium [10], which were supplemented with 5 mg/dm^3 2.4-D and 5 mg/dm^3 of zeatin and then solidified with agar. The process of callus origination was observed in a growth chamber at 26°C with light 16 hours a day. Over a 4 week period, the ability of the seedlings to develop callus on various media was observed.

After a 4 week incubation period, the initial callus tissue (of cothyledone origin) was transferred onto a number of media: MS [9], SH [11], NN [12], LS [13] and Mill. [14], modified with stable content of growth regulators (5.00 mg of 2,4 D and 5.00 mg of zeatin). Apart from these modifications of the basic media, several growth variations were used in the incubation of *R. hirta* callus, where the solidifying agent varied; it was either agar or Phytagel (manufactured by Sigma). Moreover, cultures were established on stationary-liquid media, with membrane rafts manufactured by Sigma, used to keep the tissue on the surface of the medium (Table 2).

Growth of callus tissue of *R. hirta* on the media, modified as described above, was observed for a period of 4 weeks and callus growth was evaluated with the use of the formula quoted by Klein [13],

$$G_f = [(Mc - Mi) : Mi] \times 100.$$

Mc — callus mass after a 4 week incubation period
Mi — mass of the inoculum
G_f — growth factor

The value of G_f , the % content of water in the callus and the macroscopic information were then used to develop optimum conditions for the propagation *in vitro* of callus of *R. hirta*. In addition, the content of anthocyanin pigments in the tissues obtained on various media was identified with the use of HPLC, thus providing assessment of the suitability of each growth modification for the production of anthocyanins *in vitro* (Table 2). All experiments with different growth media were repeated 4 times. Both the percentage of water and the growth factor were calculated on the basis of weight of fresh tissue from 15 baby food jars. The experiments described above led to the development of an original two-phase growth system which allows increased anthocyanin production in the callus of *Rudbeckia hirta* L. The two-phase growth system is made up of two experimental media:

I — modified Schenk-Hildebrandt medium (phase of tissue growth)
II — modified Miller's medium (phase of anthocyanin production) (Table 1).

TABLE 1
COMPOSITION OF TWO-PHASE GROWTH SYSTEM

Component	Content of each component in mg/dm^3	
	I stage — SH medium	II stage — Mill. medium
1	2	3
KNO_3	2,500.00	1,000.00
NH_4NO_3	–	1,000.00
$Ca(NO_3)_2 \times 4H_2O$	–	500.00
$CaCl_2$ anhydr.	151.00	–
KCl anhydr.	–	65.00
$MgSO_4$ anhydr.	195.40	35.00
KH_2PO_4	–	300.00
$NH_4H_2PO_4$	300.00	–
$Na_2EDTA \times 2H_2O$	20.00	–
$Na_2FeEDTA \times 2H_2O$	–	32.00
$FeSO_4 \times 7H_2O$	15.00	–
$MnSO_4 \times H_2O$	10.00	6.50
$ZnSO_4 \times 7H_2O$	1.00	2.70
H_3BO_3	5.00	1.60
KJ	1.00	0.80
$Na_2MoO_4 \times 2H_2O$	0.10	–
$CoCl_2 \times 6H_2O$	0.10	–
$CuSO_4 \times 5H_2O$	0.20	–
Myo-inositol	500.00	100.00
Saccharose	30,000.00	30,000.00
Glycine	–	2.00

1	2	3
Cysteine	–	10.00
Thiamine x HCl	5.00	1.00
Riboflavin	0.50	–
Pyridoxine x HCl	–	0.10
Nicotinic acid	5.00	0.50
Adenine	–	1.00
2,4-D	5.00	5.00
Zeatin	5.00	5.00

The *R. hirta* tissue was incubated on SH media (phase I) for 2 weeks, at 26°C, with light intensity of 5000 lx. The biomass was then transferred onto Miller's medium (phase II) and maintained at 26°C with light intensity of 5000 lx for 3 weeks. Throughout the experiment stationary-liquid media were used, with membrane rafts (manufactured by Sigma) to keep the tissue on the surface of the medium. The tissues of *R. hirta* grown on the two-phase system underwent phytochemical analysis.

2.2. Phytochemical analysis

Chromatography: PC, Whatman No.1, mobile phases: S_1 = n-butanol:glacial acetic acid:water (4:1:5) -organic phase; S_2 = glacial acetic acid:water (2:98); S_3 = hydrochloric acid:formic acid:water (7.1:31.4:61.5); S_4 = n-butanol:glacial acetic acid:water (4:1:3); S_5 = n-butanol:pyridine:water (6:4:3); S_6 = n-butanol:pyridine:water (3:3:1); S_7 = pyridine:ethyl acetate:glacial acetic acid:water (5:5:1:3); TLC-Cellulose DC-Fertigplatten (10x20 cm); mobile phases: S_8 = n-amyl alcohol:glacial acetic acid:water (10:3:1); S_9 = formic acid:hydrochloric acid:water (10:1:3); S_{10} = acetic acid:hydrochloric acid:water (10:3:1); S_{11} = glacial acetic acid:water (3:2:0); S_{12} = benzene:methanol:glacial acetic acid (45:8:4); S_{13} = benzene:dioxane:glacial acetic acid (90:25:4).

Isolation and identification of anthocyanins: 450 g of dried callus of *R. hirta*, which had been propagated with the use of optimised two-phase growth system (SH/Mill media — Table 1), was powdered and then extracted exhaustively with a mixture of methanol and hydrochloric acid (1000:8.6). The extraction of the anthocyanin compounds was carried out with 5 portions of the solvent for 5 hours, at room temperature. The acid methanol extracts were then condensed at reduced pressure at the temperature of 35°C to form a syrup-like residue. It was in turn diluted in water modified with hydrochloric acid to pH=3, and then extracted with chloroform to remove ballast substances. The solvent was removed (distilled at reduced pressure, temp. 35°C), and the syrup-like residue (30 g) was dissolved in methanol modified with hydrochloric acid (1000:8.6). Chromatographic analysis (PC,TLC,HPLC) of the purified anthocyanin fraction obtained from *R. hirta*

callus showed the presence of 12 anthocyanin compounds. The following step was the isolation and identification of dominant substances in the pigments complex (compounds **1** and **2**).

Compound **1** (cyanidin-3-0-(6-0-malonyl-)β-D-glucopyranoside), which was the main component in the anthocyanin complex in *R. hirta*, was obtained as a result of separation of the anthocyanin complex on a cellulose column (phase S_8). Fractions 21-30, which contained this compound, were combined and then purified in a column filled with Sephadex LH-20, washed away with methanol:hydrochloric acid (1000:8.6). This produced 40 mg of compound **1**.

Compound **2** (cyanidin-3-0-β-D-glucopyranoside) in amorphous form (16 mg) was obtained as a result of preparative paper chromatography (PC) with mobile phase S_3. The compound was then purified in a column filled with Sephadex LH-20, washed away with methanol:hydrochloric acid (1000:8.6). This produced 12 mg of pure, amorphous compound **2**.

*Compound **1**:* dark red amorphous substance. Fluorescence: UV-bright red; daylight -red; daylight (following treatment with 25% solution of NH_4OH) -navy blue.

PC Rf: S_1=0.18; S_2=0.75; S_3=0.85; TLC Rf: S_8=0.18; S_9=0.75; HPLC t_r (min): gradient I programme = 9,15-identical with standard sample of cyanidin-3-0-(6-0-malonyl-β-D-glucopyranoside).

^{1}H NMR (10%v/v TFA-90% DMSO-d_6) δ:8.88 (1H, *s*, H-4); 8.21(1H, *dd*, *J* = 2.0; 8.7 Hz, H-6'); 7.99(1H, *d*, *J* = 2.0 Hz, H-2'); 7.02(1H, *d*, *J* = 8.7 Hz, H-5'); 6.89(1H, *d*, *J* = 2.0 Hz, H-8); 6.72(1H, *d*, *J* = 2.0 Hz, H-6). Glucose moiety: 5.37(1H, *d*, *J* = 8.0 Hz, H-1); 4.44(1H, *d*, *J* = 11.2 Hz, H-6_b); 4.11 (1H, *dd*, *J* = 7.4; 11.2 Hz, H-6_a); 3.81(1H, *dd*, *J* = 7.4; 9.2 Hz, H-5); 3.52(1H, *dd*, *J* = 8.0; 8.8 Hz, H-2); 3.40(1H, *dd*, *J* = 8.8; 9.2 Hz, H-3); 3.23(1H, *dd*, *J* = 9.2; 9.2 Hz, H-4); 3.35 - 3.36 (range of — 2H, malonyl-CH_2-signals).

$UV_{\lambda max}^{MeOH + 0,1\%HCl}$ = 295; 318; 528 — identical as standard for cyanidin-3-0-(6-0-malonyl-β-D-glucopyranoside).

*Acid hydrolysis of comp. **1*** (0,5N H_2SO_4; 1h., 100°C) — aglycone of comp. **1** — dark red amorphous substance. Fluorescence: UV-bright red; daylight-red; PC Rf: S_1=0.64; S_2=0.09; TLC Rf: S_8=0.74; S_9=0.12; S_{10}=0.53; S_{11}=0.75, HPLC t_r (min): gradient I programme = 49.02; *UV of comp. **1**. aglycone*: $UV_{\lambda max}^{MeOH + 0,1\%HCl}$= 264;508 — identical with standard sample of cyanidin.

*Sugar of comp. **1***: PC Rf: S_5 = 0.39; S_6 = 0.34; S_7 = 0.15 — identical with standard sample of glucose.

*Alkaline hydrolysis of comp. **1*** (1N KOH; 1h, room temp.) — glucoside of comp. **1**- red amorphous substance. Fluorescence: UV-bright red; daylight-red; PC Rf: S_1 = 0.24; S_2 = 0.26; S_3 = 0.29; S_4 = 0.47; TLC Rf: S_8 = = 0.28; S_9 = 0.29, HPLC t_r (min): gradient I programme = 34.41; *UV of comp. **1**. aglycone*: $UV_{\lambda max}^{MeOH + 0,1\%HCl}$ =277; 519 identical with standard sample of cyanidin-3-0-monoglucoside.

*Acid residue of comp. **1***: TLC Rf: S_{12} = 0.13; S_{13} = 0.6 — identical with standard sample of malonic acid.

Compound **2**: brown red amorphous substance. Fluorescence UV–red; daylight–red; daylight (following treatment with 25% solution of NH_4OH)–navy blue.

PC Rf: S_1 = 0.24; S_2 = 0.26; S_3 = 0.24; S_4 = 0.47; TLC Rf: S_8 = 0.28; S_9 = 0.29; HPLC t_r(min): gradient I programme = 34.41-identical with standard sample of cyanidin 3-0-(β-D-glucopyranoside).

^{1}HNMR (10%v/v TFA – 90% DMSO – d_6) δ: 8.88(1H, *s*, H-4); 8.23(1H, *dd*, *J* = 2.2;8.6 Hz, H-6'); 8.00(1H, *d*, *J* = 2.2 Hz, H-2'); 7.02(1H, *d*, *J* = 8.0 Hz, H-5'); 6.90(1H, *brs*, H-8); 6.69 (1H, *d*, *J* = 2.0 Hz, H-6). Glucose moiety: 5.34(1H, *d*, *J* = 7.8 Hz, H-1); 3.71(1H, *d*, *J* = 10.3 Hz, H-6_b); 3.49 (3H, *m*, H-2, H-5, H-6a); 3.37(1H, *dd*, *J* = 9.2; 9.2 Hz, H-3); 3.22(1H, *dd*, *J* = 9.2;9.0 Hz, H-4).

$UV_{\lambda max}^{MeOH + 0,1\%HCl}$ =277;519 identical with standard sample of cyanidin-3-0-(β-D-glucopyranoside).

Acid hydrolysis of comp. **2***:* aglycone of comp.**2**: dark red amorphous substance. Fluorescence: UV-red; daylight-red; PC Rf: S_1 = 0.64; S_2 = 0.09; TLC Rf: S_8 = 0.74; S_9 = 0.12; S_{10} = 0.53; S_{11} = 0.75, HPLC t_r (min): gradient I programme = 49.02; *UV of comp.* **2***. aglycone*:

$UV_{\lambda max}^{MeOH + 0,1\%HCl}$ = 266;508 identical with standard sample of cyanidin.

Sugar of comp. **2** : PC Rf: S_5 = 0.39, S_6 = 0.34, S_7 = 0.15 — identical with standard of cyanidin.

Other compounds (comp. **3** and comp. **4**) were identified by direct comparison with authentic samples.

Quantitative analysis of anthocyanins: The plant material for research (callus tissues) was dried (10 mg sample), pulverised and extracted at room temperature with a mixture composed of methanol:hydrochloric acid (1000:8:6). The acid methanol extracts were combined, condensed at reduced pressure and then diluted in 25 mL methanol (Merck). The solutions were filtered through 0.45 μm fliters (J.T.Baker) and injected onto a chromatographic column. The HPLC conditions were as follows; column: LiChrospher RP-18 (5 μm, 250 × 4 mm I.D) (Merck, Darmstadt, Germany), mobile phase: solvent A (water:formic acid, 90:10) and solvent B (methanol:acetonitrile:water:formic acid 22.5:22.5:45:10) with the following gradient I programme: from 0-1min-100%A; from 1-30-min-/ from 0% to 30% B in A (linear gradient); from 30-60 min –30% B in A (isocratic elution); flow rate: 1.0 mL min^{-1} at room temp.; detection at λ=530 nm (sensitivity 0.008 AUFS).

3. Results and discussion

In order to produce callus tissue of *Rudbeckia hirta* L., sterile seedlings divided into 0.5 cm segments were placed on two kinds of media, MS and G-5, supplemented with a 5 mg addition of 2,4-D (2,4-dichlorophenoxyacetic acid) and zeatin. After a week of growth, initial formation of callus tissue from explants (of cothyledone origin) was observed. It was also observed that with G-5 medium only 30% of the cothyledones formed callus, whereas

with MS medium this tendency was visible in all cothyledone segments. Moreover, the callus formed on G-5 medium had a slimy consistency and it withered quickly. On the contrary, the tissue formed on MS medium, although greyish-transparent, without chlorophyll, was good for further transfers.

The initial research into the maintenance of callus of *R. hirta* led to the conclusion that the G-5 medium, which has low mineral content [10], was not appropriate to maintain callus cultures of *R. hirta*. It was also concluded that callus tissue of this plant requires mineral rich media (MS medium) [9].

Further research focused on developing optimum conditions for continuous growth of callus tissue and conditions for anthocyanin synthesis. A number of media commonly used for *in vitro* purposes were tested. The media were selected for their variable composition, in terms of basic minerals, vitamins and amino acids. The media were supplemented with identical quantities of growth hormones, used at the initial stage only, to eliminate their potential direct influence on growth parameters and anthocyanin production. In view of the above considerations, MS medium was used, as it was assumed that the high content of NO_3^-, K^+ and NH_4^+ ions would enhance initial growth of the tissue. LS medium, which differs from MS only by the presence of a single vitamin (thiamine hydrochloride), was used to verify the impact of the vitamin factor on the growth and metabolism of callus tissue of *R. hirta*. The experiment also included two media low in mineral content (NN and Miller) [12,14] and the SH medium, rich in both minerals and organic components (500 mg of inositol) [11].

Research showed a significant impact of the mineral content not only on the growth and development of the investigated callus tissue, but also on anthocyanin compounds which it produced. The most significant growth was observed on mineral rich media, i.e. MS, LS and SH (Table 2).

It is worthy of note that of these media, by far the strongest growth was observed on SH medium (Wp = 581 — liquid variation XIII), which could imply positive role of inositol. Results were different for mineral poor media, i.e. NN and Mill. These showed very poor growth (Wp = 217 — option IV-NN; Wp = 190.4 — option V-Mill.) (Table 2). Moreover, the macroscopic observations indicated, that towards the end of the 4^{th} week of incubation, the tissues grown on NN and Mill. media withered away and could not be used for transfers. Taking that into consideration, the two media may be eliminated from further research as inappropriate for continuous growth of *R. hirta*. At this stage of research no relation was detected between the composition of the media and the water content of the biomass (Table 2). Generally, the water content in *R. hirta* callus was within the 94.3% - 96.7% range.

TABLE 2

EFFECT OF THE CHANGE OF THE GROWTH MEDIUM ON THE GROWTH FACTOR (G_f), % OF WATER IN TISSUES AND ANTHOCYANINS CONTENT IN CALLUS OF *Rudbeckia hirta* L. (n=15 TESTS)

Type of growth medium	Growth factor (G_f)	Percentage of water in tissues	Percentage content of anthocyanins			Gelling agent
			Sum	Cyanidin-3-malonyl-glucoside (comp. **1**)	Cyanidin-3-O-glucoside (comp. **2**)	
MS	321.0	95.8	1.61	0.42	0.39	agar
LS	378.4	96.1	1.60	0.41	0.39	
SH	480.1	95.2	1.60	0.44	0.40	
NN	217.4	95.4	0.72	0.01	0.01	
Mill.	190.4	94.3	2.16	0.72	0.32	
MS	447.8	94.9	1.62	0.44	0.38	Phyta-gel
LS	411.3	95.9	1.60	0.40	0.37	
SH	570.2	96.1	1.68	0.45	0.40	
NN	346.6	96.7	0.69	0.01	0.01	
Mill.	281.8	95.1	2.16	0.74	0.32	
MS	392.3	96.0	1.69	0.46	0.40	Liquid-stationary
LS	424.7	96.5	1.62	0.44	0.41	
SH	581.0	95.6	1.72	0.47	0.41	
NN	311.0	96.6	0.74	0.019	0.02	
Mill.	304.6	94.9	2.18	0.80	0.37	

MS — Murashige and Skoog medium
LS — Linsmaier and Skoog medium
SH — Schenk and Hildebrandt medium
NN — Nitsch and Nitsch medium
Mill. — Miller's medium

In the course of the experiment the media were tested in the third (III) variations (Table 2), which differed in the degree to which each medium was solidified as well as in the solidifying agent. In consequence, the stationary-liquid media were selected for further research. They showed best growth factors and also, maintaining the tissues on membrane rafts washed by the nutrient liquid made it easier to separate the callus from the medium. This part of the experiment proved that the degree to which the medium was solidified had a significant impact on tissue growth (Table 2).

Concurrently with the research into biomass growth of *R. hirta*, the tissue samples produced on the various media underwent qualitative and quantitative chromatographic analysis (HPLC) for the presence of anthocyanin pigments. The macroscopic observations of callus produced on various media with the exception of Miller's showed that anthocyanin production was limited

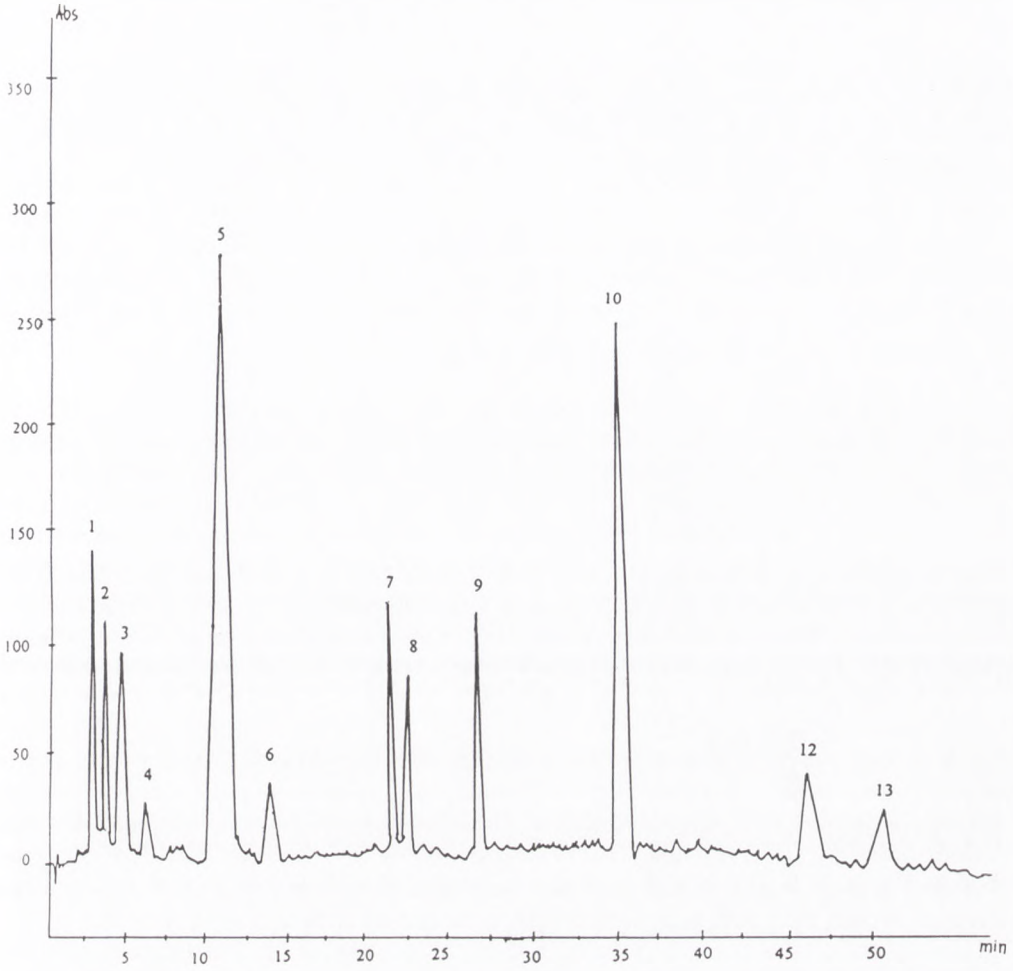

Fig. 1. HPLC of anthocyanins from callus cultures of *Rudbeckia hirta* L. maintained on Miller's medium (gradient elution).

Peaks: 5 = cyanidin-3-O-(6-malonyl-β-D-glucopyranoside)-comp. **1**; 10 = cyanidin-3-O-(β-D-glucopyranoside)-comp. **2**; 12 = malvidin-3-O-glucopyranoside-comp. **3**; 13 = free cyanidin-comp. **4**.

to surface areas of the biomass. MS, LS and SH media, which are mineral rich, produced callus tissue which contained a 12 element anthocyanin complex, in quantities of approx. 1.61%. (HPLC analysis). The main components of the complex were: comp. **1**: cyanidin-3-0-(6-0-malonyl-β-D-glucopyranoside) and compound **2**: cyanidin-3-0-(β-D-glucopyranoside) (Table 2). These two compounds were present in more or less equal quantities, regardless of the medium used. The lowest content of anthocyanins was identified on NN medium (0.72%). Paradoxically, Mill. medium, which is also mineral poor, yielded

an exceptional increase in the content of anthocyanin compounds, at 2.18% (a complex made up of 12 components). Also, a change in the proportions of the individual components of the complex was noted (Fig. 1). In the case of Miller's medium the dominant component of the complex proved to be compound **1**: cyanidin-3-0-(6-0-malonyl-β-D-glucopyranoside) (0.8%). The content of compound **2**: cyanidin-3-0-(β-D-glucopyranoside) dropped to 0.37%. The tests which were carried out did not provide conclusive evidence as to why Miller's medium, which caused progressive necrotic changes in the tissue, provided such powerful stimulation for anthocyanin production. It could be related to high content of amino acids in this medium, cystein in particular, which was not present in the other media (Table 1). In fact, cystein is not a direct precursor of anthocyanin pigments, but being a limited source of nitrogen it could influence the production of secondary metabolites [15].

It has already been mentioned here, that macroscopic observations of callus incubated on Mill. medium showed that biosynthesis of pigments occurred in the entire volume of the tissue and was not limited to the surface area. This, however, was related to the progressive necrotic changes in the tissue and rendered Mill. medium useless for continuous growth of callus. Observations of anthocyanin production on various media together with the tissue growth results led to the development of a two-phase system made from SH medium, which ensures best callus growth, and Mill. medium, which stimulates anthocyanin production (Table 1). This system allowed maximum tissue growth (2 week growth period on SH medium) and then biosynthesis of anthocyanins at the level of 2.18% (incubation on Mill. production medium — 3 weeks). Thus the advantages of both media were combined. Literature on the subject indicates that it is very difficult to develop conditions which would ensure good tissue growth and at the same time dynamic production of secondary metabolites — hence the idea of two-phased systems [16]. Such systems have not been yet used in anthocyanin synthesis and the solution developed here is original.

The tissues which were propagated with the use of the two-phase system underwent phytochemical analysis to isolate and identify the main components of the anthocyanin complex. Chromatography (TLC, PC and HPLC) showed the presence of 12 anthocyanin compounds in the complex (Fig. 1). Preparative thin layer and column chromatography led to the isolation of 2 main compounds: comp. **1** and **2**. These were identified with the use of spectroscopic methods (^{1}H NMR, UV) and classical methods (acid and alkaline hydrolysis, chromatography) to be cyanidin-3-0-(6-0-malonyl-β-D-glucopyranoside) — compound **1** and cyanidin-3-0-(β-D-glucopyranoside) — compound **2**. Two more compounds were identified against standard samples in the anthocyanin complex: comp. **3**: malwidin-3-0-glucoside and comp. **4**: free cyanidin. The other compounds in the 12 component complex were not identified, because they were only present in small quantities and standard samples were not available.

Summing up, the biotechnological research led to the development of an original two-phase system which can produce highly vital callus biomass of

R. hirta, capable of producing anthocyanins at the level of 2.18%. This level of production in an *in vitro* tissue may be considered high when compared to the natural plant which only produces anthocyanins in tubular flowers, in minimal quantities (0.28% of cyanidin-3-monoglucoside) [6]. It can be therefore said that the product of the *in vitro* research is an entirely new plant material which may serve as a rich source of anthocyanins.

In the course of phytochemical research, two main components of the pigment complex were isolated. One of them was identified as cyanidin-3-0-(6-0-malonyl-β-D-glucopyranoside), an ester compound, which is more durable than simple anthocyanin glucosides and free anthocyanidines [17,18].

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Stimulation of anthocyanin production by two-phase growth system, in callus of *Rudbeckia hirta*

Summary

Biotechnological research to achieve and then increase anthocyanin production in callus tissue of *R. hirta* L., involved testing a number of growth media, modified with growth regulators. The evaluation of the growth of propagated tissues, their water contents and in particular their ability to produce anthocyanins, led to the development of an original two-phase growth system. This new system has the advantages of both growth-stimulating media and pigment production

media. As a result of the research, callus tissue was obtained on a two-phase growth medium, which was made of modified Schenk-Hildebrandt medium (growth phase) and Miller's medium (production phase). The callus synthesised a 12-component anthocyanin compound complex, which constituted 2,18% of dry mass. This is a considerable amount compared to 0,28% in the natural plant.

Phytochemical analysis (TLC, HPLC, PC, UV, ^{1}HNMR) of the anthocyanin complex isolated from callus produced with the two-phase system proved that the dominating compounds in the pigment complex were: cyanidin-3-0-(6-0-malonyl-β-D-glucopyranoside) and cyanidin-3-0-(β-D-glucopyranoside).

Key words:

Rudbeckia hirta, Asteraceae, in vitro cultures, two-phase growth system, growth factor, anthocyanin accumulation, malonated anthocyanins.

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