Conditions for efficient induction and maintenance of callus cultures *Vaccinium corymbosum var. Bluecrop* and its accumulation of polyphenolic compounds

Wanda Dembińska-Migas
Maria Łuczkiewicz
Piotr Migas
Wojciech Cisowski
Department of Pharmacognosy
Medical University
Gdańsk

1. Introduction

Vaccinium corymbosum var. Bluecrop (Ericaceae) which is the subject of this research is a cultivar created through crossing hybrids (Jersey x Pioneer with Stanley x June) obtained from *Vaccinium corymbosum* L. and other species of the *Vaccinium* genus (1).

The above plant has became especially widely cultivated in recent years, because of its tasty fruit, rich in vitamins and antocyanin pigments. Therefore, there is a wealth of publications on biotechnology, most of which aim at developing various conditions for micropropagation of *Vaccinium corymbosum var. Bluecrop* (2,3). No publications, however, have been found which would concentrate on the attempts to obtain and maintain callus cultures from the above mentioned plant, with the aim to achieve production of pharmacologically active compounds. Also, as for phytochemical research on naturally cultivated *Vaccinium corymbosum var. Bluecrop*, it has to be noted that no analysis of chemical composition of this cultivar has been carried out so far. Moreover, it was concluded from publications review that only the chemical composition of the basic plant, *Vaccinium corymbosum*, has been partially researched. The research, however, only pertained to the fruit of this plant, identifying in it 14 anthocyanin compounds (4), flavonoids (quercetin-3-O-glucoside and myricetin-3-O-glucoside) (5), a number of organic acids (including benzoic acid, malic acid and citric acid) (6), vitamins (B1, B2, C, PP, A), inorganic salts (Co, Fe, P) as well as protein and fat (6,7).

This paper is part of a comprehensive research programme carried out at the Institute of Pharmacognosy in the Department of Pharmacy, Medical University of Gdańsk, with the aim of finding new natural sources rich in benzo-γ-piron derivatives within the *Ericaceae* family. These compounds have a variety of valuable pharmacological properties but obtaining them from wild or cultivated plants is often difficult because of the climate conditions and the changing seasons of the year (8,9). Thus, obtaining tissue material from *in vitro* cultures which would be rich in the componunds mentioned above makes it possible to become independent of the environmental conditions and to carry out extended control of the secondary metabolite biosynthesis process.

In view of the above, the goal of this paper was to initiate callus cultures from natural cultivated plants of *Vaccinium corymbosum var. Bluecrop*, and then to achieve optimum conditions for maintaining callus cultures of the tissues obtained. A preliminary assessment as to the possibility of obtained callus tissues for the production of the searched-for metabolites was also intended.

2. Materials and methods

2.1. Tissue cultures

Cuttings of *Vaccinium corymbosum* var. *Bluecrop* were obtained in 1991 from Horticultural Seed and Cuttings Centre (Centrum Nasiennictwa Ogrodowego i Szkółkarstwa) in Ożarów near Warsaw, in Poland. *In vitro* experiments were carried out on two types of experimental media: Zimmerman & Broome (2) (Table 1) and Shenk & Hildebrandt (SH) (10), supplemented with 3% sucrose, 5 mgl^{-1} thiamine, 0.5 mgl^{-1} riboflavine, 5.0 mgl^{-1} nicotinic acid, 500 mgl^{-1} myo-inositol (chemical substances by Sigma comp.). All experimental biotechnological research was carried out on stationary media solidified with agar (0.7%). The pH of the media was always adjusted to 4.9 before autoclaving. Adventitious buds with sections of internode served as explants used to start callus culture. The above biological material was surface-disinfected with 0.1% $HgCl_2$ (45 min) and rinsed three times with sterile double distilled water. For explanting, the shoot tips were trimmed to a length of 5 mm and placed horizontally onto the medium. In order to initiate callus, 4 explants were placed per baby food jar with c.a. 20 ml of Zimmerman & Broome medium with variable content of plant growth regulators (Table 2). Then the formation of undifferenciated callus tissue depending on the experimental medium used was observed over a 4 week period.

After a 4 week incubation period the callus obtained was transferred to Schenk-Hildebrandt medium with variable content of plant growth regulators (Table 3). The growth of callus depending on the regulator modification was observed over a 4 week incubation period. On completion of the growth period the value of growth factors was determined for callus tissues, calcu-

lated according to Klein's formula: Growth factor: $(G_r) = \frac{W_g - W_o}{W_o} \times 100$; W_g — the mass of the fresh callus, in grams, after a period of growth (4 weeks), W_o — the mass of the fresh callus, in grams, at the beginning of incubation (the mass of the inoculum) (11). Moreover, the content of water in the obtained biomass was determined (Table 3). This information, together with macroscope data, were used to determine the optimum conditions for maintaining callus culture of *Vaccinium corymbosum var. Bluecrop* biomass. All experiments, with changed content of plant growth regulators in each medium, were repeated 4 times. Both the percentage content of water in tissues and growth factors were calculated on the basis of the weight of the fresh tissues from 15 baby foods jars. Then the results underwent statistical assessment with the t-Student test (Tabele 3). Callus tissue was incubated in baby food jars in environmental controlled growth chamber (17 h photoperiod, 120 mmol $m^{-2}s^{-1}$ Fluorescent TLD Philips lamp, 60-70% humidity, 26/22°C).

2.2. Phytochemical analysis

313 g of dried, powdered callus grown in optimised SH medium was exhaustively extracted with chloroform („purification"), and then with methanol in Soxhlet aparatus. Initial chromatography analysis (TLC — thin layer chromatography, PC — planar chromatography) confirmed the presence of poliphenolic compounds (flavonoids and phenolic acids) in the methyl alcohol extract obtained.

The basic methanol extract was first purified using the classical method (12,13,14) and then it underwent isolation processes in order to isolate flavonoid compounds. These compounds were separated using column chromatography packed with powdered cellulose (Whatmann) and Sephadex LH 20 (Fluka) in a sequence of mobile phases (results not given). In this way three flavonoid compounds were obtained: 1 (7 mg) and 2 (26 mg) in crystalline form and compound 3 (3 mg) — as an amorphous yellow sediment. Phenolic acids in the basic methanol extract were analysed after removal of flavonoids.

Qualitative analysis of flavonoids was based on determining the melting point temperatures for the compounds obtained and comparing them with various standards (TLC, HPLC — high preformance liquid chromatography and PC analysis). Moreover, spectroanalysis (UV — Pye Unicam 750 instrument and IR — Perkin Elmer 1600 SPE — IR) was carried out for compounds 1, 2 and 3.

The qualitative analysis of phenolic acids was carried out in the methanol extract, using cochromatography with standard substances (TLC, HPLC and PC). Qualitative and quantitative analysis of flavonoids and phenolic acids was carried out with the use of LiChrospher columns 100-RP 18 (250 mm x 4 mm, 5 μm) in room temperature, HPLC method (HPLC Knauer System/model 64-00, UV detector/model 87-00 fitted with Rheodyne RM-7125

valve with 20 ml loop, controlled by a computer/computer software HPLC Knauer, version 211A). Isocratic elution and the following mobile phases were used for flavonoids: F_1 — $H_2O:MeOH:CHCl_3:HCOOH$ = 78:6:15:1 (λ = 254 nm); for phenolic acids: F_2 — $H_2O:CH_3CN:H_3PO_4$ = 85:13,8:2 (λ = 280 nm).

Calibration for quantitative analysis:

Stock solutions of comp 1 (quercetin-3-O-glucoside), comp 2 (quercetin-3-O-galactoside), comp 4 (gallic acid) and comp 5 (protocatechuic acid) were prepared by dissolving 2 mg of these compounds in 10 ml of methanol. The volumes injected (20 μl) corresponded to ammounts of 1, 2, 4 and 5 in the range 1-4 μg. A calibration graph was obtained by plotting peak area (y) against concentration of standard solutions (x):

	regression equation	correlation coefficient
comp 1	y = 35.200 x - 0.080	r = 0.999
comp 2	y = 34.106 x - 0.333	r = 0.999
comp 4	y = 0.079 x + 0.020	r = 0.999
comp 5	y = 4.252 x - 0.020	r = 0.999

3. Results and Discussion

3.1. Analysis of growth of callus tissues

In this experiment the basic Zimmerman & Broome (2) (Table 1) medium was used in order to initiate the growth of callus cultures of *Vaccinium corymbosum var. Bluecrop*. The choice of this medium followed from publications describing its use in experiments involving micropropagation of Highbush blueberry (2,3). The content of plant growth regulators was modified in the basic Zimmerman & Broom medium through supplementing IAA with two auxins (NAA and 2,4-D). In order to obtain conditions for sufficient growth, other cytokinins (kinetin and zeatin) were used instead of 2iP (Table 2). The above modifications enabled callus formation of the explants inoculated to the medium, as opposed to stem regeneration typical of the original Zimmerman & Broome medium (2).

In the experiment, the best results in terms of callus tissue formation were achieved for low concentration (0,2 mgl^{-1}) of 2,4-D in the medium. Under these conditions (media I and II — see Table 2) 100% of the explants formed callus tissue in the 1st week of incubation. During the following 2 weeks, the callus tissue totally covered the initial stem sections on the above mentioned media. Unfortunately, the achieved tissue juvenility was very poor.

TABLE 1

CONTENT OF BASIC ZIMMERMAN AND BROOME MEDIUM IN mg/dm^3

Component	Amount mg per liter
NH_4NO_3	160.00
$(NH_4)_2SO_4$	198.00
$Ca(NO_3)_2 \times 4H_2O$	708.00
KNO_3	202.00
KH_2PO_4	408.00
$MgSO_4 \times 7H_2O$	370.00
$FeSO_4 \times 7H_2O$	55.70
Na_2EDTA	74.40
$MnSO_4 \times H_2O$	16.90
$ZnSO_4 \times 7H_2O$	8.60
H_3BO_3	6.20
KJ	0.83
$Na_2MoO_4 \times 2H_2O$	0.25
$CoCl_2 \times 6H_2O$	0.025
$CuSO_4 \times 5H_2O$	0.025
Myo-inositol	100.00
Adenine sulfate $\times$ $2H_2O$	80.00
Thiamine $\times$ HCl	0.40
IAA	4.00
2iP	15.00
Sucrose	30,000.00
Agar	5,500.00

The pH is adjusted to 4,8 before adding agar
IAA — indole-3-acetic acid
2iP — 6-γ-γ-dimethylaminopurine

The frequency of callus induction was decreased as 2,4-D concentration in the medium increased to 1,0 mgl^{-1} (Table 1). Similarly, NAA could be used to initiate callus tissue, but only at 1,0 mgl^{-1} concentration. However, with these parameters only 30% of the explants produced small callus which darkened quickly and could not be used as inoculum. Complete absence of callus production was noted at 0,2 mgl^{-1} concentration of this auxin in the medium.

While the experiment showed significant effect of the type and concentration of auxins, in the growth medium, on callus tissue production of *Vaccinium corymbosum var. Bluecrop*, the use of cytokinins did not seem to have any significance in this process.

TABLE 2
FREQUENCY OF EXPLANT INDUCTION OF *Vaccinium corymbosum var. Bluecrop*
IN RELATION TO THE COMPOSITION OF ZIMMERMAN & BROOME MEDIUM

No. of initial Zimmerman & Broome	Concentration of plant growth regulators in mgl^{-1} in Zimmerman & Broome medium				Percent of explant producing
medium	2.4-D	NAA	Kinetin	Zeatin	callus tissue
I	0.2	—	2.0	—	100%
II	0.2	—	—	2.0	100%
III	1.0	—	2.0	—	85%
IV	1.0	—	—	2.0	90%
V	—	0.2	2.0	—	—
VI	—	0.1	2.0	—	30%
VII	—	0.2	—	2.0	—
VIII	—	0.1	2.0	—	30%

In view of the low juvenility of callus tissue produced at the initial stage, further experiments aiming to optimise prolonged callus production used Schenk & Hildebrandt medium. The medium has been successfully used by the authors of this paper in cultivating callus cultures of other species in the *Ericaceae* family. The basic medium was modified in the experiment through varied content of plant growth regulators in order to determine the influence of phytohormones on tissue growth parameters (Table 3).

The values of the growth factor show that the complex made up of 0.5 mgl^{-1} 2,4-D and 0,2 mgl^{-1} of zeatin proved to best stimulate callus tissue growth (medium A — Table 3).

Unchanged content of cytokinin in the experiment makes it possible to propose, that 2,4-D most satisfactorily influenced the growth and juvenility of the callus. The other auxins (IAA and IBA) proved less advantageous. When IAA was used in the medium, not only callus growth but also its juvenility were highly unsatisfactory. Addition of IBA to the medium, to various concentration levels, slightly improved the quantity of biomass obtained, however, occurrences of roots on the surface of callus tissue were occasionally noted. The capability for root formation failed to disappear in the tissue within next subcultures.

It was also observed during the experiment, that the varying content of zeatin in the medium significantly modulated water content in the cultivated callus. Media with increased zeatin content (2,0 mgl^{-1}), regardless of the auxin used, produced tissue with notably lower water content (Table 3).

The experiment resulted in determining the composition for the SH medium which would condition prolonged and satisfactory growth of callus tissue of *Vaccinium corymbosum var. Bluecrop* (medium A Table 3). In 20 subcultures of homogeneous callus tissue grown onto the above medium, 313 g of dry biomass was obtained. Following the basic premise of this

TABLE 3

THE WATER CONTENT AND GROWTH FACTOR OF *Vaccinium corymbosum var. Bluecrop* CALLUS BIOMAS MAINTAINED ON SH MEDIUM WITH VARIOUS PLANT GROWTH REGULATORS (STATISTICAL ASSESSEMENT) (n = 15 tests)

Media number	Content of plant growth regulators in SH medium [mg^{-1}]		Growth factor (G_f) (avg. value X)	Standard deviation (±SD)	Variance significance level (p)	Content of water in tissues (avg.value X)	Standard deviation (±SD)	Variance significance level (p)
	2,4-D	Zeatin						
A	0.5	0.2	970.4	0.022	$>10^{-7}$	95.4%	0.036	>10-7
B	5.0	0.2	830.2	0.017	insignificant	98.2%	0.052	$>10^{-7}$
C	0.5	2.0	940.2	0.022	$>10^{-7}$	91.3%	0.054	>10-7
D	5.0	2.0	800.0	0.024	$>10^{-7}$	91.0%	0.033	>10-7
	IAA	Zeatin						
E	0.5	0.2	340.1	0.021	$>10^{-7}$	94.0%	0.019	>10-7
F	5.0	0.2	380.0	0.026	$>10^{-7}$	93.8%	0.028	>10-7
G	0.5	2.0	300.1	0.017	$>10^{-7}$	89.2%	0.030	insignificant
H	5.0	2.0	350.0	0.046	$>10^{-7}$	91.7%	0.027	insignificant
	IBA	Zeatin						
I	0.5	0.2	420.4	0.019	$>10^{-7}$	96.7%	0.019	>10-7
J	5.0	0.2	510.2	0.033	insignificant	97.2%	0.046	$>10^{-7}$
K	0.5	2.0	480.7	0.040	10^{-7}	91.8%	0.023	>10-7
L	5.0	2.0	530.2	0.029	10^{-7}	89.9%	0.027	>10-7

paper, the biological material obtained in this way underwent a phytochemical analysis to detect the presence of poliphenolic compounds (flavonoids and phenolic acids) in order to assess the ability of the obtained callus tissue to produce that certain group of secondary metabolites.

3.2. Assessment of *Vaccinium corymbosum var. Bluecrop* to produce selected poliphenolic compounds

No information has been found in the available publications as to the presence of flavonoids or phenolic acids in the naturally cultivated *Vaccinium corymbosum var. Bluecrop*. Therefore, the phytochemical analysis of the plant material in question was based on data related to the chemical composition of the basic species, *Vaccinium corymbosum*. Quercetin-3-O-glucoside detected in the fruit of this plant was also found and isolated (7 mg) in the callus tissue of *Vaccinium corymbosum var. Bluecrop* subcultured on optimised SH medium (medium A — Table 3). Quantitative HPLC analysis of this substance showed, that quercetin-3-O-glucoside (comp.1) is present in the analysed biomass in the amount of 0,0047% (Table 4). Much higher concentration (0,014%) of another flavonoid compound — quercetin-3-O-galactoside (comp. 2) was identified in the callus of *V. corymbosum var. Bluecrop*. It is worthy of note that these substances cannot be found in the naturally cultivated plant and is typical for the obtained tissue material. Moreover, it was noted that the analysed callus did not synthesise myricetin-3-O-glucoside, which was found in the fruit of naturally cultivated *V. corymbosum*. As for the third flavonoid compound found in the callus of *V. corymbosum var. Bluecrop*, its low content makes it only possible to say that it is an undetermined glicoside derivative of quercetin (Fig. 1).

TABLE 4

QUANTITATIVE HPLC ANALYSIS OF COMPOUNDS 1, 2, 4 AND 5 IN THE CALLUS CULTURES OF *Vaccinium corymbosum var. Bluecrop*

N° compound	Amount of compounds (% dry material) n = 15	Standard deviation (%)
1	0.0047	0.0215
2	0.014	0.0042
4	0.072	0.0284
5	0.0073	0.0127

Phenolic acids found in the obtained callus tissues also underwent phytochemical analysis. These compounds have not been analysed in the naturally cultivated plant. The qualitative analysis (TLC, HPLC) of the methanol extracts obtained from callus of *V. corymbosum* proved the presence of gallic

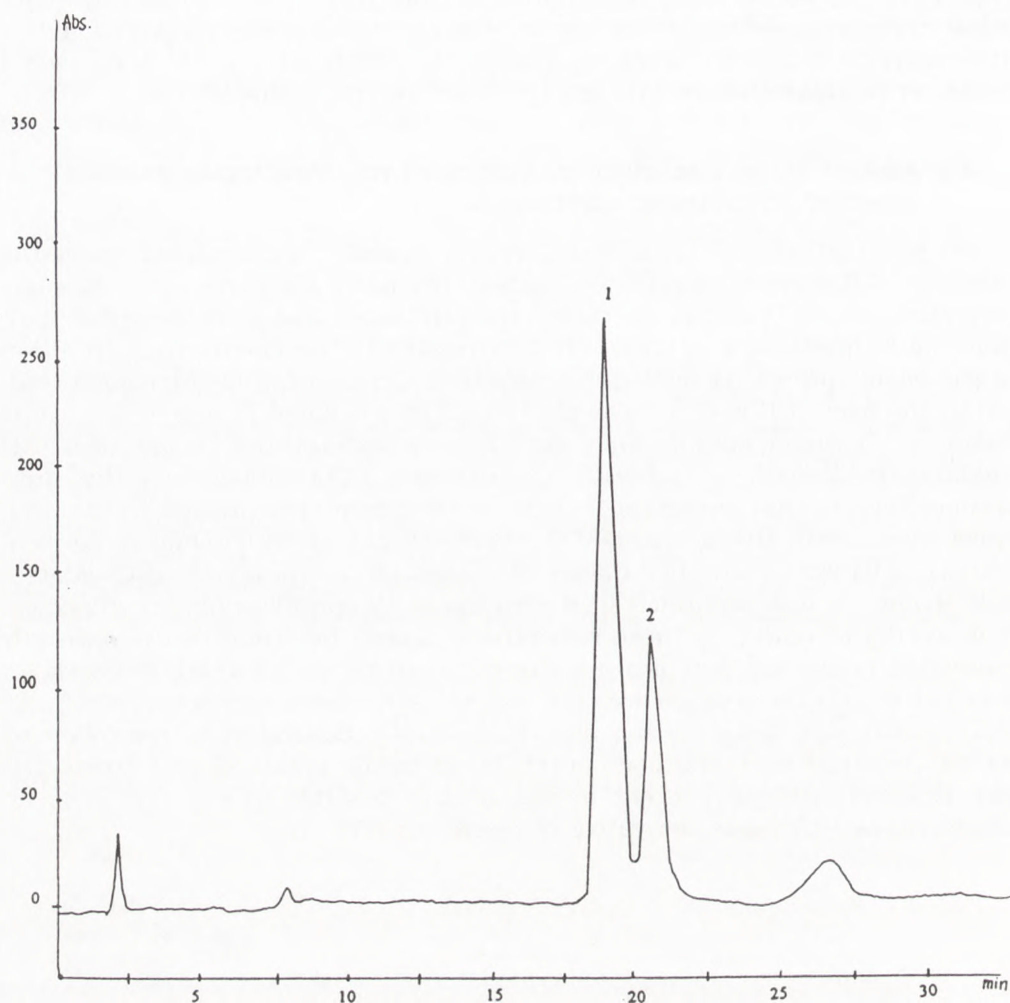

Fig. 1. HPLC of flavonoids from the callus culture of *Vaccinium corymbosum var. Bluecrop*. Peaks: 1 — quercetin-3-O-glucoside-comp 1; 2 — quercetin-3-O-galactoside — comp 2.

acid, protocatechuic acid, p-hydroksybenzoic acid, ferulic acid and p-coumaric acid in the callus material cultivated on optimised SH medium (Fig. 2). In addition, gallic acid (0,072%) and protocatechuic acid (0,0073%) were identified in the callus during the quantitative analysis (Table 4). These compounds were found to be dominating in the phenolic acids complex in the material.

The conditions for separation of quercetin-3-O-glucoside and 3-O-galactoside which were established during the research, were then used to determine the qualitative and quantitative composition of flavonoid compounds in callus

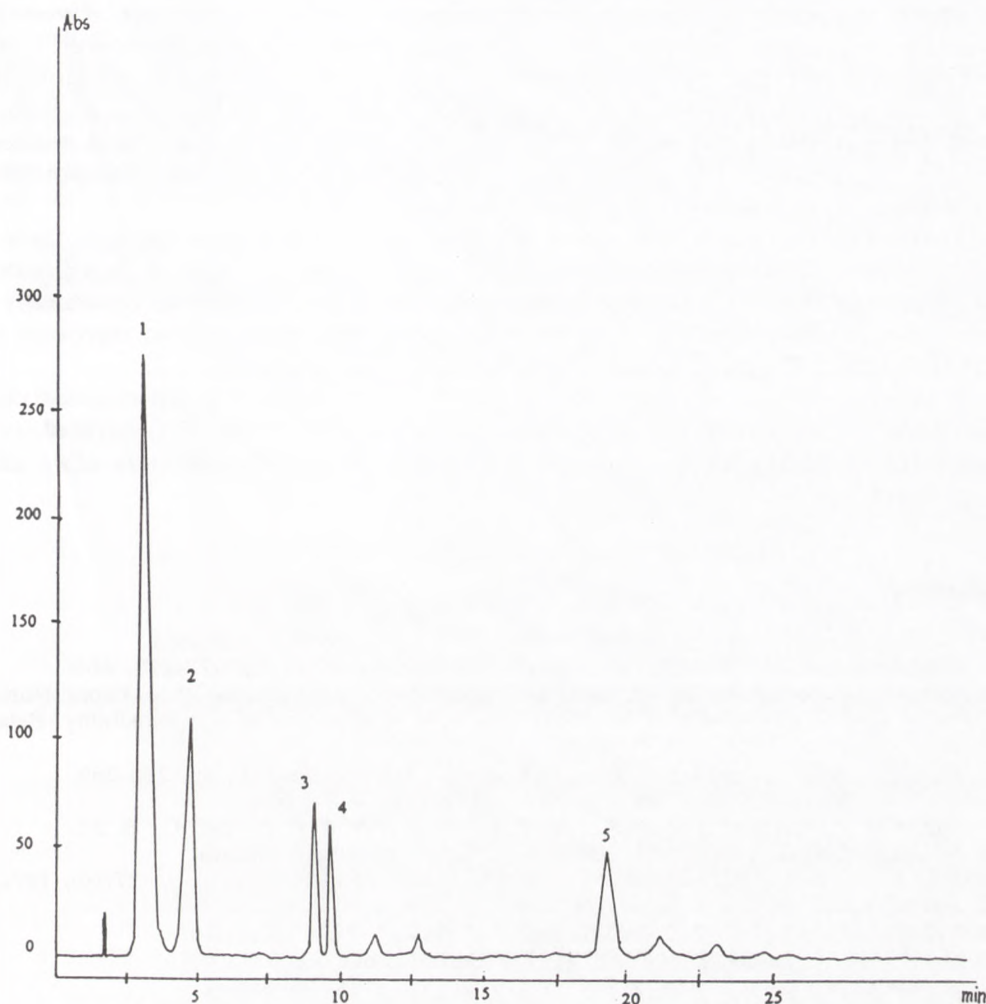

Fig. 2. HPLC of phenolic acids from the callus culture of *Vaccinium corymbosum var. Bluecrop.* Peaks: 1 — gallic acid — comp 4; 2 — protocatechiuc acid — comp 5; 3 — p-hydroksybenzoic acid — comp 6; 4 — ferulic acid — comp 7; 5 — p-coumaric acid — comp 8.

tissues cultivated on SH medium, enriched with various hormonal compositions (Table 3). This analysis was to establish whether varying addition of plant growth regulators had any influence on the qualitative and quantitative composition of the researched secondary metabolites in the callus.

The chromatographic analysis showed no qualitative differences between the flavonoid complex content in callus tissues subcultured on SH media with different content of plant growth regulators (Fig. 1). The variance of the quantitative content of the researched compounds was not statistically significant to indicate influence of any given medium (results not given).

Phytochemical analysis of callus tissues of *V. corymbosum var. Bluecrop* which was to determine the content of poliphenolic compounds proved that the tissue material cultivated *in vitro* can synthesise significant amounts of the secondary metabolites researched here. Especially in the case of flavonoid compounds the callus of *V. corymbosum var. Bluecrop* can be a source of both quercetin-3-O-glucoside and 3-galctoside; both of these compounds have interesting pharmacological properties (9).

Furthermore, taking into account the high value of the growth factor together with high productivity of quercetin-3-O-galactoside, a compound not found in the naturally cultivated plant, the tissue material obtained *in vitro* may be considered to be a new source of materials rich in flavonoids, created artificially as a result of biotechnological research.

It is also worthy of note, that at the present stage of *in vitro* research, no impact of growth regulating substances present in the SH medium on biosynthesis of flavonoid compounds was shown in callus tissues of *V. corymbosum var. Bluecrop.*

Literature

1. Podbielkowski Z., (1985), *Słownik roślin użytkowych*, PWRiL, Warszawa.
2. Zimmerman R. H., Broome O. C., (1979), Hort. Science, 14 (3), 477-478. Abst.
3. Zimmerman R. H., Broome O. C., (1980), *Bluberry micropropagation*, Proc. Cronf. Nursery Prod. Fruit Plants Through Tissue Culture — Applications and Feasibility, Betsville, 44-47.
4. Ballinger W. E., Eleandor P. J., (1970), J. Am. Soc. Hortic. Sci., 95, 283-385.
5. Bilyk A., Sapers G. M., (1986), J. Agric. Food Chem., 34, 585.
6. Buthiene Z., Buthus U., (1987), Liet. TSR Mosklu Akad. Darb. Ser. C., 3, 23.
7. Rejman A., Pliszka K., (1984), *Borówka wysoka*, PWRiL, Warszawa.
8. Beyeler S., Testa B., Perrissoud D., (1988), Biochemical Pharmacology, 37(10), 1971-1979.
9. Spilková J., Hubik J., (1988), Pharmazie in unserer Zeit, 17(1), 1-9.
10. Schenk R. V., Hildebrandt A. C., (1972), Can. J. Bot., 50, 199.
11. Zenkteler M.,(1984), *Hodowle komórek roślinnych*, PWN, Warszawa.
12. Borkowski B., Miłkowska K., (1996), Herba Polonica, XLII (2), 118-126.
13. Cisowski W., Dembińska-Migas W., Łuczkiewicz M., (1993), Polish J. Chem., 67, 829.
14. Mabry T. J., Markham K. R., Thomas M. B., (1970), *The systematic identification of flavonoids*, Springer-Verlag, Berlin-Heidelberg-New York.

Conditions for efficient induction and maintenance of callus cultures *Vaccinium corymbosum var. Bluecrop* and its accumulation of polyphenolic compounds

Summary

Zimmerman & Bromme and Schenk & Hildebrandt media with unchanged content of plant growth regulators in the basic medium, were used to develop the optimum conditions for efficient production of callus tissue of *Vaccinium corymbosum var. Bluecrop*. It has been determined that, of the auxins used, it was 2,4-D which has the most beneficial effect on callus tissue growth.

Zeatin was found to regulate the water content of the tissue obtained. Quercetin-3-O-glucoside, as well as quercetin-3-O-galactoside were identified and analysed quantitatively in the callus tissue from *in vitro* cultures. Moreover, the following phenolic acids were identified in the callus with the use of chromatography: ferulic acid, p-hydroxybenzoic acid, p-coumaric acid, as well as gallic acid and protocatechuic acid which were also analysed quantitatively in the researched tissue material. Phytochemical monitoring of the propagated tissues for different production media (HPLC analysis) did not show any relationship between the plant growth regulators used and the analysed secondary metabolites.

Key words:
Quercetin-3-O-galactoside, Quercetin-3-O-glucoside, modified media, growth factor, HPLC, *Ericaceae*.

Address for correspondence:
Wojciech Cisowski, Katedra i Zakład Farmakognozji, Akademia Medyczna, Al. Gen. J. Hallera 107, 81-416 Gdańsk-Wrzeszcz.