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OBTAINING, TESTING AND VALUATION OF SOME VEGETABLE EXTRACTS FROM MEDICINAL SPECIES

OBTINEREA, TESTAREA SI EVALUAREA UNOR EXTRACTE VEGETALE DIN SPECII MEDICINALE

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Rezumat. *Tehnicile de cultivare in vitro a ţesuturilor şi celulelor vegetale sub influenţa fitohormonilor, aplicate speciilor medicinale, devin metode uşoare şi rapide de inducţie şi selecţie a metaboliţilor valoroşi. Se ştie că sub influenţa condiţiilor de cultură artificială se induc variaţii somatice la nivelul materialului biologic cultivat. Cercetarea noastră şi-a propus să evalueze variabilitatea somatică prin testarea conţinutului de polifenolici şi antioxidanti la liniile tisulare de castravete amar (Momordica charantia) selectate in vitro. De asemenea, am testat capacitatea antioxidantă, conţinutul de proteine şi antociani ale unor linii tisulare de afin (Vaccinium myrtilus) obtinute de la populaţiile locale. Rezultatele noastre conduc la concluzia că echilibrul hormonal şi genotipul influenţează puternic variabilitatea proprietăţilor biochimice la nivelul liniilor tisulare selectate in vitro. Acestea reprezintă un material biologic foarte valoros pentru obţinerea produselor de biosinteză cu utilizări farmacologice.*
Cuvinte cheie*: Momordica charantia, Vaccinium myrtilus linii celulare, continut antioxidant si antocianic*

Abstract. *In vitro cultivation techniques of vegetable tissues and cells under the influence of phytohormones, applied to medicinal species, become easy and fast methods of induction and selection of valuable metabolites. It is known that under the influence of the artificial culture conditions, somatic variations at the level of the cultivated biological material are inducing. Our research aimed to evaluate the somatic variability by testing the polyphenolic and antioxidant content on the tissue lines of bitter cucumber (Momordica charantia) selected in vitro. We also tested the antioxidant capacity, protein and anthocyanic content of some blueberries (Vaccinium myrtilus) tissue lines provided from local populations. Our results lead to the conclusion that hormonal balance and genotype strongly influence the variability of biochemical properties at the level of tissue lines selected in vitro. These represent a very valuable biological material for obtaining biosynthesis products with pharmacological uses.*
Key words*: Momordica charantia, Vaccinium myrtilus tissue lines, antioxidant, anthocyanic content*

Introduction

Momordica charantia L. is a medicinal plant successfully used as an alternative therapy for diabetes for a long time, and as an antiviral and antineoplastic agent (Agarwal, Kamal, 2004; Basch, et al., 2003). The productivity and the content of the interested metabolites could be improved using biotechnological methods. One of the most important metabolites is protein P, a polypeptide with molecular weight of about 11,000 Dalton consisting of 166 amino acids. We evaluated the antioxidants and poliphenols content and the protein fingerprint from the selected bitter cucumber tissue lines, to demonstrate the somatic variability induced in *Momordica charantia* tissue culture under *in vitro* conditions influence.

The bilberry plants have therapeutic importance as: hypoglycemic, hypotensive, antiseptic, cholagogue, vasoprotectives of capillaries (eye, brain, peripheral), antidiarrheal, antioxidant (Martz et. al, 2010). The reported results highlighted the fact that, although in a number of species cells grown in vitro synthesize higher amounts of secondary metabolites than intact plants, there are still numerous problems determined by the instability of cell lines, low yields, low growth rate and the industrialization of production (Poiana at al., 2008; Guo et al.,2022).

Materials and methods

We used *Momordica charantia* cotyledonal calluses, sub-cultivated *in vitro* in three successive cycles, on liquid or solid medium to obtain sufficient quantities of biological material for analysis (Agarwal, Kamal, 2004; Malic et al., 2007). Tissue lines and production of metabolites was done on Murashige-Skoog solid medium (MS), with two auxines: naphthaleneacetic acid (NAA), indoleacetic acid (IAA) and two citokinines: benzylaminopurine (BAP) and kinetin (KIN), combined in five different experiments (Table 1).

Table 1. Hormonal variants to *Momordica charantia* calluses

Fitohormon	Cantitatea mg/l				
	V1	V2	V3	V4	V5
Nphthaleneacetic acid (NAA)	0,5	1	1,5		
Indoleacetic acid (IAA)				1	
Benzylaminopurine (BAP)	0,5	1	1		1
Kinetin (KIN)				1	1

Ten cell lines, obtained with different hormonal variants, were tested for total antioxidant capacity (TAC) using FRAP method (Strain, 1999) and total phenol content using Folin-Ciocalteu method (Ianculov et al., 2010). Absorption determination for FRAP and total phenol content was made using SmartSpec spectrophotometer by Bio-Rad.

Because local blueberry populations (*Vaccinium myrtilus*) are a valuable biological material in terms of active principles, we studied *in vitro* behavior and the possibility of inducing and selection of useful somatic variations in producing secondary metabolites, in three genotypes of blueberries (local populations from central-western Romania). Caluses obtained *in vitro* (in six subcultures) on the WPM (Woody Plant Medium) environment under the influence of the 1.5 mg/l ANA + 1.5 mg/l BAP and three different concentrations of adenosine sulfate (AS) (40 mg/l; 60 mg/ L and 80 mg/l AS) were selected for the proliferative capacity and the synthesis of anthocyanins in the populations of Arișeni, Retezat and Valea Sebeșului. At the level of the selected tissue lines, the antioxidant activity, total protein, total anthocyanins were studied.

Determining total antioxidant capacity. The extracts obtained from calus and foliar tissues from the mother plants were used. To determine the antioxidant capacity of these extracts, we used a quick method, modified by Szőllősi and Varga, (2002), the FRAP method (Feeric Reducing Antioxidant Power).

The total protein content for selected tissue lines from the three blueberries was determined by the Lowry method (Ianculov, Filimon, 2003). The data obtained from these determinations were still used to calculate the enzymatic activity. The calculation relationship applied to obtain the values corresponding to the total protein content: total protein content (mg/ml) = (E x 400)/(0.001985 x 1000), where:

E = extinction in 675 Nm; 400 = the degree of dilution; 0.001985 = calibration curve;

1000 = factor for transforming values from μg into mg.

Determination of the total content of anthocyanins by spectrophotometry. Anthocyanic compounds were extracted from blueberry samples using an acidified methanol solution 1% hydrochloric acid, at 4 ° C. The centrifugal extract has been analyzed at variable wavelength spectrophotometer (UV-Viz 5 Thermo Spectronic) in the 350-700 Nm field, identifying the wavelength for which the absorbance is maximum (λ = 525 Nm). The total anthocyanins expressed as Cianidin-3-glucoside were determined, using the molar extinction coefficient 2.69 x 104 according to the formula: C (mg/g) = ($\bar{A}$ x M x 103) / (ε x 104 x G). For Cianidin-3-glucoside the molecular mass and the extinction molar coefficient are: M = 449,2; ε = 2.69 x 104 (Silva et al., 2017).

Results and discussions

1. Results regarding polyphenolic and antioxidant content on the tissue lines of bitter cucumber (*Momordica charantia*)

In the conditions of subcultivation of the cotiledonar calus of bitter cucumber under the influence of the various hormonal combinations, on liquid or solid medium we selected ten tissue lines. For each line the antioxidant capacity and the phenols content were determined (Table 2).

Table 2. Antioxidant capacity and polyphenols content for different lines of *Momordica charantia*

	Samples	Antioxidant capacity TAC (µmol Fe^{2+}/g)	Phenols (µmol gallic acid/g)	Sample position in the gel
1	Callus MS liquid 0,5 ANA+ 0,5 BAP	1.75	2.19	1
2	Callus MS solid 0,5 ANA+ 0,5 BAP	1.96	2.28	10
3	Callus MS liquid 1 ANA+ 1 BAP	0.69.	2.83	2
4	Callus MS solid 1 ANA+1 BAP	2.63	4.01	3
5	Callus MS liquid 1,5 ANA+ 1 BAP	1.80	2.89	8
6	Callus MS solid 1,5 ANA+1 BAP	2.75	4.12	9
7	Callus MS liquid 1 BAP+1 KIN	1.35	3.32	4
8	Callus MS solid 1 BAP+1 KIN	1.62	3.53	5
9	Callus MS liquid 1 AIA+ 1 KIN	2.14	2.57	6
10	Callus MS solid 1 AIA+ 1 KIN	2.25	2.70	7
	Cotiledon control	*1.12*	*2.38*	

The antioxidant capacity was higher for all of the cell lines, compared to the cotiledon control. The highest antioxidant capacity (TAC) and polyphenols values were identified for the cell lines induced on solid medium supplemented with ANA and BAP. A high antioxidant capacity was obtained on the hormonal balance AIA and KIN and a high phenolic content on the BAP and KIN medium. The comparison between all of the experimental variants pointed out that the cultivation on solid medium increased the total antioxidant capacity and also the phenolic content due to a better oxigenation of the plant tissue.

The size for each protein fraction was evaluated using the ratio (Rf) between the migration distance for the band and the buffer solution, compared with the molecular marker. For all of the analyzed cell lines seven bands were visualized, with sizes between 18000 and 110000 Da (F1-F7).

Depending on the presence (1) or absence (0) of the polymorphic bands the matrix of similarities was established and the dendrogram was drawn using the cluster method (Table 3; Fig. 1).

The first cluster was compsed from the cell lines R1 and R10, with a total similarity; these lines being produced with the same hormonal balance (ANA and BAP), with the same concentrations, on liquid respectively solid medium. The values for the antioxidant capacity and phenolic content were slightly higher on solid medium compared with the liquid one. In the same cluster the cell lines R8 and R9 were present, showing a difference of 15%. They were produced on 1.5 mg/l ANA and 1 mg/l BAP hormonal balance, on liquid respectively solid medium. Their antioxidant capacity and phenolic contents were higher on solid medium, with maximum values compared with all of the other experimental values.

Table 3. The similarities coefficients between calluses

Regenerant	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
R1	**1.00**	0.71	0.85	0.42	0.42	0.71	0.57	0.71	0.85	1.00
R2	0.71	**1.00**	0.85	0.71	0.71	0.71	0.57	0.42	0.57	0.71
R3	0.85	0.85	**1.00**	0.57	0.57	0.57	0.42	0.57	0.71	0.85
R4	0.42	0.71	0.57	**1.00**	1.00	0.71	0.57	0.42	0.28	0.42

R5	0.42	0.71	0.57	1.00	**1.00**	0.71	0.57	0.42	0.28	0.42
R6	0.71	0.71	0.57	0.71	0.71	**1.00**	0.85	0.71	0.57	0.71
R7	0.57	0.57	0.42	0.57	0.57	0.85	**1.00**	0.57	0.42	0.57
R8	0.71	0.42	0.57	0.42	0.42	0.71	0.57	**1.00**	0.85	0.71
R9	0.85	0.57	0.71	0.28	0.28	0.57	0.42	0.85	**1.00**	0.85
R10	1.00	0.71	0.85	0.42	0.42	0.71	0.57	0.71	0.85	**1.00**

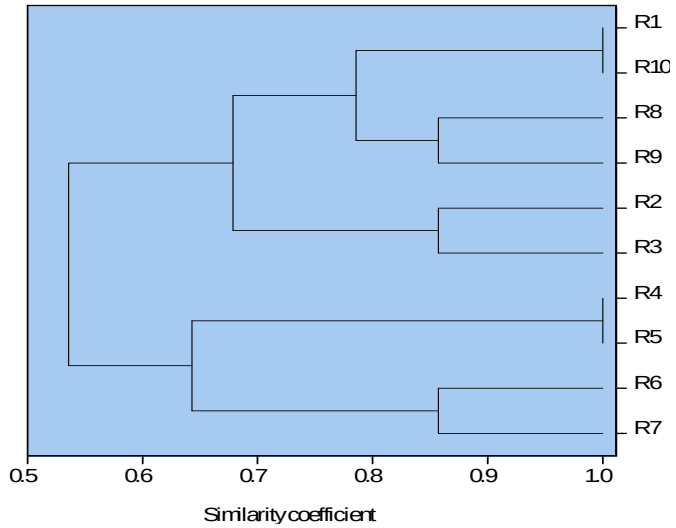

Fig. 1. URGMA clustering of the *Momordica charantia* callus

The cell lines R2 and R3 with a 85% similarity formed the second cluster and they had a total difference of aproximately 30% compared to the first cluster group components. These lines were produced on ANA 1 mg/l and BAP 1mg/l hormonal balance, on liquid and solid medium. The differences between the culture systems were very high.

2. Experimental results regarding the evaluation of total anthocyanin contents in *Vaccinium myrtilus* calluses

The tissue lines selected from three local populations (Arieseni, Retezat and Valea Sebeșului) were analyzed for the total content of anthocyanins expressed as cyanidin a 3-glucoside equivalents (C3GE), compared to control samples (leaves, shoots). These lines were selected from the callus culture on solid WPM medium, under the influence of the hormonal balance 1.5 mg/l ANA with 1.5 mg/l BAP and three different concentrations of AS (40, 60 and 80 mg/l).

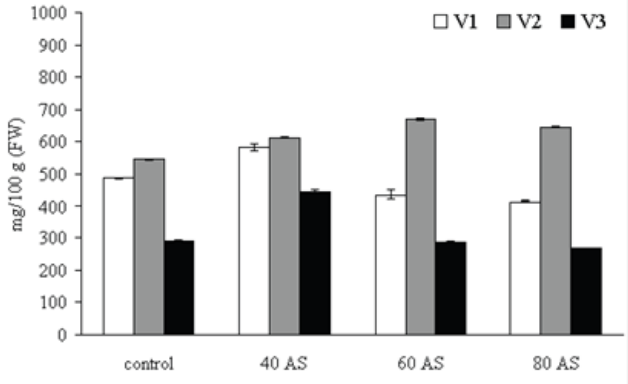

Fig. 2. Experimental results on the total anthocyanin content (mg C3GE / g) of the bilberry calluses on solid WPM medium for the population Arieșeni (V1), Retezat (V2) și Valea Sebeșului (V3)

It was found that, depending on the genotype, the anthocyanin content could transiently increase in the Retezat population (V2) as a function of AS concentration. The analysis of the callus composition and the control samples shows remarkable differences between the Retezat population and Valea Sebeșului, strengthening the belief that the genotype significantly influences the synthesis of anthocyanins. And the concentration of AS favorably influences this character. The Arieseni and Valea Sebeșului populations had higher values in terms of anthocyanin content on the medium supplemented with 40 mg/l AS, compared to the control. The selected tissue lines produce higher amounts of anthocyanins compared to control samples, in all three populations, as a result of AS intake. The values obtained by us were consistent with those obtained by other researchers for total anthocyanin content expressed as cyanidin 3-glucoside equivalents (C3GE) (Stevenson, Scalzob et al., 2012).

Determination of total protein content by the Lowry method

Selected tissue lines from the three populations were evaluated for total protein content compared to controls (young shoot and leaf blade fragments taken from blueberry plants from the three areas).

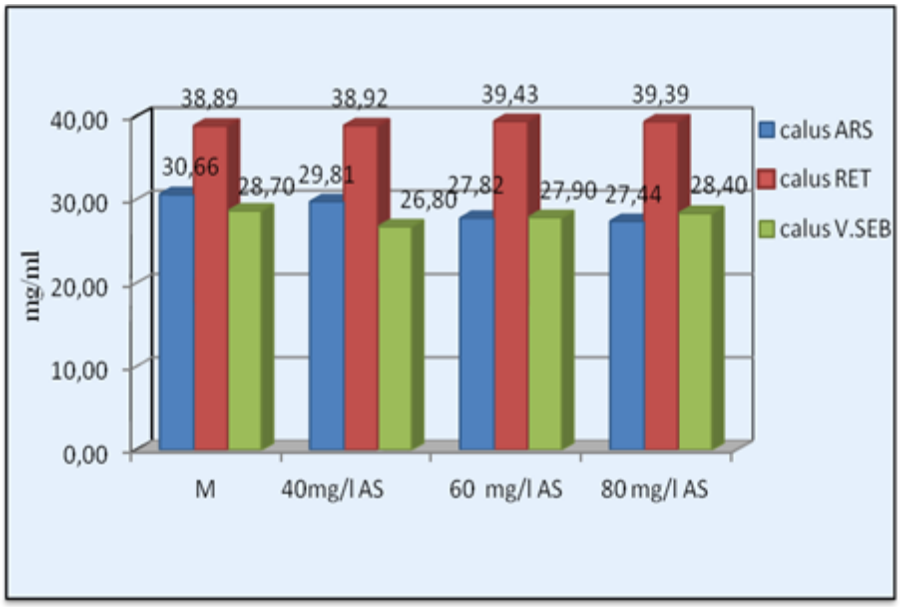

Fig. 3. Total protein content (mg/ml), to the level of the tissues lines selected from the Arieșeni population (V1), Retezat (V2) and Valea Sebeșului (V3), raported to the martor (leaf and shoots fragments)

The tissue lines selected from the Retezat population recorded superior values compared to those obtained from the control, for all three concentrations of AS added to the WPM culture medium with 1.5 mg/l ANA and 1.5 mg/l BAP. For the concentration of 60 mg/l AS applied to the culture medium, the value obtained in terms of protein content was maximum, namely 39.43 mg/ml (fig. 3). These results demonstrate that genotype strongly influences total protein content, and concentrations of 60 mg/l AS and 80 mg/l AS are favorable for protein accumulation.

Experimental results regarding the evaluation of antioxidant capacity using FRAP method.

In the Arieșeni, Retezat and Valea Sebeșului populations, good results were obtained regarding the callus growth capacity in the subculture and the synthesis of anthocyanin pigments (red callus).

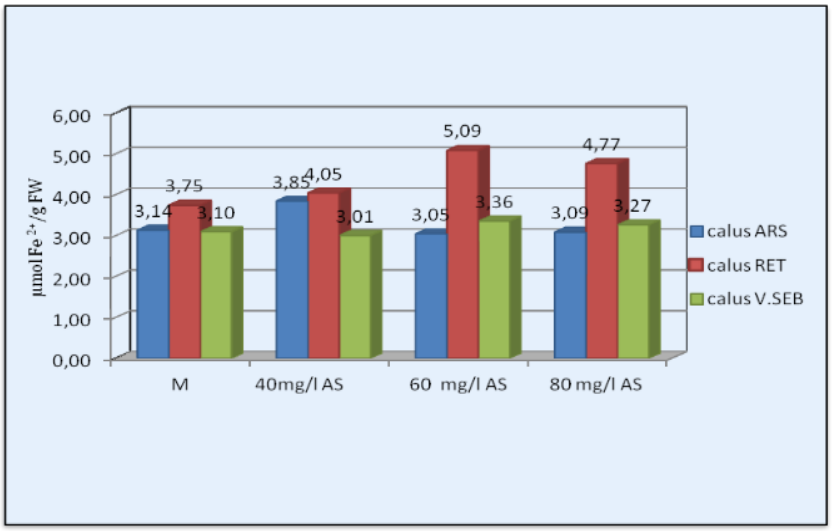

Fig. 4. The antioxidant capacity of tissue lines selected from the population of Arieseni (V1), Retezat (V2)and Valea Sebeșului (V3), compared to the control (mother plant)

Significant differences between populations in terms of antioxidant capacity were recorded in the case of the application of the hormonal variant 60 mg/l AS (Fig. 4). The tissue line from the Retezat population cultivated on WPM medium with 1.5 mg/l ANA + 1.5 mg/l BAP + 60 mg/l AS, presented the highest antioxidant capacity (5.8 µmol Fe2+/g) compared with the rest of the population.

In this study, the limits of the values recorded for the antioxidant capacity (3.78-5.8 µmol Fe2+/g) were consistent with the values reported by Simões et al. (2012): 7.41-13.69 µmol Fe2+/g from fruits and with those reported by other researchers (Bunea et al., 2011).

Conclusions

The antioxidant capacity was higher for all of the cell lines, compared to the cotiledon control, emphasizing the advantages of the cell cultures for the metabolites producing.

The highest antioxidant capacity (TAC) and polyphenols values were identified for the cell lines induced on solid medium supplemented with ANA and BAP.

The cells cultivated on solid medium had a superior biosintetic capacity for the antioxidants and phenolic compunds, compared with the liquid medium.

Blueberry callus culture becomes an alternative method of producing plant material rich in anthocyanins and mineral salts, which can be used as a source of food supplements.

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