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Variability study of the some biochimical traits at *Momordica charantia* cell lines

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Abstract

The aim of this paper was the study of the somatic variability sources, innducing in Momordica charantia cell culture, by the evaluation of antioxidants and polyfenols content and proteic fingerprint. Based on correlation between UPGMA proteic clustering of the bitter melon cell lines and their antioxidants and polyfenols content, we can conclude that the phytohormons have a strongly influence on variability of polyfenolic and antioxidant content. Hormonal balance ANA + BAP showed the most biosynthesis potential of this active substances and superior level of proteic fraction polymorphism.

Keywords : antioxidants and polyfenols content, proteic fingerprint, *Momordica charantia* cell lines

Introducere

Momordica charantia L. is a medicinal plant originating from the tropical Asian area, successfully used as an alternative therapy for diabetes for a long time, and recently proposed as an antiviral and antineoplastic agent (Basch, Gabardi, & Ulbricht, 2003). In our country's temperate climate conditions the plant yield is low, but the productivity and the content of the interested metabolites could be improved using biotechnological methods. One of the most important metabolites is protein P- insulin, a polypeptide with molecular weight of about 11,000 Dalton consisting of 166 amino acids. Clinical study study revealed that the polypeptide-p-ZnCl2 produced blood sugar lowering effect (Khanna and Mohan, 1973) reported that besides the fruits, p insulin was also found in seeds and tissue cultures of *Momordica charantia*.

One of the most important biotechnological techniques is *in vitro* culture which allows the cultivation of cells and tissues from very different plant species to obtain cell mass (callus) which can be used as a source of secondary metabolic products and to induce specific morphogenetic processes. Unlimited proliferative potential and the high informational plasticity of the plant somatic cells are important traits underlying the totipotency expression for *in vitro* culture.

It is known that a plant cell callus consists of somatic undifferentiated cells with a homogeneous histological structure. This tissue could be a valuable biological material due to its genetic instability and the polyploidization possibility under certain culture conditions and it could be the basis of different cell lines with special traits and even plants regeneration.

The aim of these paper was to establish the factors which induce the somatic variability in *Momordica charantia* cell culture through the evaluation of the antioxidants and poliphenols content and the protein fingerprint for the selected cell lines.

Material and method

In vitro culture

As biological material *Momordica charantia* cotyledonal calluses were used, sub cultivated *in vitro* in three successive cycles to obtain sufficient quantities of biological material for analysis (Agarwal, Kamal, 2004; Malic, Zia, Riaz-ur-Rehman, Chaudhary, 2007).

The proliferation of undifferentiated tissue and production of metabolites was done on Murashige-Skoog medium (MS), both liquid and solid, with two auxine: naphthaleneacetic acid (NAA), indoleacetic acid (IAA) and two citokinine: benzylaminopurine (BAP) and kinetin (KIN), combined in five different experiments (Table 1).

Table 1 Hormonal variants

Fitohormon	Cantitatea mg/l				
	V1	V2	V3	V4	V5
Naphthaleneacetic 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 and total phenol content using Folin-Ciocalteu method. Absorption determination for FRAP and total phenol content was made using SmartSpec spectrophotometer by Bio-Rad.

Evaluation of total antioxidant capacity (TAC)(FRAP method adapted)

Reagents: Acetate buffer: 300mM/L, pH 3.6 (3.1g sodium acetate $3H_2O$ and 16 ml conc. acetic acid per 1L of buffer solution); 10mM/L TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM/L HCl; 20mM/L $FeCl_36H_2O$ in distilled water. FRAP working solution: 25ml acetate buffer, 2.5mL TPTZ solution and 2.5 mL $FeCl_3$ solution. The working solution must be always freshly prepared. Aqueous solution of known Fe (II) concentration was used for calibration, in a range of 0.1-1.0 mM/L. For the preparation of calibration curve 1ml aliquot of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 μM/mL aqueous Fe (II) as Mohr salts solution were mixed with 5 ml FRAP working solution; FRAP reagent was used as blank. The absorption was read after 10 min. at 25 °C at 593 nm, 1cm lights path, and the calibration curve was drawn. One ml from diluted 1/10 plant ethanolic extracts was mixed with the same reagents as described above, and after 10 min. the absorption was measured. All determinations were performed in triplicate. Total antioxidant capacity in plant methanol extracts in Fe (II) equivalents was calculated. Correlation coefficient (r^2) for calibration curve was 0.959 (Strain, 1999).

The amount of phenolic compounds

The following reagents were used: 2.0M Folin-Ciocalteu phenol reagent, gallic acid and anhydrous carbonate. The content of total phenolic compounds in plant ethanolic extracts was determined by Folin-Ciocalteu method (1927) (**3**). For the preparation of calibration curve 1ml aliquot of 0.16, 0.32, 0.60, 1.20, 2.0, 2.8 μM/ml aqueous gallic acid solution were mixed with 10 ml Folin-Ciocalteu reagent (diluted ten-fold) and 9 ml (7.5%) sodium

carbonate. The absorption was read after 2 h at 20 °C at 750 nm and the calibration curve was drawn. One ml from diluted 1/10 plant ethanolic extracts was mixed with the same reagents as described above, and after 2 h the absorption was measured for the determination of plant phenolics. All determinations were performed in triplicate. Total content of phenolic compounds in plant ethanol extracts in gallic acid equivalents (GAE) was calculated. Correlation coefficient (r^2) for calibration curve was 0.975 (Folin and Ciocalteu, 1927).

The protein analysis using polyacrilamide gel electrophoresis

The method SDS-Poliacrylamide Gel Electrophoresis (SDS-PAGE) was used for protein variability identification

The following reagents were used: 40% acrylamide; 0.39% bisacrylamide; running buffer 121.14 g Tris in 1000 ml distilated water, pH 8.8; loading buffer: 141.1 g glicină, 30 g Tris, 10 g SDS, distilated water to 1000 ml, pH 8.3; 10% SDS; 10% APS; tetramethylethylenediamine (TEMED), 2% tricloracetic acid; 1% Coomasie Brilliant Blue; staining solution: 55g tricloracetic acid, 65 ml acid acetic glacial, 180 ml ethanol in 25 ml solution.

The analysed samples consisted of 15µl fresh extract obtained from 1g of triturated callus or suspension cells. For protein separation a 10 % acrylamide gel was used: 8 ml – acrylamide 40%, 10 ml bisacylamide 0.39%, 0.32 ml SDS 10%, 12 ml running buffer, pH 8.8; 0,8 ml APS 10% and 16 µl TEMED.

The gel run at room temperature, 200 V, was fixed in trichloroacetic acid solution 2% and was stained with Coomassie Brilliant Blue (Schägger H, von Jagow G., 1987).

Results and discussions

The *Momordica charantia* cotyledonal calluses, obtained in the first experiments were sub-cultivated on liquid or solid medium, supplemented with different hormonal balances. Thus, it was possible to select ten cell lines which were analyzed further on.

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*	

In general 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 protein fractions, extracted from the selected ten cell lines were separated in 10% polyacrilamide gel (Fig. 1). To evaluate the molecular weight the „Broad Range Protein Molecular Weight Marker" (Promega) was used.

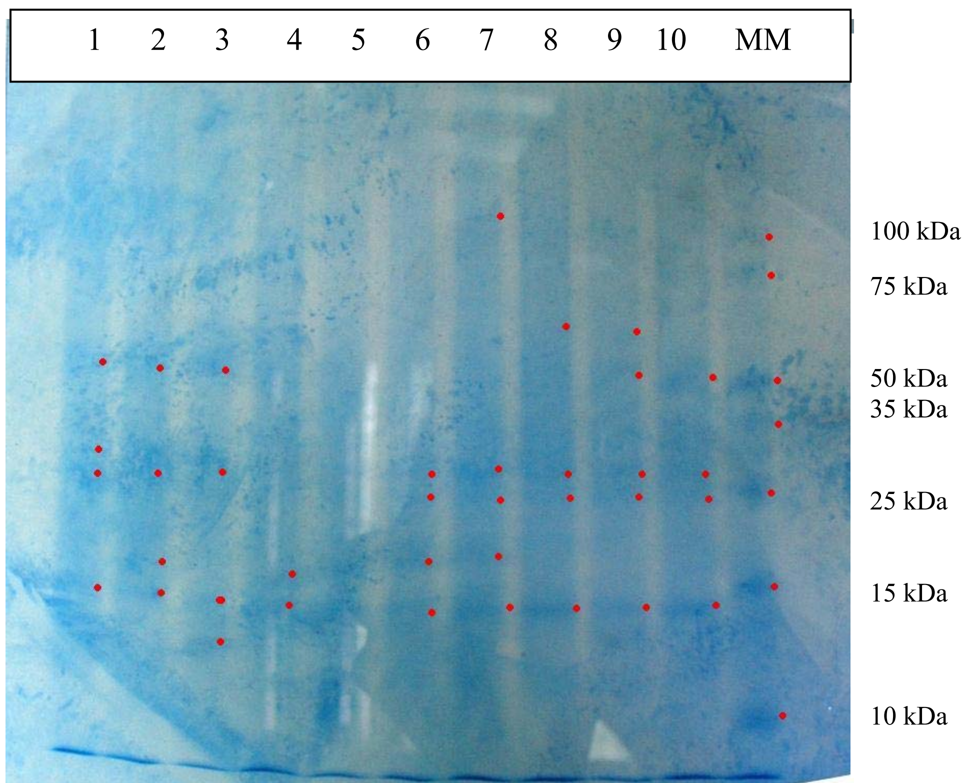

Fig. 1 The PAGE analysis for the 10 *Momordica charantia* cell lines

1	Callus MS liquid 0,5 ANA+ 0,5 BAP
2	Callus MS liquid 1 ANA+ 1 BAP
3	Callus MS solid 1 ANA+1 BAP
4	Callus MS liquid 1 BAP+1 KIN
5	Callus MS solid 1 BAP+1 KIN
6	Callus MS liquid 1 AIA+ 1 KIN
7	Callus MS solid 1 AIA+ 1 KIN
8	Callus MS liquid 1,5 ANA+ 1 BAP
9	Callus MS solid 1,5 ANA+1 BAP
10	Callus MS solid 0,5 ANA+ 0,5 BAP
MM	molecular marker - *Broad Range Protein Molecular Weight Marker* (Promega)

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). The presence or the absence of each band was scored for each cell line (Table 3) (presence – 1; absence -0)

Table 3 The results of scoring bands per individual and per locus

The protein fraction	Molecular weight (MM) Da	Cell line									
		R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
F1	110000	0	0	0	0	0	0	1	0	0	0
F2	64000	0	0	0	0	0	0	0	1	1	0
F3	54000	1	1	1	0	0	0	0	0	1	1
F4	34000	1	0	0	0	0	1	1	1	1	1
F5	31000	1	1	1	0	0	1	1	1	1	1
F6	20000	0	1	0	1	1	1	1	0	0	0
F7	18000	1	1	1	1	1	1	1	1	1	1

Of the seven proteic bands visualized on the gel, six were polymorphic and one was monomorphic (18000 Da), resulting a polymorphism rate of 85.71%. The lower frequency was observed for the 110000 Da and 64000 Da bands. The total polymorhism (PIC- polymorphic index content) presented values ranging from 0.180 for 110000 Da fraction and 0.500 for the 20000 Da one with an average value of 0.383 (**Table 4**). Total value of the polymorphism generated by the protein fingerprint was 2.300.

Table 4 Polymorphism (PIC) and frequency of proteic bands (p_i) in the studied *Momordica charantia* cell lines

	Proteic bands (Da)							Average
	110000	64000	54000	34000	31000	20000	18000	
p_i	0,100	0,200	0,500	0,600	0,800	0,500	1.000	0.529
PIC	0,180	0,320	0,500	0,480	0,320	0,500	0.000	0.383

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 5; Fig. 2).

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.

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.

Table 5. The similarities coefficients between regenerants

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**

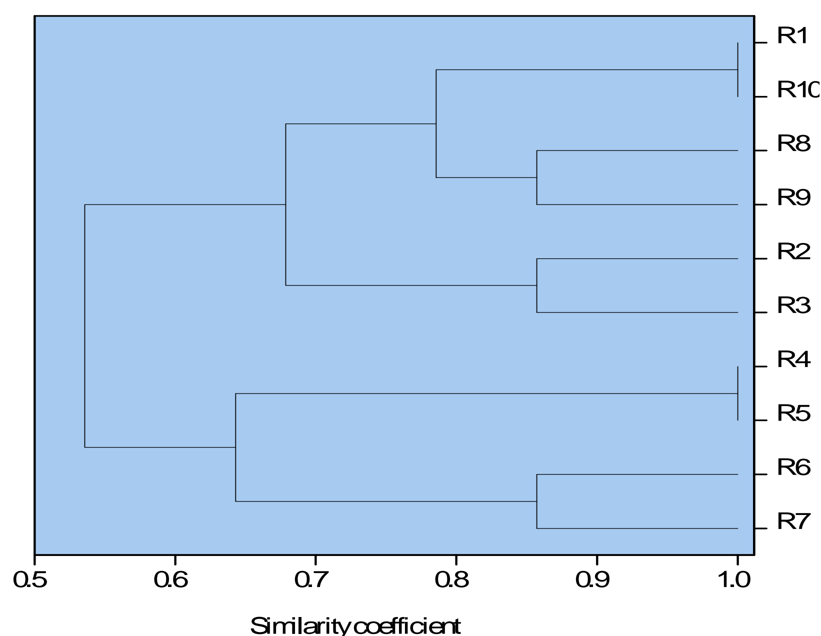

Fig. 2 URGMA clustering of the *Momordica charantia* regenerants

In the third cluster the cell lines R4 and R5 (BAP 1 mg/l and KIN 1 mg/l) were included, with a total similarity, toghether with the cell lines R6 and R7 (AIA 1 mg/l and KIN 1 mg/l) from which a difference of 35% was pointed out. The cell lines from the third cluster presented a similarity of aproximately 52.5% compared with the other analyzed cell lines. Regarding the phenolic content it turned out that the association of the both citokinins determine a high capacity of the phenol biosynthesis and reduce the antioxidant capacity.
An equilibrate synthesis of the both compunds was obtained in AIA and KIN presence.

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.

The cell lines produced on medium suplemented with ANA and BAP had a similar protein fingerprint due to close protein synthesis processes.

The kinetin presence induced different fingerprints, the cell lines being included in a different cluster.

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References

1. AGARWAL M., KAMAL R., *In vitro* clonal propagation of *Momordica charantia* L. , Indian Journal of Biotechnol., 3, (2004), p.426-430.
2. BASCH, E., GABARDI, S., & ULBRICHT, C. *Bitter melon (Momordica charantia): A review of efficacy and safety.* American Journal of Health-System Rharmacy, 60(15), (2003), 356–359
1. FOLIN, O. AND CIOCALTEU, V., *On tyrosine and tryptophane determination in proteins*, Journal of Biological Chemistry, **27**, (1927), 627-650.
2. KHANNA R., MOHAN S., *Isolation and identification of diosgenin and sterols from fruits and in vitro cultures of Momordica charantia Linn.* Indian Journal of ExRerimental Biology, (1973) 11: 58-60
3. MALIC S., ZIA M., RIAZ-UR-REHMAN., CHAUDHARY F., *In vitro plant regeneration from direct and indirect organogenesis of Momordica charantia* L., Pakistan Journal of Biological Sciences, 2007, ISSN 1028-8880.
4. SCHÄGGER H, VON JAGOW G., *Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa*., Anal Biochem. 166 (2): (1987), 368–379
5. STRAIN, J.J., Ferric Reducing/ Antioxidant Power Assay: Direct Measure of Total antioxidant Activity of Biological Fluids and Modified Version for Simultaneous Measurement of Total Antioxidant Power and Ascorbic Acid Concentration. Methods in enzymology. vol. 299: 1999, 15-23. FRAP Assay