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CHEMOPREVENTIVE FUNCTIONAL FOOD THROUGH SELENIUM BIOFORTIFICATION OF CAULIFLOWER PLANTS

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ABSTRACT: The aim of this work was to develop a biotechnological approach for production of cauliflower as safe functional food, with an optimal content of chemopreventive compounds, by a protective biofortification, through selenium application together with betaine and spraying adjuvants. In the control and treated cauliflower plants we determined the amount of total selenium, glucosinolates (sulforaphane) and SAH (S-Adenosyl-homocysteine). We also assayed the chemopreventive effects of compounds formed in the treated cruciferous plants through *in vitro* tests, using human colorectal tumor cell line (CaCo2). Extracts of plants treated with selenium applied together with betaine and spraying adjuvant were significantly more active on reduction of tumoral cell viability than the extract of control plants. Cauliflower plants, obtained after our treatments for protective biofortification, were used to feed rabbits, for 10 days. The ingestion of biofortified cauliflower did not modify the hematological and biochemical parameters on the laboratory animals.
Keywords: cauliflower, selenium, biofortification, chemopreventive compounds, functional food

INTRODUCTION:

Cruciferous vegetables have been proved to determine beneficial effect on human health and were considered among the main class of functional food (Kaur and Das, 2011). These beneficial effects on the health of human subjects are determined by their high content on vitamins (Dominguez-Perles et al., 2014; Singh et al., 2007), phenolic antioxidants (Podsędek, 2007; Singh et al., 2006), mineral nutrients with high bioavailability (Jahangir et al., 2009), but especially on sulfur compounds, particularly glucosinolates (GLS), (Ishida et al., 2014; Sarikamis, 2009). Epidemiological evidence has associated the frequent consumption of cruciferous vegetables with decreased cancer risk (Jeffery and Keck, 2008; Murillo and Mehta, 2001). Isothiocyanates, degradation products of glucosinolates, which occur naturally in a variety of cruciferous vegetables, are some of the most bioactive components of cruciferous vegetables, which were correlated with chemopreventive effects / cancer decrease (Gupta et al., 2014; Hayes et al., 2008; Juge et al., 2007; Sarikamis, 2009).

In this work, we propose a selenium protective biofortification technology for cruciferous crops, in order to obtain functional food containing safe and constant level of selenium and *Brassicaceae* chemopreventive compounds. Selenium is known for its chemopreventive effects (Costantini et al., 2011; Hatfield et al., 2014; Zeng and Combs, 2008). Also, selenium biofortification was shown to determine physiological modifications in treated plants, which lead to an increase of edible yield quality (Malagoli et al., 2015). However, selenium have a very narrow physiological windows, the difference between the recommended daily human dietary intake for chronic diseases prevention and that producing pathophysiological effects being very small (Rayman, 2012; Rocourt and Cheng, 2013; Wrobel et al., 2016). Our protective biofortification biotechnology thus has also a practical relevance for public health, related to supplementation of the food chain with safe selenium levels. We have tested the development and application of some products as new inputs in the selenium protective biofortification of cruciferous plants, by which to re-balance the sulfur metabolism, maintaining the formation of the optimal level of chemopreventive selenocompounds (Oancea et al., 2015a). The proposed technology – treatments with selenium salt, spraying adjuvants and betaine – was applied on a cauliflower crop, because it is a well-studied cruciferous vegetable regarding health effects, including those linking cauliflower-containing diets to cancer prevention (Ambrosone and Tang, 2009; Higdon et al., 2007). In order to prove the character of safe and efficient functional food of vegetables derived from our protective biofortification technology, we used multiple *in vitro* tests, which were validated through an experiment of feeding laboratory animals (rabbits) with plant products derived from cruciferous vegetables protective biofortified with selenium.

MATERIALS AND METHODS:

Plant material and experimental site. The experiment was done on the experimental field of Ecofruct Srl, Stefan-cel-Mare, and Călărași County, Romania, located at 40° 59 'N latitude, 27°40' E longitude and 54 m altitude. The averages values of multi-annual temperature, wind speed, sunshine daily duration and total precipitations for this site are: 11.5°C, 3.5ms−1, 6.8 h and, respectively, 504 mm. Soil on the experimental site is a calcaric kastanic chernozem, developed on a loess parental rock, with an average total selenium content in the upper soil horizon (0-20 cm) of 67 µg/kg, lower with almost 40% than the

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average soils content considered as unaffected by Se deficiencies (Lăcătuşu et al., 2010).

Cauliflower crop (*Brassicaceae Family / Cruciferae - Brassica oleracea L. Botrytis group* cv. Adelanto F1) was established by seedling transplantation. Transplanting was done in rows at a distance of 70 cm between rows and 25 cm between plants in the row, according to the cultivation technology for cauliflower. In the experimental field we practiced drip irrigation, in accordance to calculated evapotranspiration. The soil was fertilized with inorganic fertilizers (N – 160 kg/ha, P – 120 kg/ha, K – 120 kg/ha), applied 5 days before cauliflower seedlings transplantation. The climatic conditions for 2015 period of cauliflower vegetation were characterized by higher monthly temperatures (+1.3°C in May; +0.4°C in June; +2.7°C in July) and lower monthly precipitations (-31.5 mm in May; -22.7 mm in June; -34,9 mm in July) than the average multi-annual.

Treatments application. Two treatments were carried out, with six different variants of products. Treatments were applied by foliar spraying, using an applicator with nozzle pump adjusted so as to distribute about 30 mL of treatment solution per plant. Experimental treatment variants (V1-V6) were randomly distributed in the experimental field.

Solutions used for treatment of cauliflower crop in field:

- V1 –10 µM sodium selenate (Sigma) in distilled water;
- V2 - 10 µM sodium selenate and 1% spraying adjuvant (obtained by SC Tesospec SRL);
- V3 - 10 µM sodium selenate, 1% spraying adjuvant and 1 mM betaine (Sigma);
- V4 - 5 µM sodium selenate (Sigma) in distilled water;
- V5 – 1% spraying adjuvant in water;
- V6 - 1% spraying adjuvant and 1 mM betaine

The spraying adjuvant which we used in our experimental treatments was produced from rapeseed oil, by trans-ethylation with an excess of ethanol, in the presence of potassium hydroxide / potassium ethanoate, neutralization of excess alkali with oleic acids, and addition of lecithin.

Plant extracts. Freshly harvested cauliflower (1 g) was homogenized in 4 ml acidified water, pH 6, at 45 °C, for 2.5 h. Sulforaphane was extracted by addition of 20 ml dichloromethane and incubation at room temperature, for 1 h and then, was purified using solid-phase extraction (Campas-Baypoli et al., 2010). The extract was evaporated to dryness, in vacuum, using a rotary evaporator (Heidolph VV Micro, Germany) and the residue was dissolved in 1 ml acetonitrile. For S-Adenosyl-homocysteine (SAH) extraction, fresh plant samples (5 g) were subjected to two-step procedure, first with 5 ml methanol, precooled at -20 °C, then with 5 ml isopropanol, precooled at -20 °C (Nikiforova et al., 2005). The obtained supernatants were further combined and filtered through 0.22 µm membrane filter.

HPLC analysis of glucosinolates and S-Adenosyl-homocysteine (SAH) was carried out on a HPLC Agilent 1200 system with diode array detector. Prior to injection into HPLC apparatus, the samples were filtered through 0.45 µm PTFE membrane filter. Their separation was performed in Zorbax XDB C18 column (150 x 4.6 mm i.d.), at flow rates of 0.6 mL/min and 0.2 mL/min, respectively and elution under isocratic conditions, using water/acetonitrile gradient mobile phase (Struys et al., 2000). Their detection was carried out at 202 nm and 258 nm, respectively, and their peak retention times were compared with those obtained for sulforaphane and S-Adenosyl-homocysteine (SAH) external standards (Sigma Aldrich). Calibration curves were built in the range of 5-100 µg/mL for sulforaphane and 2.5-50 µg/mL for SAH and were used to quantify their concentration in cauliflower plant extracts.

Selenium content was determined in dried plants after calcination at 450 °C. The analysis was performed by vapor-generation atomic absorption spectroscopy of the hydrogen selenide formed after boron hydride reducing procedure. The results were reported as µg/g dry weight (d.w.) of sample.

In vitro antitumoral activity. Caco-2 human colon adenocarcinoma cell line was obtained from ECACC. Cells were grown in MEM medium supplemented with 2 mM glutamine, 1% non-essential amino acids, 10% fetal bovine serum (FBS) and 1% antibiotics mixture (penicillin, streptomycin, and neomycin) and incubated at 37 °C in a humidified atmosphere of 5% CO_2. For cytotoxicity tests, cells were seeded in a 96-well plate, at a concentration of $5x10^4$ cells/ml. After 24 h of cultivation in standard conditions, the medium was replaced with fresh medium containing V1-V6 treated plant extracts and control plant extract, in 100–2500 µg/ml range of concentration and the plates were cultivated in an incubator, for 24 h and 72 h, respectively. Sample cytotoxicity was evaluated by MTT assay, as previously described (Craciunescu et al., 2007) and cell morphology was observed at an inverted phase light microscope (Nikon).

In vivo tests on laboratory animals. Cauliflower treated with V3 variant was introduced in laboratory animals (rabbits breed New Zealand) feeding ratio. For 10 days, the rabbits received cauliflower in food (between 50 and 200 g/day), in order to determine the maximum quantity tolerated/animal. All animal experiments were performed according to the code of animal welfare ethics and did not involve killing animals, but only monitoring their progress and collecting of blood samples, in which hematological and biochemical parameters were determined. Animals were separated by sex, housed in air-conditioned rooms, at a temperature of 22 ± 2 °C and a relative humidity of 65 ± 5%. Water was provided at the discretion and ¼, ½ and ¾ of the calculated demand in diet was replaced with fresh cruciferous vegetable material, mixed with regular concentrated feed. Hematology was performed at Autohematology Analyzer equipment, Mindray model BC-2800 VET, Serie RR 92100358 by counting of white blood cell, lymphocytes, monocytes, granulocytes, total hemoglobin and hematocrit. Biochemical tests for liver (transaminases GOT and GPT) and kidneys (uric acid,

creatinine and urea) were performed with Reflovet Plus equipment, Serial No. 6001045, at Spiru Haret Faculty of Veterinary Medicine clinic. These parameters were considered relevant for biological activity of vegetal functional food (Liu et al., 2009)/.

Statistics. Three replicates were used for all experiments. The results were presented as mean ± standard deviation (SD). Significant statistic differences were considered for $p<0.05$ after performing Student's *t*-test on paired samples. . The Excel software (Office 365 - Excel 2016, Microsoft, Redmont, WA, USA) was used to make calculations and to draw figures.

RESULTS AND DISCUSSION:

At three and six weeks after cauliflower crop setting, the plants were treated with six different variants of experimental biostimulant mixtures (V1-V6), and after another 3 weeks, they were harvested (Figure 1). The obtained production of treated cauliflower was compared with the untreated plants one (control). The results are presented in Table 1.

Fig. 1. Different stages of cauliflower plants from the experimental treatments

Table 1.

Production of cauliflower plants treated with the six variants of biostimulant mixtures

Variant of applied treatment	Production (t/ha)
Control (untreated plants)	22.19 ± 1.42
Treatment V1, 10 µM sodium selenate (Sigma) in distilled water	22.71 ± 0.88
Treatment V2, 10 µM sodium selenate and 1% spraying adjuvant	22.75 ± 1.27
Treatment V3, 10 µM sodium selenate, 1% spraying adjuvant and 1 mM betaine	23.06 ± 2.08
Treatment V4, 5 µM sodium selenate (Sigma) in distilled water	22.87 ± 1.32
Treatment V5, 1% spraying adjuvant in water	22.74 ± 1.73
Treatment V6, 1% spraying adjuvant and 1 mM betaine	22.75 ± 1.17

We have observed no damages produced by the applied treatments on cauliflower plants. The obtained results showed that the applied treatments have caused a slight increase in production, which demonstrates the biostimulant character of the used mixtures. These results are in accordance with those obtained for cabbage plants (from the same *Cruciferous vegetables* family as cauliflower), treated with the same mixture of selenium salt, spraying adjuvants and betaine (Oancea et al., 2015b). In plants treated with selenium, there can be noticed an overuse of the metabolic pool of S-Adenosyl-methionine, a common compound of the metabolic pathways of selenium and sulfur in plants (Gao et al., 2015; Lyi et al., 2007; Matich et al., 2012). The added betaine acts as an osmoprotectant (Ashraf and Foolad, 2007), being in the same time donor of methyl groups, for recovery of methionine from homocysteine (Baburina and Shevyakova, 1995). The treatment solutions were applied by foliar spraying. In order to improve the foliar absorption, we have used spraying adjuvants (ethylic esters of fatty acids from rape with additional ethanol, glycerol, potassium oleate, lecithin), products that are designed to increase the penetrability of plant hydrophobic cuticle.

Samples of V1-V6 treated cauliflower plants were analyzed for determination of selenium, sulforaphane (as degradation product of glucosinolates) and S-Adenosyl-homocysteine (SAH) amounts. The obtained results are presented in Table 2.

Table 2.

Total selenium, sulforaphane and S-Adenosyl-homocysteine (SAH) content in cauliflower samples from field crop

Experimental treatment	Total selenium (µg/g d.w.)	Sulforaphane (µg/g d.w.)	SAH (µg/g d.w.)
Control (untreated plants)	0.153 ± 0.007*	185.12 ± 7.53	2.95 ± 0.14
Treatment V1, 10 µM sodium selenate (Sigma) in distilled water	0.169 ± 0.008*	213.43 ± 9.10*	3.88 ± 0.22*
Treatment V2, 10 µM sodium selenate and 1% spraying adjuvant	0.188 ± 0.008*	274.52 ± 9.25*	4.86 ± 0.19*
Treatment V3, 10 µM sodium selenate, 1% spraying adjuvant and 1 mM betaine	0.117 ± 0.006*	159.28 ± 6.34*	4.55 ± 0.24*
Treatment V4, 5 µM sodium selenate (Sigma) in distilled water	0.112 ± 0.006*	188.44 ± 7.94	2.99 ± 0.16
Treatment V5, 1% spraying adjuvant in water	0.095 ± 0.005	180.25 ± 8.11	2.85 ± 0.18
Treatment V6, 1% spraying adjuvant and 1 mM betaine	0.091 ± 0.005	187.36 ± 8.42	3.04 ± 0.15

*p<0.05, compared to distilled water treated plant extract (control)

Analyzing these results, we have concluded that the best treatment variant is V3 (10 µM sodium selenate, 1% spraying adjuvant and 1mM betaine), which allows an increase of chemopreventive compounds amount in treated plants. This conclusion sustain our initial controlled conditions findings (Oancea et al., 2015a), that betaine could compensate Se effects on sulfur metabolism, maintaining the level of sulforaphane in treatments applied on cauliflower plants. Equilibrate formation of selenium and sulfur bioactives in *Brassica* crops treated with this Se-based biostimulant composition could provide better characteristics as functional food for this vegetable. The protective and stimulating effects of biofortification with Se were also indicated in previously studies on cereals and vegetables (Feng et al., 2013; Hanson et al., 2003).

In order to demonstrate the chemopreventive action of the compounds formed in cauliflower crops treated with the experimental mixtures, we have used *in vitro* tests in tumor cell culture (Caco-2 human colon adenocarcinoma cell line). We have tested concentrations of cauliflower extracts in the range of 100-2500 µg/mL and their cytotoxicity was assessed by MTT assay, after 72 h of cultivation. Comparing the values obtained for each extract, we have observed that the highest antitumoral activity was exhibited by V3 extract (obtained from plants treated with 10 µM sodium selenate, 1% spraying adjuvant and 1 mM betaine) (Figure 2).

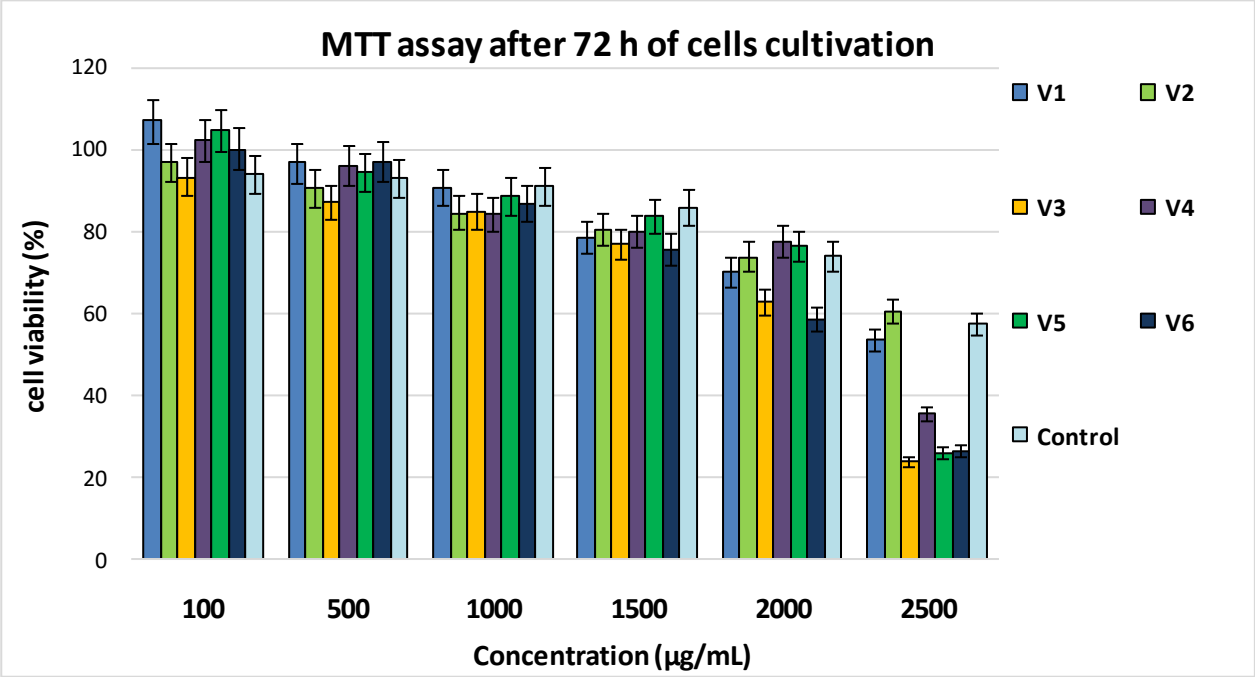

Figure 2. Caco-2 adenocarcinoma cell viability after cultivation in the presence of cauliflower plant extracts, for 72 h, evaluated by MTT assay. Values are expressed as mean of three determinations ± SD and reported to the control plant extract, considered 100% viable.

For V3 treatment, the calculated cell viability was 76.91% for 1500 µg/mL cauliflower extract concentration, 62.78% for the concentration of 2000 µg/mL and 23.97% for the concentration of 2500 µg/mL. Hematoxylin–Eosin staining of the tumoral cells cultivated in the presence of 2500 µg/mL of cauliflower extracts obtained from plants treated with V1-V6 treatment mixtures allowed observations of cell morphology and proliferation (Fig. 3). For V3, V5 and V6 variants, it was evidentiated a low cell density and cells with modified morphology, presenting round and multinucleated cells with granular cytoplasm (Fig. 3 B-D).

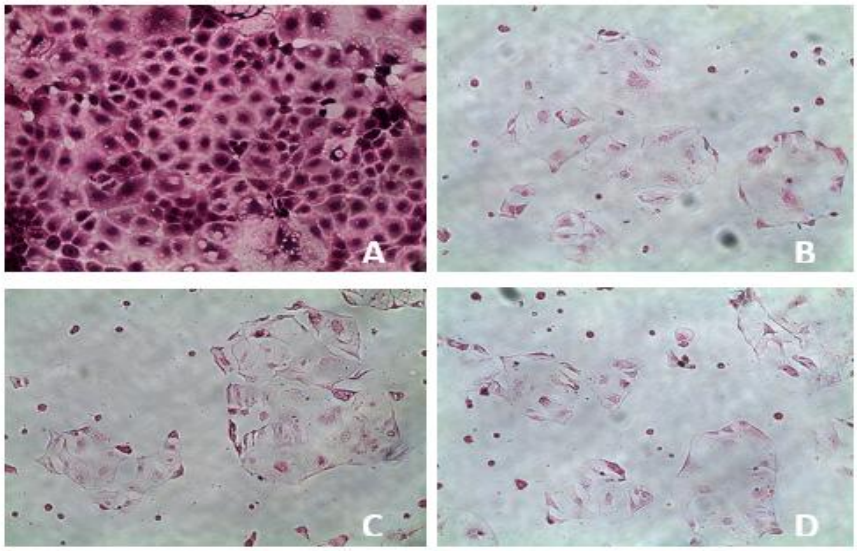

Figure 3. Morphology of Caco-2 tumoral cells cultivated in the presence of 2500 µg/mL extracts of cauliflower treated with V3 (B), V5 (C) and V6 (D), for 72 h. Control (untreated) cell culture presented a normal morphology (A) (Hematoxylin–Eosin staining, x10)

The cytotoxicity evaluation of cauliflower extracts obtained from treated plants on Caco-2 tumoral cell line showed that these extracts have an antitumoral effect. The most efficient variant for cell proliferation inhibition was V3. Our results are in accordance with previous studies demonstrating that some compounds (i.e., sulforaphane) from *Brassicaceae* plants could inhibit the proliferation in human colon cancer cell lines (Pappa et al., 2006).

Preliminary *in vivo* tests performed in order to demonstrate the functional food qualities of cauliflower plants treated with biostimulant mixtures were made on New Zealand rabbits, according to the code of animal welfare ethics. Animals were divided in 3 groups, each of 10 rabbits. Each group received cauliflower treated with V3 mixture variant in diet, as follow: group 1 – 50 g/day, group 2 –100 g/day and group 3 –200 g/day, during 10 days. After this period, blood samples were collected from each rabbit and hematological and biochemical parameters were determined. The results are presented in Table 3.

Table 3

Hematological and biochemical parameters of lab animals receiving biostimulated cauliflower plants in their regular diet, for 10 days

	Hematological parameters					
	WBC	**Lymph**	**Mon**	**Gran**	**HGB**	**HCT**
Reference range	5.2-13.5 x 10^9/L	3.2-9.0 x 10^9/L	0.1-0.6 x 10^9/L	2.0-7.5 x 10^9/L	105-170 g/L	31.0-46.0 %
Animal group						
Group 1 (50g/day)	6.1	2.5	0.2	4.3	110.5	37.7
Group 2 (100g/day)	9.2	2.6	0.3	6.2	120.6	42.6
Group 3 (200g/day)	8.0	3.2	0.3	4.4	119.3	41.9
	Biochemical parameters					
	GOT	**GPT**	**Creat**	**Urea**		
Reference range	6.7-54.2 U/L	8.2-32.2 U/L	<=1.8 mg/dL	<=54 U/L		
Animal group						
Group 1 (50g/day)	15.7	35.4	0.99	53.0		
Group 2 (100g/day)	28.0	35.0	1.38	41.4		
Group 3 (200g/day)	22.1	13.9	1.35	37.4		

Results represent the average of three determinations for each animal.

During the experiment, we have observed that animals did not lose weight and showed no clinical signs of disease. Results from table 3 showed that no major alterations occur in rabbits blood parameters. We can conclude in this experiment stage that cauliflower introduced in rabbits daily diet do not modify their health status.

CONCLUSIONS:

We have elaborated a new biostimulant composition based on selenium, spraying adjuvants and betaine for foliar application on cauliflower plants and we have established its optimal concentration that allows to obtain good yields. We have determined that the optimal treatment variant (10 µM sodium selenate, 1% spraying adjuvant and 1 mM betaine) determine, as a consequence, the accumulation of chemopreventive compounds (glucosinolates and SAH). The treated

plant extracts were tested *in vitro* on tumoral cell line, in order to demonstrate their chemopreventive action and our results have indicated that the cauliflower V3 extract had the most pronounced antitumoral activity at a concentration of 2500 µg/mL. Testing the same V3 treated cauliflower plants *in vivo* on laboratory animals, we have observed no major changes in their blood composition and health status.

We can conclude that cauliflower treated with V3 biostimulant composition, based on selenium, can be considered a functional food, due to its increased chemopreventive compounds content.

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