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SELENIUM BIOFORTIFICATION TREATMENT OF CAULIFLOWER ENHANCES THEIR CONTENT IN CHEMOPREVENTIVE COMPOUNDS AND *IN VITRO* ANTITUMORAL ACTIVITY

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Abstract

Cruciferous vegetables are known as food with chemopreventive effect due to their high content in bioactive compounds, such as mineral nutrients, including selenium, antioxidants, vitamins and glucosinolates, which were shown to inhibit cancer cell growth, both in vitro and in vivo testing. The aim of this study was to evaluate the effect of a new selenium-based composition, applied on experimental field conditions to cauliflower plants (Brassica oleracea L.)*, on their chemopreventive compounds level and antitumoral activity. Treated plants, cultivated both in normal watered and water stress conditions, were compared for total selenium and sulforaphane contents, determined by electrothermal atomic absorption spectrometry (ETAAS) and high performance liquid chromatography (HPLC), respectively. In vitro cytotoxicity of cauliflower extracts was evaluated in NCTC fibroblast cell line, while their antitumoral activity was tested in Caco-2 human adenocarcinoma cell line using MTT colorimetric assay. The results indicated that the applied biofortification treatments increased the selenium intake, allowed formation of bioactive glucosinolates and enhanced the antitumoral activity of cauliflower plants cultivated in both normal watering and water stressed conditions. In conclusion, this new biotechnological approach on cauliflower cultivation, using a treatment with a novel selenium-based composition, could be considered promising step for obtaining functional food from cauliflower crops.*

Key words*: antiproliferative activity, cauliflower, glucosinolate, selenium, sulforaphane.*

INTRODUCTION

Selenium (Se) is an important element for human and animal nutrition because it plays critical roles in a variety of physiological processes (Rayman, 2012). Statistical studies on human subjects have revealed constant correlations between the physiological response, determined by the expression of major selenoproteins and seleno-chemopreventive compounds, and the risk of mortality from chronic diseases, including cancers (Bleys et al., 2008; Rocourt and Cheng, 2013). The dietary intake of Se on a specific area is determined by the mean value of Se in soil. Worldwide such value is 383 ± 255 µg/kg, when not affected by deficits or excesses (Kabata-Pendias and Pendias, 2001).

In Romania, Se level in soil stands at the deficit limit. Various pathologies caused by Se deficiency were reported for animals from different regions of the country (Serdaru et al., 2003; Lăcătușu et al., 2012). Compared to the international known mean value, the Se content is reduced with 30% - 63% in different regions of Romania (Lăcătușu et al., 2010; Lăcătușu et al., 2012). A low level of Se in soil reduces Se dietary intake and indicates the need of supplementation, to achieve the optimal level of Se, beneficial for reducing the risk of

chronic diseases (Mehdi et al., 2013; Steinbrenner et al., 2013). However, Se supplementation treatment for a better human health shall be related also to the very narrow Se physiological window, wherein the difference between the recommended daily human dose for prevention of chronic diseases and the dose producing pathophysiological effects is very small (Oancea et al., 2014).

Selenium agronomic biofortification, i.e. Se-treatment applied during plants cultivation period, have been considered an effective solution for producing functional foods, beneficial for both animal and human health. Such biotechnology applied to largely consumed vegetables show several advantages, like supplementation by controlled levels of highly bioavailable seleno-compounds and a wider availability to different categories of people at risk of chronic diseases, including those with low income (White and Broadley, 2009; Fageria et al., 2012).

On the other hand, Se biofortification treatments allow the valorization of Se protective and stimulatory effects on plants (Feng et al., 2013). Experimental studies have shown that Se is a beneficial microelement for plants, stimulating their growth (Hartikainen and Xue, 1999; Sajedi et al., 2011) and playing a role in plant protection against infestation caused by insects or phytopathogenic agents (Hanson et al., 2003), oxidative stress (Xue et al., 2001) and hydric stress (Wang et al., 2011). It was reported that Se-based treatments applied to plants have also improved their response to drought stress, involving both water and oxidative stress (Kuznetsov et al., 2003; Yao et al., 2009). Due to this protective effects against biotic and abiotic stresses selenium was included among inorganic compounds acting as plant biostimulants (Du Jardin, 2015).

Consumption of cruciferous vegetables increased lately, due to their high content in bioactive compounds, such as mineral nutrients, including Se, antioxidant compounds, vitamins and glucosinolates, which form a unique class of sulfur compounds (Samec et al., 2016). The known role of chemopreventive food was attributed to cruciferous mainly due to studies indicating that glucosinolates have acted as potent inducers of phase II enzymes, which inactivated carcinogenic metabolites and inhibited cancer growth in vitro and in vivo (Park et al., 2014; Tortorella et al., 2015). Vegetables from *Brassica* spp., such as broccoli, Brussels sprouts and cabbage were the main crops on which Se biofortification treatments were applied (White and Broadley, 2009). However, per our knowledge, few studies were done on cauliflower Se biofortification (Avila et al., 2014, Oancea et al. 2015).

We developed previously a new composition for selenium biofortification treatment, intendent not only to increase accumulation of (organo)selenium compounds on *Brassica* crops, but also to enhance protective effects of selenium treatment on cultivated plants, especially against water stress (Oancea et al., 2014, Oancea et al., 2015).

The aim of the study was to investigate this new selenium-based composition, applied on experimental field conditions to cauliflower plants, on accumulation of chemopreventive compounds (Se, glucosinolates) into edible parts, cytotoxicity and antitumoral activity of plant extracts and cauliflower plant resistance to water stress.

MATERIALS AND METHODS

Biological material. Seedlings of cauliflower (*Brassica oleracea* L. var. *botrytis* cv. Adelanto F1) were transplanted and cultivated on an experimental field, located on Ştefan cel Mare, Călăraşi, Romania (40° 59' N latitude, 27°40' E longitude, 54 m altitude), according to the recommended cultivation technology. The calcaric kastanic chernozem soil was fertilized with 160 kg ha^{-1} N, 120 kg ha^{-1} P and 120 kg ha^{-1} K, 5 days before cauliflower seedlings transplantation. The total selenium content in the upper soil was 67 µg/kg, representing 40% lower value than the average content in soils unaffected by Se deficiencies (Lăcătuşu et al., 2010). The transplants were placed on 25 cm, in rows done at 70 cm one from another.

During 9 weeks of cultivation, there were recorded 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 multi-annual average.

Plant treatment. Plants received two subsequent treatments with the new selenium based mixture, consisting of 10 μM Na_2SeO_4 (Sigma), 5 mM betaine (Sigma) and 1% spraying adjuvant (Teso Spec Srl). The treatments were applied by foliar spraying, at 3 and 6 weeks after crop establishment by seedling transplant. The spraying adjuvant was obtained from rapeseed oil by transesterification in the presence of potassium hydroxide, neutralization of excess alkali with oleic acid, and final addition of lecithin and nonionic emulsifier (Vladulescu et al., 2012). Plants were grown in normal watering conditions (watered daily, at 80% field capacity) and in water stress conditions (watered once every two days, at 80% field capacity. After 9 weeks of cultivation, normally watered and water stressed cauliflower crops were separately harvested and weighed, for the establishment of the marketable yields. Controls were obtained from untreated plants, cultivated in similar conditions and without Se-based treatment (Table 1).

Table 1. Experimental treatments done on field grown cauliflower

Sample label	Water supply	Se -based mixture used for cauliflower treatment
C1	normal watering, control	-
C2	normal watering	10 μM Na2SeO4 + 5 mM betaine + 1% spaying adjuvant
C3	water stressed control	-
C4	water stressed	10 μM Na2SeO4 + 5 mM betaine + 1% spaying adjuvant

Determination of total Se content. Total Se content was measured using an atomic absorption spectrometer (Agilent AA-1475, with Vapor Generation Accessory, VGA 76, and Agilent Se- hollow cathode lamp). The measurements were undertaken after electrothermal atomization of each sample in a graphite oven SR EN ISO 15586:2004. The results were reported in μg/g dry weight (d.w.).

Analysis of sulforaphane content. Sulforaphane extraction was performed using the method described by Campas-Baypoli et al. (2010). Briefly, fresh cauliflower plants were weighed (0.15 g) and incubated with 4 ml of acidic water (pH 6) for 2.5 h at 45°C. The mixture was extracted with 20 ml dichloromethane and the resulting solution was filtered through Whatman no. 5 paper. The sulforaphane was purified with Chromabond SPE silica gel (SiOH) columns. Prior to use, the silica gel column was conditioned with dichoromethane after which the organic extract was loaded. The column was washed with ethylacetate and the sulforaphane was eluted with methanol. The methanol extract was dried at 45°C using a rotary evaporator and re-dissolved with 1 ml acetonitrile. The resulting solution was filtered with a PTFE membrane of 0.45 μm and stored at -4°C until HPLC analysis.

The chromatographic analysis was performed using an Agilent 1200 HPLC system, equipped with a photodiode array detector. HPLC identification and quantification of sulforaphane was carried out using a Zorbax XDB C18 (4.6 x 150 mm) column (Agilent) and 70% acetonitrile as mobile phase, at a flow rate of 0.6 ml/min. Twenty microliters of sample were injected into the HPLC system and the sulforaphane was detected at 202 nm.

Standard solutions of sulforaphane were prepared in acetonitrile in the range of 5-100 μg/ml. The chromatograms were processed with ChemStation Agilent software and the sulforaphane was quantified from the peak areas, in correlation with sulforaphane standard concentration. Calibration curves were built for concentrations ranging between 5-100 μg/ml.

Cell culture experiments. Total extracts of control and treated plants were obtained by incubation of fresh cauliflower plants (30 g) in deionized water, at 45°C, for 24 h. The samples were centrifuged at 2500 rpm, for 10 min and the supernatant was sterile filtered through 0.2 μm membranes. The resulting solutions were stored at -20°C until cell culture analysis.

In vitro experiments were performed using a normal cell line of mouse fibroblasts (NCTC clone L929) and a tumor cell line derived from human colorectal adenocarcinoma (Caco-2), provided by ECACC. The cells were maintained in MEM culture medium containing 10% fetal calf serum (FCS) and antibiotics, at 37°C, in humid atmosphere with 5% CO_2. For the experiment, cells were seeded in 96-wells

culture plates, at a cell density of $5x10^3$ cells/well, for 24 h, to allow cell adhesion. Then, different concentrations (0-2 mg/ml) of cauliflower extracts were added in each well and the plates were incubated in standard conditions, for 72 h.

Evaluation of cytotoxicity and antiproliferative activity. Cytotoxicity and antiproliferative activity of cauliflower extracts were evaluated using MTT assay, as previously described (Moldovan et al., 2008). Briefly, at the end of incubation period, the culture medium from each well was replaced with 500 µl MTT solution (0.25 mg/ml) in fresh culture medium and the plates were incubated in standard conditions (5% CO2 air, 37°C), for 3 h. After discarding the culture medium, 500 µl isopropanol were added to dissolve formazan crystals by gently shake, at room temperature, for 15 min and the optical density (OD) was read at 570 nm using a microplate reader (Tecan, Austria). The results were reported as cell viability percent from control sample (cells incubated in culture medium), considered 100% viable. The samples were tested in triplicate.

Statistical analysis. The results were expressed as mean of 3 values ± standard deviation (SD). Statistical analysis of the results was performed using paired Student's t-tests. Significant differences were considered at values of $p<0.05$.

RESULTS AND DISCUSSIONS

Crop yield of Se-treated cauliflower field plants

Normally watered and water stressed cauliflower crops were separately harvested after 9 weeks of cultivation (Figure 1) and weighed to calculate the obtained yields.

Figure 1. Se-treated cauliflower plants, cultivated in normal watered (A) and water stressed (B) conditions

The cauliflower crop yield variation was analyzed related to normal watering or water stress conditions of cultivation. It was observed that similar crop yields were obtained for Se - treated cauliflower plants, in both normal watering conditions (equivalent to 23.80 tones ha^{-1}) and water stressed (equivalent to 23.40 tones ha^{-1}) conditions. In turn, the corresponding controls presented a lower yield in water stressed conditions (18.62 tones ha^{-1}) than in normal watering conditions (24.10 tones ha^{-1}). These values allowed us to conclude that the Se-based treatment mixture provided protection of cauliflower plants against hydric stress.

The protective effect of Se-based biofortification was also reported for other vegetables and cereal crops (Hanson et al., 2003; Feng et al., 2013).

Total Se content in biofortified cauliflower plants. In our study, a biostimulant mixture of 10 µM sodium selenate, 5 mM betaine and 1% spraying adjuvant was selected for the treatment of cauliflower plants, based on previous experiments (Oancea et al., 2015; Oancea et al., 2016). The mixture was planned to contain sodium selenate, as the main form of inorganic Se used for crops biofortification (Hawkesford and Zao, 2007), betaine, as a plant osmoprotectant and a modulator of S-Adenosyl-Methionine cycle, overused by selenium assimilation (Oancea et al., 2015) and a spraying adjuvant, based on methyl esters of rapeseed fatty acids, as an enhancer of foliar fertilizers penetrability. A parallel experiment involving normal watered and water stressed plants was performed, as described in "Experimental part" section, in order to observe the effect of Se-based treatments on plant response to water stress. After 9 weeks of cultivation, the results of total Se content analysis showed that, in normal watered plants, the level of total Se content significantly increased ($p<0.05$) (1.35-fold) compared to control plants (Table 2). Similar variation of total Se content was obtained for cauliflower field plants cultivated in water stressed conditions, the level of Se being significantly increased ($p<0.05$) (1.34–fold) in treated plants compared to untreated control plants (Table 2).

The registered values were slightly lower than those of plants cultivated in normal watering conditions, indicating the positive effect of Se-based treatment on cauliflower plants

cultivated in water stress conditions. Other studies reported that foliar application of sodium selenate increase the fruit yield in olive trees cultivated under water stress conditions (Proietti et al., 2013) and the antioxidants level in the leaves of lettuce (Rios et al., 2008).

Table 2. Variation of Se content in Se-treated cauliflower field plants, determined by electrothermal atomic absorption spectrometry*

Sample	Selenium content (µg/g d.w.)	
	Normal watering	Water stressed
Control, field grown plants	0.080 ± 0.004	0.076 ± 0.003
Se-based treatment, field grown plants	0.108 ± 0.010*	0.102 ± 0.005*

*Results represent mean of 3 determinations ± SD.

Sulphoraphane content in Se-treated cauliflower plants. 4-methylsulfinybutyl glucosinolate (glucoraphanin) and its hydrolysis product, sulforaphane, are the most studied compounds with chemopreventive activity in *Brassica* phytochemicals research (Samec et al., 2016). In our study, sulforaphane was extracted from cauliflower plants, grown in field conditions, treated with Se-based (biostimulant) mixture and cultivated in normal and water deficit conditions. Identification of sulforaphane in treated plant extracts was performed by HPLC analysis and comparison of the retention time with that of the standard solution. As indicated in (Figure 2) and (Figure 3), sulforaphane peak was present in the recorded profiles at ≈4.9 min, in both treated plants cultivated in different conditions of water stress.

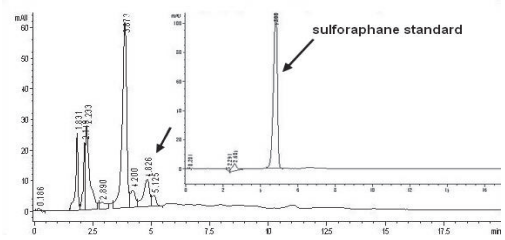

Figure 2. HPLC identification of sulforaphane in Se-treated cauliflower plants cultivated in normal watering conditions

Sulforaphane content in each cauliflower sample was determined using the method of plotting the calibration curve of sulforaphane standard by linear regression analysis of the integrated peak area versus concentration.

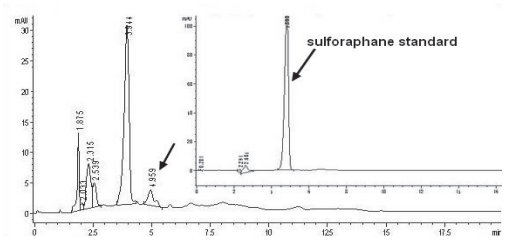

Figure 3. HPLC identification of sulforaphane in Se-treated cauliflower plants cultivated in water stressed conditions

The results of quantitative calculations obtained for all cauliflower plant extracts are presented in (Table 3). The values of sulforaphane content obtained for cauliflower plants treated with Se-based mixture, in normal watering and water stressed conditions are presented in (Table 3).

Table 3. Content of sulforaphane in cauliflower plant extracts*

Sample	Sulforaphane (µg/g d.w.)	
	Normal watering	Water stressed
Control, field grown plants	34.83 ± 5.88	27.65 ± 5.44
Se-based treatment, field grown plants	25.39 ± 4.12	18.20 ± 4.11

*Results represent mean of 3 determinations ±SD.

The results indicated that the treatment did not significantly ($p>0.05$) affect the accumulation of glucosinolates degradation product. The Se-treated plants presented sulforaphane values like those of corresponding control plants.

Previous studies reported the content of six main glucosinolates in 7-day-old cauliflower seedlings treated with 50 µM sodium selenate and indicated a significant variation in two of three cauliflower cultivars (Avila et al., 2014). The differences in total glucosinolate levels in relation to cultivars were explained by their genotype or genotype-environment interaction (Farnham et al., 2004).

The biochemical results of this study indicated that the used of Se-based mixture induced high levels of total Se and allowed accumulation of glucosinolates in cauliflower field plants cultivated in both normally watered and water stressed conditions. This is probably due to betaine from the treatment mixture, which could influence and compensate the cross-talk between selenium and sulphur metabolism. Hsu et al. (2011) also showed that it was possible to produce Se-biofortified broccoli that

concomitantly accumulated high levels of Se and glucosinolates.

In vitro cytotoxicity of cauliflower extracts. The cytotoxicity of cauliflower extracts was tested in a normal cell line of fibroblasts to determine the biocompatible range of concentrations for *in vitro* experiments. The results reported to the cell viability of untreated fibroblast cells (considered 100% viable) are presented in (Figure 4, Figure 5). It was observed that the extracts of Se-treated plants and cultivated in normal watering conditions induced a decrease in cell viability of fibroblast cells proportional with the tested concentrations. Still, they were biocompatible in the range of 0-1500 µg/ml, with values of cell viability higher than 75%. Only at 2000 µg/ml extract concentration it was recorded a decrease in cell viability up to 64.92%. The same trend was recorded for control plants and for the Se-treated plants cultivated in water stressed conditions. The Se-treated plants induced significantly higher ($p<0.05$) cell viability, in comparison with that of control plants, at certain values of concentration (Figure 3). This is the first study reporting the cytotoxicity of cauliflower extracts in a cell line of normal fibroblasts.

In vitro antiproliferative activity of cauliflower extracts. The antitumoral activity of several vegetables was previously demonstrated as accumulation of bioactive food components, like glucosinolates and methyl Se amino acids (Shankar et al., 2013; Bera et al., 2013).

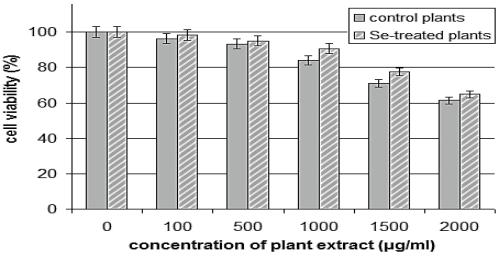

Figure 4. Effect of plant extracts from cauliflower cultivated in normal watering conditions on NCTC fibroblast cells viability after 72 h of cultivation, evaluated by MTT assay.

In our study, the effect of Se biofortification of cauliflower field plants on their capacity to inhibit adenocarcinoma cells growth was investigated in cell culture experiments.

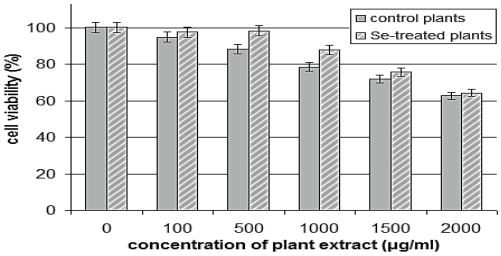

Figure 5. Effect of plant extracts from cauliflower cultivated in water stressed conditions on NCTC fibroblast cells viability after 72 h of cultivation, evaluated by MTT assay. Values are expressed as mean of three determinations ± SD and reported to the control, considered 100% viable

After 72 h of cultivation of Se-treated cauliflower extracts in tumor cell culture, the normally watered variant has induced a decrease in cell viability below 75% at concentrations of 1500 µg/ml (68.64%) and 2000 µg/ml (60.62%) (Figure 6). The water stressed plants presented lower values of cell viability, reaching 61.35% and 52.91% at concentrations of 1500 µg/ml and 2000 µg/ml, respectively (Figure 7). These values were significantly lower ($p<0.05$) than those of control plants tested at the same concentrations.

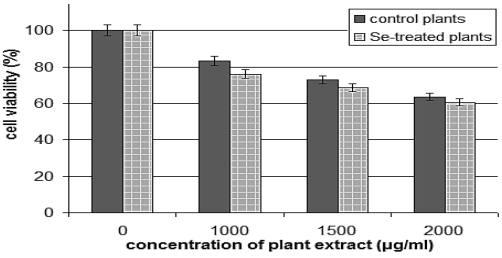

Figure 6. Effect of plant extracts from cauliflower cultivated in normal watering conditions on Caco-2 human adenocarcinoma cells viability after 72 h of cultivation, evaluated by MTT assay

These results showed that Se treated cauliflower plants presented a higher antitumoral activity than control plants. This activity is probably due to the high content of total Se and glucosinolates, which we determined in the cauliflower plants during the biochemical screening. Previously, it was reported that broccoli sprouts treated with selenate presented an enhanced antiproliferative effect in human prostate cancer cell lines, in a dose-dependent manner (Abdulah et al., 2009). Also, Se-

enriched broccoli extracts induced a greater growth inhibition of human colon cancer cells than untreated extracts (Tsai et al., 2013).

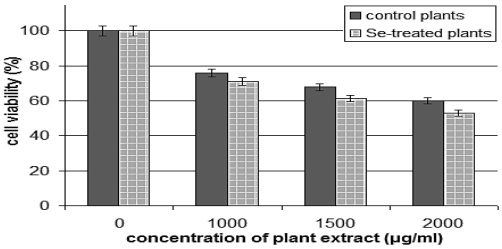

Figure 7. Effect of plant extracts from cauliflower cultivated in water stressed conditions on Caco-2 human adenocarcinoma cells viability after 72 h of cultivation, evaluated by MTT assay.

Our new Se-based composition enhance the biostimulant effect of selenium, related to an improved response to water stress and to a higher quality of the treated plants. Plant stimulants are characterized by the effects on nutrients uptake and nutrient use efficiency, response to abiotic stress and edible yield quality (Du Jardin, 2015). This new Se-based composition reduce the effects of water stress on cauliflower yield, increase the level of the main chemopreventive compounds and the antitumoral effects.

CONCLUSIONS

We have used a mixture of 10 µM Na2SeO4, 5 mM betaine and 1% adjuvant as biostimulant composition for treating cauliflower crops in the field.

The obtained results demonstrated that the proposed approach of Se-based biofortification is protective against drought cultivation conditions, resulting in increased plant tolerance to water stress, probably by modifying plant physiological processes. In the same time, the treatment had stimulatory effect, resulting in an increased Se intake by treated cauliflower plants and allowing accumulation of bioactive glucosinolates. Equilibrate formation of Se and glucosinolates in cauliflower crops treated with this Se-based biostimulant composition could provide characteristics of functional food for this vegetable. The selected cyto-compatible concentrations of biofortified cauliflower extract presented higher antitumoral activity in Caco-2 adenocarcinoma cell line. The new biotechnology consisting of Se-based biostimulant treatment of cruciferous field crops could be further tested using in vivo experimental models.

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