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Original Article

Growth, Photosynthetic Pigments, Phenolic, Glucosinolates Content and Antioxidant Capacity of Broccoli Sprouts in Response to Nanoselenium Particles Supply

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Abstract

Improving the nutritional quality of plants has emerged from the fact that macro- and micro- nutrients are limited in various agricultural areas. The aim of our study was the biofortification of broccoli sprouts with selenium nanoparticles (NSePs) and evaluation of growth parameters, assimilator pigments content, total phenols, glucosinolates content along with antioxidant capacity, in order to boost value added output, such as improved nutrition and food functionality. NSePs were prepared by reduction of $NaHSeO_3$ using glucose as reducing agent, and characterized from structural and morphological point of view. The growth of broccoli seedlings was dependent on NSePs concentration. The treatment with 10 and 50 ppm NSePs caused a slight increase in total biomass, by contrast with 100 ppm treatment. Chlorophyll content, total carotenoid and total phenols content was not affected by the treatment of broccoli sprouts with different concentrations of NSePs. Instead, the content of individual glucosinolates varied between the samples, depending on the levels of NSePs. The highest antioxidant capacity was obtained for 100 ppm NSePs concentration. The effective uptake of NSePs was further demonstrated by FTIR spectroscopy and Hyperspectral Microscopy. NSePs did not induce any toxicity on broccoli sprouts. Moreover, broccoli supply with NSePs may target higher nutritional impact and health benefits.

Keywords: *Brassica oleracea*; DPPH; FRAP; FTIR; glucosinolates; HPLC; hyperspectral microscopy; selenium

Introduction

The research focused towards improving the nutritional quality of plants has emerged from the fact that macro- and micro- nutrients are limited in various agricultural areas, depending on the soil topography, climate and agricultural or industrial utilization (Stolfa *et al*., 2017).

Selenium is essential for life and has attracted growing interest in both human health and agricultural field of science (Reilly, 2006) due to its ability to annihilate toxic effects induce by heavy metals or ionizing radiation exposure (Bassem *et al*., 2012; Hassanin *et al*., 2013). One of the possibilities to increase the selenium intake is by the diet of people, or used them as agricultural fertilizers (Ducsay *et al*., 2006; Jiang *et al*., 2015). Selenium uptake by plants, from the soil, is strongly related to the form in which this element occurs in the soil: elemental selenium, selenite, selenate, in association with other elements or in organic forms (Khoei *et al*., 2017).

There are many publications that investigated the bioavailability of different inorganic forms of Selenium (Sajedi *et al*., 2011; Barickman *et al*., 2014; Trolove *et al*., 2018; Islam *et al*., 2018) for plants but for the first time, the bioavailability of Selenium in the form of nanoparticles in the broccoli sprouts and its effect on the bioactive compounds was investigated.

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In food products (both vegetable and meat), selenium occurs in combination with proteins, being demonstrated that in terms of dairy products, selenium levels are negatively correlated with fat content (Reilly, 2006). It seems that plants that are rich in sulphur (such as members of Liliaceae family-onions and garlic, Cruciferae familiy-cabbage and broccoli) are expected to reach high level of accumulated selenium (Abdulah *et al*., 2009). Years ago, it was generally considered about elemental selenium to be biologically inert, but recently, some researchers proved that nanoSelenium (NSe) has similar bioavailability to other selenium forms (Zhang *et al*., 2008) and reported that NSe not only has a higher efficiency in up-regulating seleno-enzymes, but also seems to be less toxic comparing with selenite. These results indicated that NSe can serve as an antioxidant with reduced risk of toxicity, showing a better absorption into plants, animals, humans and microorganisms (Cavalu *et al*., 2017). However, the uptake of Selenium by plants depends on several factors: plant capacity to accumulate, soil composition and environmental factors (El-Ramady *et al*., 2015). The agronomic biofortification with Selenium supplemented fertilizers to target the greatest nutritional impact and health benefits is a result of rapid emerging biotechnologies.

Glucosinolates (GLS) (β-thioglucoside-N-hydroxy-sulphates) are very important plant secondary metabolites, specific to Brassica crops, like broccoli (*Brassica oleracea* var. italica) (Vicas *et al*., 2013). The most abundant GLS in broccoli are glucobrassicin, neoglucobrassicin, glucoraphanin, glucoiberin (Tian *et al*., 2005; Barbieri *et al*., 2008). The GLS have demonstrated chemoprotective effects against different types of cancer, and their level has been found to be greater in sprouts than in mature plants (Tarasevičienė *et al*., 2009).

The aim of our study was the supply with NSePs of broccoli sprouts, the evaluation of growth parameters, the content of photosynthetic pigments, total phenols, glucosinolates and antioxidant capacity, in order to enhance value added output, such as improved nutrition and food functionality. To our knowledge, there are no references in literature regarding the influence of NSePs addition during germination of Brassica seeds; only few works were reported, in which inorganic Selenium forms were added to the seeds during germination period (Ávila *et al*., 2014; Piekarska *et al*., 2014; Bachiega *et al*., 2016).

Materials and Methods

Preparation and morphological characterization of selenium nanoparticles

NSePs were prepared by chemical reduction of $NaHSeO_3$ solution with freshly prepared 0.25% glucose solution (Cavalu *et al*., 2018). The ratio between selenium salt and glucose was 4:1 (mol/mol). The mixture was heated to 120 °C for 15 min (until the characteristic red colour of nano-Se was achived) and, after cooling, was centrifuged at 6000 rpm for 10 min. The supernatant was removed, and the red NSePs were washed with distilled water, followed by repeated centrifugation (4 times). Finally, distillated water was added to obtain a colloidal solution and stored at room temperature. Dynamic Light Scattering (DLS) was applied to colloidal solution, using ZEN 3690 (Malvern Instruments), in order to determine the average particle size and size distribution. AFM microscopy (SPM/AFM 5500 Keysight Technologies) was applied in order to observe the morphology and surface topography of the drop-coated film of NSePs.

Plant material and experimental design

The research was performed using broccoli seeds (*B. oleracea* L. botrytis subvar. Cymosa, Agrosel Company, Romania). Seeds were germinated in plastic box (25 x 20 cm), on filter paper, 250 seeds per box, sprinkled every day with different concentration of NSePs (10, 50 and 100 ppm) and distilled water as a control. The germination was carried out on a plant growth chamber with controlled temperature (25 °C) and photoperiod (16 h of light, 8 h in the dark) during 9 days. After germination, broccoli sprouts were harvested, freeze-dried and stored at -20 °C until the investigation.

Percentage of germination was determined after 48 h from the start of the experiment. Samples from three replicates were harvested after 9 days. The roots, shoots and leaves were separated and weighted immediately after harvesting. Values were expressed in grams per 20 sprouts. The biomass of fresh broccoli sprouts was calculated by weighing the entire broccoli sprouts (root, shoot and leaves) and expressed as grams per 20 broccoli sprouts. In order to extract photosynthetic pigments, 50 mg of fresh broccoli sprouts leaves were homogenized with 5 ml DMF (N,N-dimethylformamide) following the protocol described by Sharma *et al*. 2011.

Extraction and HPLC analysis of GLS from broccoli sprouts

The extraction method of GLS from broccoli sprouts was made according to the EEC Regulation N1864/90. Shortly, duplicated samples (200 mg powder) were place in a hot water bath (80 °C), left for 5 min and then extracted with 5 ml hot ethanol 70% for 3 min using Silent Crusher M (Heidolph) homogenizer, at 5000 rpm. Then, the mixtures were centrifuged at 5000 rpm for 20 min. Aliquots of 1 ml supernatant were loaded twice on a mini-column filled with 0.6 ml DEAE-Sephadex A- 25 anion-exchange resin, conditioned with 25 mM acetate buffer pH 5.6. After washing with 3 ml acetate buffer, volume of 200 µl purified sulphatase were loaded on each mini-column, left overnight at room temperature and the desulfo-GLS were eluted with 3 ml of ultra-pure water, then filtered through a 0.45 ml filter and analyzed by HPLC. A known amount of glucotropaeolin (200 µl from a solution containing 1mg/ml) was added to each broccoli sprouts samples before the first extraction, as an internal standard for the HPLC analysis.

The quantification of GLS from broccoli sprouts extracts was performed by an HPLC-PDA system (Shimadzu Corporation, Scientific Instruments, Kyoto, Japan) equipped with a CBM- 20A controller, LC-20 AD pump, a DGU-20A degaser, a SIL-20 AC autosampler, CTO-20 AC column oven and a SPD-M20A photodiode array detector. Desulfo-GLS were separated on a Platinum

(C 18) 100 A column (250×4.6 mm, 5 µm), at 30 °C, using a flow rate of 0.5 ml/min and an injection volume of 20 µl. The data were processed using Labsolution version 5.10.153 (Shimadzu) software. The mobile phases consisted of water (eluent A) and acetonitrile (eluent B), using a gradient program as follows: 1 min 1% B; 22 min linear gradient up to 22% B; 10 min linear gradient down to 1% B. Elution of desulfo-GLS was monitored at 229 nm. The desulfo-GLS was identified by retention time using the standards and UV-Vis spectra. The content of individual and total GLS, expressed in µmol/g dry weight (dw) was calculated using glucotropaeolin as an internal standard, considering the response factors of the other desulfo-GLS relative to the desulfo-glucotropaeolin.

The total phenols content and antioxidant capacity of broccoli sprouts

In order to determine the total phenols content and antioxidant capacity, the powder of each sample (20 mg) was suspended in methanol (1 ml), sonicated for 10 minutes, centrifuged at 5000 rpm for 15 min and the supernatant was used for the analysis. The total phenols content was determined by using modified Folin-Ciocalteu method (Singleton *et al*., 1999). Briefly, aliquots of 100 µl Brassica sprouts extracts were mixed with 1700 µl distilled water, 200 µl Folin-Ciocalteu reagent (diluted 1:10, v/v) and 1000 µl of 7.5% Na_2CO_3 solution. The mixture was incubated in the dark, at room temperature for 2 h. The absorbance was measured at 765 nm, and the results were expressed in mg gallic acid equivalents (GAE)/g dw.

Evaluation of the ferric reducing antioxidant power (FRAP) of broccoli sprouts extracts was performed by Benzie and Strain method (1996). The absorption of the coloured complex (ferrous tripyridyltriazine) resulted by the reaction between Brassica sprouts extract and FRAP reagent was recorded at 595 nm and the results were expressed in µmol TE/g dw.

The free radical scavenging capacity of broccoli sprouts extracts against the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl) was performed using Brand-Williams method (Brand-Wiliams *et al*., 1995). Briefly, a volume of 200 µl of broccoli extract and 2.8 ml DPPH solution (80 µM) were mixed and the decreased in the absorbance was monitored at 515 nm for 5 minutes. The percentage of scavenging effect of extract against DPPH radicals, was calculated using the following equation:

DPPH scavenging effect (%) = [(A0-As) x 100]/A0, where, A0 is absorbance of the blank, and As is absorbance of the sample at 515 nm.

FTIR (Fourier Transform Infrared Spectroscopy) of broccoli sprouts enriched in nano-Se

FTIR spectra of broccoli sprouts leaves were recorded in the range 400-4000 cm^{-1}, using Spectrum BXII spectrophotometer (Perkin Elmer), equipped with MIRacle ATR accessory (ZnSe crystal), at scanning speed of 32cm^{-1} and spectral width 2.0 cm^{-1}. A comparison was made between the spectra recorded for different concentrations of nano-Se used in the germination period, and the reference spectrum (no selenium added).

Mapping nano-Se particles in broccoli sprouts by Enhanced Darkfield Hyperspectral Microscopy and CytoViva® unit

Broccoli leaves were mounted directly on a glass slide with a coverslip. The CytoViva® - microscope system was adapted to acquire dark field microscopy images and hyperspectral plots using an enhanced dark field transmission optical microscope (Olympus BX41) equipped with a CytoViva® unit and a hyperspectral imaging spectrophotometer unit (Headwall Photonics). The captured optical images were computed and analysed with special software (ImageJ Software). Qualitative hyperspectral analysis of the acquired images was performed using ENVI 4.8 Spectral libraries.

Statistical analysis

Based on experimental design, four samples groups were established: control (no selenium supply), Br_10, Br_50, Br_100 and analysed in triplicate. Statistical significance between the groups was determined by one-way ANOVA, Tukey's Multiple Comparison Test. A value of $p < 0.05$ was considered statistically significant.

Results and Discussion

Preparation and morphological characterization of selenium nanoparticles

Particle size and size distribution of NSe was determined by DLS measurement, as presented in Fig. 1a. The histograms demonstrates the co-existence two different specimens: the first one with average size of 100 nm and lower concentration, and second one, with average size of about 650 nm and higher concentration. The larger specimens occur because of nanoparticles aggregation. It was previously demonstrated (Zhang *et al*., 2012) that even at room temperature, NSePs tends to aggregate into larger size spheres, due to their high surface to volume ratio. AFM images of single selenium particles, spherical shape, indicates a diameter ranging from 200 nm to 700 nm, the maximum high being 220 nm, as presented in Fig. 1b. The majority of the particles was symmetrical, spherical in shape and well distributed without aggregation. The size of NSePs is considered to be in agreement with the data collected from the DLS study. However, NSePs appear larger on AFM micrographs, as they are flattened due to the contact with the substrate.

Effect of nano-Se supply on growth parameters

Under the treatment with three different concentrations of NSePs (10, 50 and 100 ppm), the germination of broccoli seeds was not affected by the presence of NSePs (data not shown). On the other hand, the growth of seedlings was dependent on Se concentration, as presented in Fig. 2. Compared to the control, the treatment with 10 and 50 ppm NSePs caused a slight increase in total biomass, while the treatment with 100 ppm caused a slight decrease, statistically significant compared with the Br_50 group (Fig. 2a). In the same time, compared to the control, 10 ppm and 50 ppm NSePs supply induced an increased weight of broccoli roots, while 100 ppm

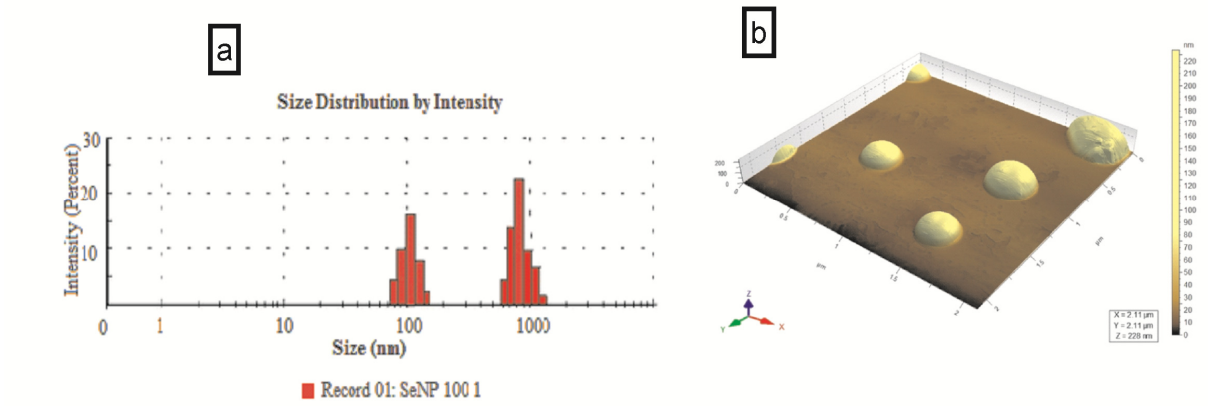

Fig. 1. a) Size distribution of NSePs b) 3D micrograph of Selenium nano-spheres

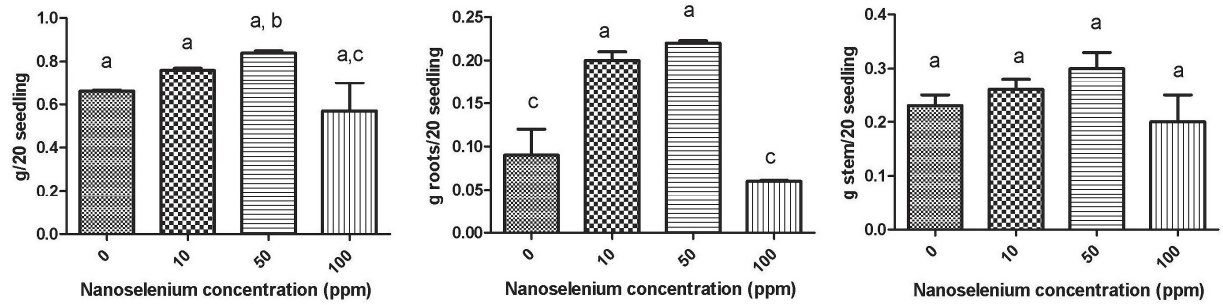

Fig. 2. Effect of NSePs particles supply on: a) Total biomass; b) Root weight; c) Shoot weight. Values are the means of three replications. Error bars represent standard deviation. Different lower case letters reflect the significantly different ($p < 0.05$) between the control and samples

induced a drastic decrease compared to the Br_10 and Br_50 groups, but not statistically significant compared to the control (Fig. 2b). We have noticed that NSePs supply had no effect on the weight of shoots (Fig. 2c). These results are in agreement with Ramos *et al.* (2010), which demonstrated that sodium selenite supply exhibited an increased shoot/root weight and biomass production.

Effect of nano-Se supply on photosynthetic pigments production

Fig. 3 exhibits the effect of different NSePs concentrations on clorophylls and total carotenoids content. A significantly increase in amount of chlorophyll a was recorded in the case of broccoli sprouts treated with 100 ppm, compared to the control. The content of clorophyll b and total carotenoids was not affected by the treatment with NSePs, as compared to the control group. The ratio chlorophyll a/chlorophyll b is a valuable indicator which provides information about the activity of the light-harvesting complex (LHC) from Photosystem II (Taran *et al.*, 2016). The inset in Fig. 3 presents the ratio chlorophyll a/chlorophyll b, indicating that no matter the concentration of NSePs, the ratio remains almost constant.

Effect of NSePs supply on total phenols content and antioxidant capacity

The major group of biologically active compounds that may contribute to the total antioxidant capacity includes different groups of polyphenols (flavonoids, hydroxyl-cinnamic acids). The treatment of broccoli sprouts with different concentrations of NSePs did not affected the content of total phenols, as shown in Fig. 4a. Some previous phytotoxicity studies showed that nanoparticles could induce stress in plants by manipulating the endogenous mechanisms (El-Ramady *et al.*, 2015). In response to these stress, plants releases various defensive compounds known as antioxidant secondary metabolites. In our study, the amount of total phenols did not show any modification comparing to the control, which suggests that NSePs treatment has no consequence in broccoli sprout stress. In Fig. 4b and c is presented the effect of NSePs treatment on antioxidant capacity of broccoli sprouts, determined by DPPH assay and FRAP assay, respectively.

Compared to the control, the antioxidant capacity of broccoli sprouts (determined by DPPH assay) from Br_10 and Br_50 groups were not significantly affected. In contrast, the group Br_100 exhibited the highest antioxidant capacity compared both to the control and other two groups. It is well known that phenols content and antioxidant capacity are directly correlated (Rychlik *et al.*, 2015). In our case, even if Br_100 group did not exhibit enhaced amount of total phenols, the antioxidant capacity showed the highest level. An explanation might be related to the fact that NSePs itself acts as a scavenger with respect to DPPH free radical, as demonstrated by Huang *et al.* (2003). The antioxidant efficacy was evaluated also by FRAP assay. Our results shown that the ability of broccoli extracts to reduce Fe^{3+} to Fe^{2+} was not influenced on the

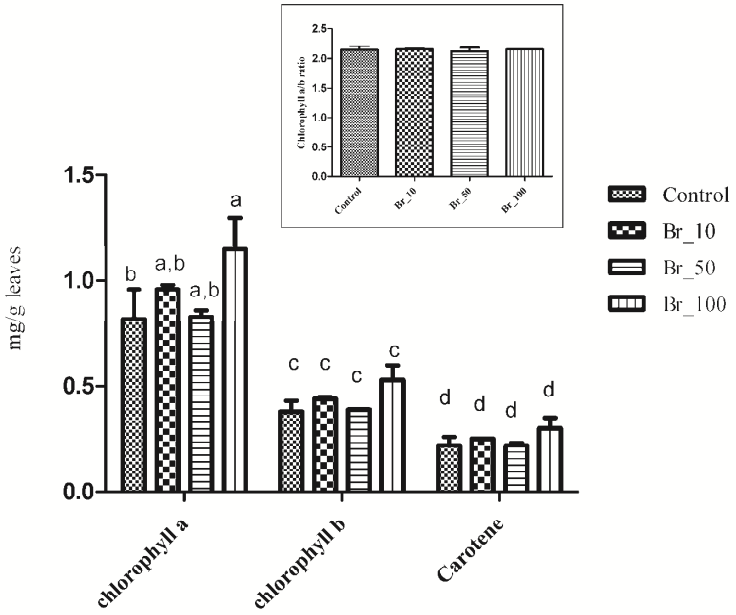

Fig. 3. Effect of NSePs treatment on green pigments content and total carotenoids. Inset, chlorophyll a/chlorophyll b ratio. Values are the means of three replications. Error bars represent standard deviation. Different lower case letters reflect the significantly different ($p < 0.05$) between the control and samples

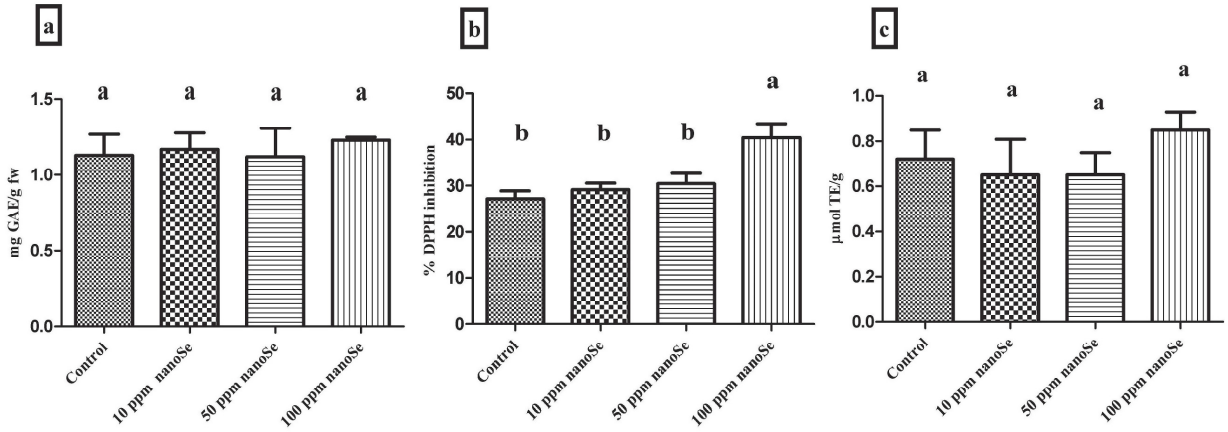

Fig. 4. Effect of NSePs treatments on: a) total phenols content; b) antioxidant capacity determined by DPPH assay; c) antioxidant capacity determined by FRAP assay. Values are the means of three replications. Error bars represent standard deviation. Different lower case letters reflect the significantly different ($p < 0.05$) between the control and samples

concentration of NSePs. This finding supports our above mentioned results, indicating that neither the concentration, or the size of NSePs used in our experiment, doesn't induced any toxicity on broccoli sprouts.

The Effect of NSePs on individual GLS in broccoli sprouts

HPLC-PDA was applied in order to determine the content of individual GLS from broccoli sprouts of 9 days old germination (HPLC chromatogram is shown in Fig. 5).

Eight different GLS were detected in broccoli sprouts (9 days old) enriched with NSePs with different concentrations (Table 1). It can be noticed that the content of individual GLS varied between the samples.

The precursor of the anticarcinogenic sulforaphane, GRA, was present in all samples, and the highest concentration was obtained in the case of broccoli sprouts supply with 100 ppm NSePs. Other aliphatic GLS, glucoerucin (GER) was found to have a decreased value in the samples with NSePs compared with the control. Instead, the indolic GLS class showed no statistical significance between the samples.

Barickman *et al.* (2014) investigated the impact of sodium selenate on glucosinolates concentrations in rapid cycling *Brassica oleracea*. Their results demonstrated that in *B. oleraceae* the increasing of selenium concentrations did not have significant decreases in glucosinolates (Fig. 5).

Table 1.Content of different GLS (µmol/g dw) separated from broccoli sprouts grown under different concentrations of NSePs

Peak Number	GLS	RT	Control	sd	10NSeP	sd	50NSeP	sd	100NSeP	sd
			mean		mean		mean		mean	
1	PRO	5.6	2.58	0.21	0.58***	0.08	2.10	0.76	2.42	0.09
2	GIB	7.9	1.93	0.34	0.45**	0.09	1.97	0.22	2.93	0.76
3	GRA	10.2	4.78	1.77	1.12***	0.61	5.59	0.81	6.90***	0.87
4	4OHGBS	16.4	0.19	0.19	0.058	0.04	0.36	0.17	0.29	0.05
5	GER	19.5	4.97	0.40	1.02***	0.35	3.00**	0.71	3.43**	0.42
6	GBS	21.3	0.25	0.1	0.08	0.01	0.22	0.06	0.31	0.05
7	MeGBS	24.8	0.32	0.01	0.18	0.03	0.35	0.02	0.35	0.07
8	NGBS	28.4	0.45	0.11	0.25	0.04	0.34	0.16	0.31	0.05

Legend: PRO- Progoitrin, GIB- Glucoiberin, GRA- Glucoraphanin, 4OHGBS- 4-hydroxyglucobrassicin, GER- glucoerucin, GBS- Glucobrassicin, MeGBS- methoxyglucobrassicin, NGBS- Neoglucobrassicin, RT- retention time. Values are the means of three replications. sd- standard deviation. Statistical significance: **p < 0.01 and ***p < 0.001.

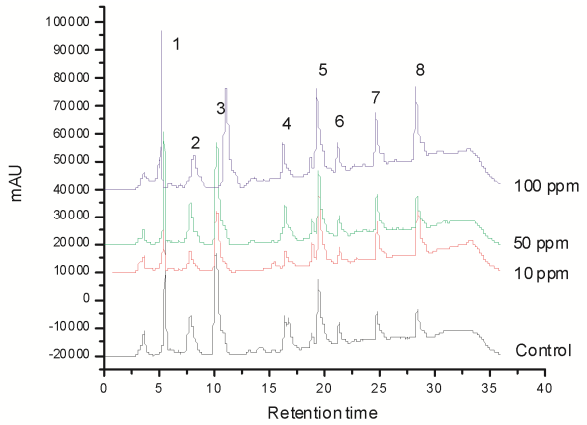

Fig. 5. HPLC profile of individual glucosinolates in broccoli sprouts (9 days old) grown under different concentrations of NSePs (10, 50 and 100 ppm). 1. PRO- Progoitrin, 2. GIB- Glucoiberin, 3. GRA- Glucoraphanin, 4. 4OHGBS- 4-hydroxyglucobrassicin, 5. GER- glucoerucin, 6. GBS- Glucobrassicin,7. MeGBS- methoxyglucobrassicin, 8. NGBS- Neoglucobrassicin

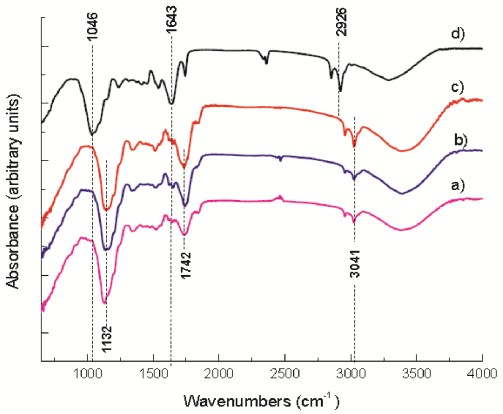

Fig. 6. FTIR spectra of broccoli sprouts leaves upon NSePs treatment: a) 100 ppm; b) 50 ppm; c) 10 ppm; d) reference spectrum

FTIR spectroscopy characterization of broccoli sprouts leaves

The FTIR spectra were recorded for each broccoli sprout specimen, corresponding to different NSePs concentrations, along with the reference spectrum (no selenium added), as displayed in Fig. 6. The overall features in this figure are dominated by the vibrational fingerprints of chlorophyll (Da Luz, 2006). The band at 1643 cm^{-1} in the reference spectrum is assigned to carbonyl stretching vibration (C=O bonds originating from ester groups), the strong band at 1046 cm-1 to carboxyl stretching mode (C-O-C), while the doublet at 2850 and 2926 cm^{-1} is assigned to methylene stretching. Comparing to the reference spectrum (no NSePs added), important structural changes are observed: the intensity of the marker bands appears to be dependent on the concentration of selenium and shifted toward higher wavenumbers, concomitant with the modification of the relative intensity. This behavior indicates that NSePs are effectively taken up by the plant and then metabolized.

Mapping nano-Se particles in situ

Using integrated hyperspectral image analysis software, the unique spectral response of nanoparticles were identified and easily mapped throughout the plant tissue. In Fig. 7a, the hyperspectral image of a leaf containing NSePs is shown as the dark reddish-brown area encircled. In Fig. 7c we have captured the spectral response for both the leaf (indicated in green) and NSePs (indicated in red). The spectral response of NSePs does share much of the spectral characteristics of the leaf due to the chlorophyll influence. However, the presence of selenium causes a shift of the spectral peak from 565 nm (in the leaf tissue) to 610 nm. Finally, the spectral library was mapped against the positive control image, and, as can be seen in the encircled area of Fig. 7b, all pixels perfectly matching the spectral response of the selenium were pseudo-colored red. Therefore, by using this high sensitivity technique, we were able to demonstrate NSePs uptaking by broccoli sprouts.

The results are in good agreement with previously reported data (El-Ramady *et al*., 2015), supporting the idea that selenium from soil is up taken by plants in the form of selenate (Se^{6+}), and then metabolized via the sulphur assimilation pathway, where selenate is reduced to selenite (Se^{4+}), which can undergo further reduction to selenide (Se^{2-}).

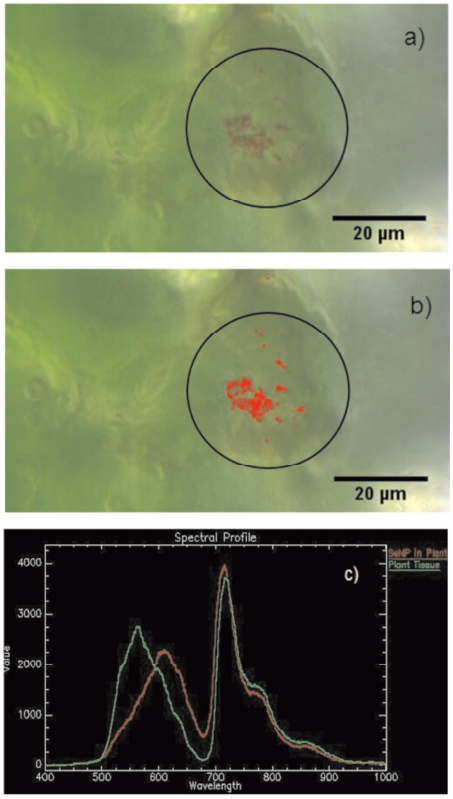

Fig. 7. Enhanced Darkfield Hyperspectral images of Se uptaken: a) 60x image of Se in broccoli leaf; b) 60x image of the leaf with Se mapped and pseudo-colored in red; c) Spectral comparison of plant tissue and Se in broccoli leaf (green line-plant tissue; red line-selenium)

Conclusions

The main goal of our study was to modify the nutritional content of broccoli sprouts, by NSePs supplementation, during germination process. Growth parameters, assimilator pigments content, total phenols content and antioxidant capacity of broccoli sprouts were evaluated. The growth of seedlings was depent on NSe concentration. The treatment with 10 and 50 ppm NSe caused a slight increase in total biomass, by contrast with 100 ppm treatment. A significantly increase in amount of chlorophyll a was recorded in the case of broccoli sprouts leaves treated with 100 ppm. The content in clorophyll b and total carotene was not affected by the treatment with nanoSe particles. The concentration of 100 ppm NSe particles was reflected in the highest antioxidant capacity. Our results demonstrated that NSe particles in concentration of 10, 50 and 100 ppm didn't induce any toxicity on broccoli sprouts. The effective uptake of NSe was further demonstrated by ATR-FTIR spectroscopy and Enhanced Darkfield Hyperspectral Microscopy coupled with CytoViva® unit. However, the complete understanding of NSe metabolism in vegetables sources requires more detailed biochemical studies and selenium uptake analysis to be conducted, especially from quantitative point of view.

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Conflicts of interest

The authors declare that there are no conflicts of interest related to this article.

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