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Article

Lettuce biofortification with selenium in chitosan-polyacrylic acid complexes

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Abstract: Selenium (Se) is an essential element of the human diet. Therefore, it is necessary to implement Se in agricultural fertilization, although it is not considered as an essential element for plants, Se provides benefits at the level of redox metabolism, increasing the resistance of plants to various stress factors. The increase of the availability of selenium with the use of biopolymer complexes was sought in Great Lakes lettuce grown in substrate pots treated with SeO_2 (5 mg L Se), Cs-PAA + Se (5 mg L Se), and Cs-PAA. The redox metabolism was modified by increasing the enzymatic activity of glutathione peroxidase. The use of Cs-PAA + Se biopolymer complexes increase selenium up to 24 mg/Kg dry weight (DW) in plant tissues.

Keywords: biofortification; antioxidants; soilless culture; nutraceutical quality; enzymatic activity; plant resistance.

1. Introduction

Selenium is essential in human diet since a low intake has been linked to a weak immune system and cognitive decline [1]. On the contrary, the optimal intake of Se entails benefits such as reducing the risk of different types of cancer, Alzheimer's disease, among others, and is necessary for thyroid function since the thyroid gland is the largest reservoir of Se in the human body, having different functions on its functioning [2]. The adequate recommended intake of Se in humans has been proposed between 50-60 µg/day, on the contrary, an intake of 350-700 µg/day may result toxic[3]. It is known that Se is related to antioxidant metabolism through its role as a cofactor of selenoenzymes[4]. Therefore, its intake promotes the synthesis of antioxidant compounds, inducing changes in the cellular redox balance. The consumption of foods with high concentrations of antioxidants contributes to the protection of the cells against oxidative stress preventing some degenerative diseases. Free radicals cause oxidative chain reactions which can be neutralized through the action of antioxidant enzymes such as catalase (CAT) and glutathione peroxidase (GPX). These enzymes increase their activity with the presence of Se in plants[5]. Since Se is a non-renewable resource, it is necessary to incorporate it into agricultural fertilization in an appropriate manner, in order to avoid wastage of the element. The use of biopolymers to encapsulate elements in order to improve their absorption by plants is a technique that can be effective for crop biofortification with Se. Biopolymers such as chitosan (Cs) and polyacrylic acid (PAA) are capable of encapsulating active ingredients using an aqueous system at room temperature[6]. The mixture between these polymers has physicochemical properties and relatively simple processing techniques that come from synthetic polymers and an adequate biocompatibility from natural polymers, this confers varied applications such as the immobilization and prolonged release of various chemical elements or bioactive agents.

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These biopolymer complexes may be used in order to avoid the loss of Se through lixiviation, adsorption to organic components of the substrate, and volatilization by microorganisms[7]. In the present work, it was sought to biofortify lettuce plants with Se (SeO_2) by applying it alone and absorbed in a complex with biopolymers, studying the impact on the plant growth, the antioxidant activity, and the accumulation of Se.

2. Materials and Methods

2.1 Preparation of Non-Stoichiometric Interpolyelectrolyte Complexes (NPEC) of Chitosan-Polyacrylic Acid (Cs-PAA) and Cs-PAA + Se

Chitosan (Cs) was supplied by Marine Chemicals (Meron, Kerala, India). Deacetylation degree was 99%, molecular weight Mv = 200,000 g/mol was determined by intrinsic viscosity in an Ubbelohde viscometer by the ASTM D2857 method using a mixture of acetic acid/sodium acetate solutions at 30 °C applying Mark-Houwink equation: $\eta = KMv^{\alpha}$, where k = 0.076 and $\alpha = 0.76$. Poly (acrylic acid) molecular weight Mw = 450,000 and selenium oxide (SeO_2) were acquired from Aldrich. Water-soluble NPEC were prepared at the Center for Research in Applied Chemistry with polycation (Cs) and polyanion (PAA)[8]. The NPEC (Cs-PAA) with the composition φ = [CS]/ [PAA] = 3 was used. The square brackets denote molar concentrations of polyions prepared by mixing water solutions of Cs and PAA at corresponding quantities of the polyelectrolytes solutions at pH 2 and then adjusting the solution with phosphate buffer at pH 7.4. Lastly, from SeO_2, 50 mg of Se per L of complex is added, amount needed to make the applications of Cs-PAA + Se.

2.2 Plant Material and Treatments

A soilless lettuce (*Lactuca sativa* L.) var. Great Lakes (FAX Seeds, Jalisco, México) crop was established in a greenhouse with polyethylene cover 50-60% relative humidity, and an average temperature of 28 °C. Lettuce seeds were sown in black polyethylene trays filled with sphagnum peat moss and perlite in a 1:1 ratio v/v. At 40 days after sow, seedlings were transplanted to black polyethylene containers with 5 L of the same substrate as in the sown, Steiner [9]nutrient solution was applied by an open drip irrigation system, with a pH adjusted daily with phosphoric acid. The treatments application started seven days after transplant (DAT) and were applied weekly during the whole production cycle (eight weeks). Four treatments were used, with two different Se sources, the Cs-PAA complex, and control, as illustrated in **Table 1**.

Table 1. Different treatments applied in the experiment during the crop production cycle.

Treatment	Content
T0	Control
T1	SeO_2 (5 mg L Se)
T2	Cs-PAA + SeO_2 (5 mg L Se)
T3	Cs-PAA

2.3 Sampling

Lettuces were harvested at 60 DAT, measured and weighted to determinate total growth and total crop yield. At the same time, samples were collected to quantify the biochemical variables and Se content, obtaining five samples by each treatment and control.

2.4 Yield and Biomass Production

In order to determine total crop yield, the lettuce heads were harvested and weighed on an analytical scale (OHAUS CORPORATION, New Jersey, USA) to determine the total fresh weight. In

order to determine the total accumulated biomass, five plants of each treatment were dehydrated in an oven at 80 °C for 48 hours. Once total dehydrated, the dry weight was registered.

2.5 Biochemical Analysis

In order to determinate nutraceutical quality, five samples were frozen at -20°C during 48 hours then freeze-dry in a Freeze Dryer (Labconco, Freezone 6, USA) at $133x10^{-3}$ mbar and -80°C during 48 hours. Once completely dried, samples were pulverized with a porcelain mortar. For the biomolecules extraction, 200 mg of powdered sample was placed in a 2 mL tube and were added 20 mg of polyvinylpyrrolidone (PVP) to stabilize the enzymes; 1.5 mL of phosphate buffer (pH 7 - 7.2) was added, homogenized by vortex for 20 seconds each sample, sonicated during five minutes, and centrifuged at 12,500 rpm at 4 °C for 10 minutes, collecting the supernatant in order to perform the analysis.

2.5.1 Total Proteins

The Bradford spectrophotometric technique[10] was used for protein quantification. 100 µL of the proteic extract were placed in an assay tube, and adding 5 mL of the Bradford reagent, and let to stand during 5 minutes. Once the incubation time passed, the absorbance was read at a wavelength of 595 nm with a UV-Vis spectrophotometer (Thermo Scientific Model G10S, Waltham, MA, USA) The results were registered and extrapolated to a calibration curve of bovine serum albumin (BSA), reporting the results in mg g^{-1}.

2.5.2 Catalase Activity (CAT) (EQ 1.11.1.6)

The enzymatic activity of catalase was quantified by a spectrophotometric technique, with two reaction times. The assay mixture consisted on 100 µL of proteic extract and 900 µL of 100 mM H_2O_2, and was added 400 µL of 5% H_2SO_4 to stop the reaction, this was carried out under stirring at 24 °C. These assays were read with a spectrophotometer (Thermo Scientific Model G10S, Waltham, MA, USA) at 270 nm; time reaction zero (T0) was recorded. For time reaction one (T1), the mixture of protein extract and H_2O_2 was stirred at 24 °C, after 1 minute 400 µL 5% of H_2SO_4 were added to stop the enzymatic activity. The remaining H_2O_2 was read with a spectrophotometer at 270 nm. For this analysis, a blank for each sample was used, consisting of 100 µL of biomolecules extract, 900 µL of phosphate buffer and 400 µL of H_2SO_4. Units of catalase activity were expressed as mM H_2O_2 min^{-1}/ total proteins [11].

2.5.3 Glutathione Peroxidase Activity (GPX) (EQ 1.11.1.9)

A spectrophotometric method[12] was used with H_2O_2 as substrate; 200 µL of biomolecules extract were placed in a test tube plus 400 µL of reduced glutathione 0.1 M and 200 µL Na_2HPO_4 0.067 M. This mixture was preheated in a water bath at 25 ° C for 5 min, then was added 200 µL of 1.3 mM H_2O_2 to start the catalytic reaction. The reaction lasted 10 min and was terminated by adding 1 mL 1% trichloric acetic acid, and the mixture was put into an ice bath for 30 min. The assay mixture was centrifuged for 10 min at 3000 rpm; 480 µL of the supernatant was placed into a cuvette, with 2.2 mL of 0.32 M Na_2HPO_4 and 320 µL of 1.0 mM 5-5'-dithiobis 2-nitrobenzoic acid (DTNB, *Sigma* product, analytical grade) were added for color development. The enzyme activity was determinate as a decrease in GSH within the reaction time, expressed as mg/L GSH min^{-1}/total proteins.

2.5.4 Glutathione(GSH)

Glutathione was quantified following a spectrophotometric technique[12], by reaction with 5,5-dithio-bis-2-nitro benzoic acid (DTNB). In a tube 480 µL of biomolecules extract were placed then added and 2.2 mL of 0.32 M Na_2HPO_4 plus 320 µL of 1.0 mM DTNB dye. It was mixed and read on a

spectrophotometer (Thermo Scientific Model G10S, Waltham, MA, USA) at 412 nm. The units were reported in mg glutathione/g dry tissue.

2.5.5 Total Phenols Content

Regarding the total phenolic extraction, 5 samples of each treatment and control were used. 200 mg of lyophilized and macerated sample were taken and 1 ml of water-acetone 1:1 solution was added, centrifuged at 10000 rpm at 4 ° C for 10 min, and the supernatant was collected to initiate the reaction. The assays were prepared using the Folin-Ciocalteu reagent, as described in **Table 2**. Subsequently, assays were let to incubate at 45 ° C for 30 minutes. The samples were read with a spectrophotometer (Thermo Scientific Model G10S, Waltham, MA, USA) at a wavelength of 750 nm and the results were recorded in $\mu g.g^{-1}$[13].

Table 2. Description of samples and blank for assay of total phenolic compounds.

Component	Blank	Sample
Extract	0	50 µL
Folin-Ciocalteu 1 M	200 µL	200 µL
Na_2CO_3 at 20%	500 µL	500 µL
Water-acetone 1:1	1 ml	1 ml
Destilated water	5 ml	5 ml

2.6 Selenium Content

Selenium was extracted using a wet digestion technique [14]. For digestion, 500 mg of the dehydrated sample were placed in a beaker, with 30 mL of nitric acid, heated during 2 hours in a heating plate until clarification of the mixture. Finally, it was collected and taken to a volume of 50 mL with deionized water and filtered with Whaltman # 11 filter paper. Mineral content was read in a plasma emission spectrophotometer (ICP, Termo Jarrel Ash ASH model 7400).

2.7 Statistical Analysis

The experimental design was completely randomized with five replicates per treatment on each variable, with one plant considered as an experimental unit. Infostat software was used, in which an ANOVA and Fisher Least Significant Difference test ($\alpha \leq 0.05$) was performed for all variables.

3. Results and Discussion

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.

3.1. Yield and Biomass Production

The treatments applied did not exert an effect on the variables related to yield and biomass, (**Table 3**). The analysis of accumulated biomass in the form of the dry weight of plant tissues is a useful indicator in order to determine the toxicity of an element. Based on the results obtained in this experiment, in which there was no significant difference between the treatments and the control, it is suggested that the applied concentrations of Se were nontoxic. The application of the Cs-PAA complexes can also be considered safe for the development of the crop. In a crop of radish plants established in soil treated with 40µM SeO_4^- per plant, the authors[15] reported a decrease in dry weight of leaves and roots by about 35% and 18% respectively. On the other hand, when cultivating

the radish plants in a soilless culture, they did not find significant differences in the dry weight of the leaves.

3.2 Biochemical variables

According to the results analyzed, the content of proteins, phenolic compounds, and glutathione, are not affected by the application of the treatments in comparison with the control, as it is detailed in **Table 3**.

Table 3. Comparison of means of the variables.

Treatment	Biomass (g DW)	PROT (mg g^{-1})	CAT (mM H_2O_2 min^{-1})	GSH (mg L^{-1})	PHEN (µg g^{-1})
Control	51.35±3.19[a¥]	2.24±0.56[a]	0.17±0.65[a]	0.81±0.02[a]	63.51±1.11[a]
SeO_2	51.67±2.43[a]	2.87±0.51[a]	2.74±0.55[a]	0.83±.01[a]	88.4±0.85[a]
Cs-PAA + SeO_2	47.27±4.12[a]	2.05±0.35[a]	3.15±1.33[a]	0.95±0.38[a]	48.76±0.68[a]
Cs-PAA	50.17±2.41[a]	3.57±0.82[a]	2.85±1.24[a]	0.97±0.58[a]	61.1±1.21[a]

¥Means with the same letter are statistically equal (LSD, p≤0.05). Mean ± standard error of the mean (n=5). PROT: proteins, CAT: catalase activity, GSH: glutathione, PHEN: total phenolic compounds.

In the enzymatic activity of catalase, the results concurred with studies carried out in tomato leaves and stems[16], and Chinese cabbage[17] were no significant difference was found cabbage leaves in conditions of low Cd content, and authors only reported an increase in CAT enzymatic activity when the Se was applied in combination with Si and under conditions of Cd toxicity. In another study with *Spirulina platensis*[18], Se applied at 150 mg/L or less, induces an increase in the activity of peroxidases enzymes, including catalase. Similarly, applying a higher Se concentration (> 175 mg/L) induces enzymatic activity but in conjunction with an increase in lipid peroxidation and a decrease in biomass and photosynthetic pigment content. Some studies[19] suggest that Se also induces an increase in the enzymatic activity of CAT when used in concentrations of 5 to 10 mg/L to reduce oxidative stress in plants of *Triticum aestivum* L. under drought stress conditions.

In the statistical analysis of the GPX activity, significant differences were found (**Figure 1**). All the treatments applied had higher values of GPX activity compared to the control. Both the SeO_2 and the Cs-PAA complex applied by themselves increase GPX activity, (729% and 789% respectively); however, when applied together as Cs-PAA + SeO_2 a higher enzymatic activity is obtained (1031% increase over control). The above results agreed with those of a study in ryegrass[20], whose authors reported an increase in GPX activity by applying Se at 1 mg kg^{-1}. Similarly, other works[21] also reported an increase in GPX activity with applications of different Se concentrations up to 150 mg/L but in conjunction with a reduction of biomass and chlorophyll when applying more than 175 mg/L in *Spirulina platensis*. Since all the treatments applied in the present work induced an increase in the GPX activity, it is suggested that both the Se and the Cs-PAA complex induce this increase by themselves, and when applied as a whole the effect is enhanced.

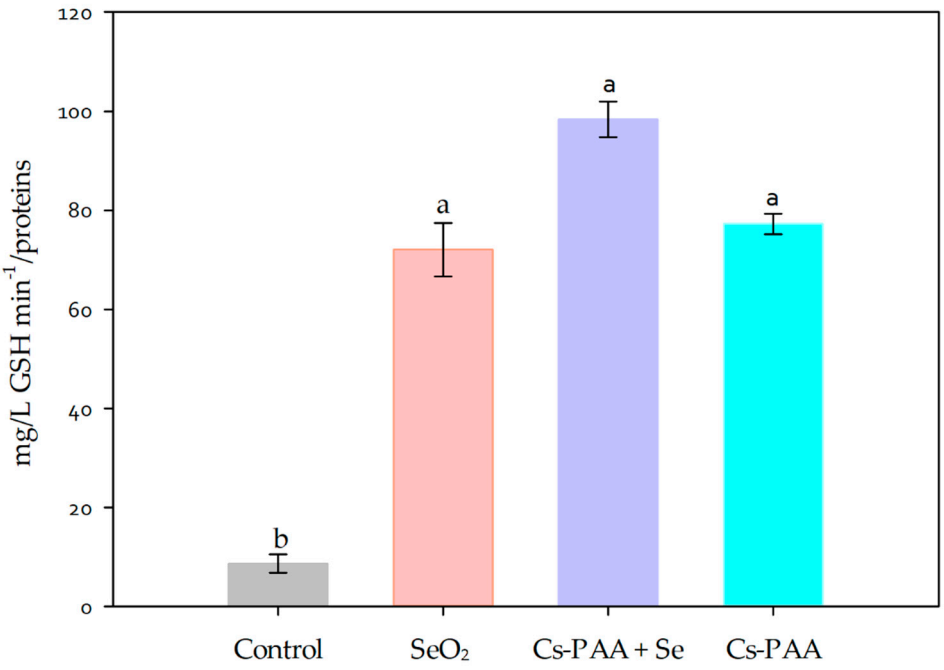

Figure 1. Means comparison of the enzymatic activity of glutathione peroxidase. Means with the same letter are statistically equal (LSD, p≤0.05).

GSH content was not affected by the treatments, in the same way as in an experiment with Chinese cabbage subjected to stress due to the high concentration of heavy metal Cd, where GSH increase when Se was applied in combination with Si; however, there was no difference when applying Se alone [17].

As in the present work, in another study[15] with radish plants using foliar applications of up to 20 mg Se per plant, no differences were found in phenolic compounds. In contrast, in an experiment under field conditions of *Allium cepa* L. Se applied 50 μg/mL^{-1} increased the content of total phenolic compounds compared to the control[22].

The evaluation of enzymatic activity of antioxidant enzymes as well as the content of non-enzymatic antioxidants is an indicator that can give a clear notion about the redox metabolism of a plant, which in turn can be translated as the nutraceutical quality of a crop by its antioxidant content. Based on this fact, the effect of Se on enzymatic and non-enzymatic antioxidants depends widely on the plant species used.

3.3 Selenium Biofortification

The treatment of SeO_2 increased Se content 236% over control, and the treatment of Cs-PAA + Se had an additional increase of 74% over the above (**Figure 2**). There was no significant difference in the accumulation of Se is the treatments of SeO_2 and Cs-PAA + SeO_2, it is possible that the difference is presented until a significant degradation of the polymer occurs, which may happen in a second growing season using the same substrate, therefore, it is feasible to use Cs-PAA + SeO_2 for biofortification.

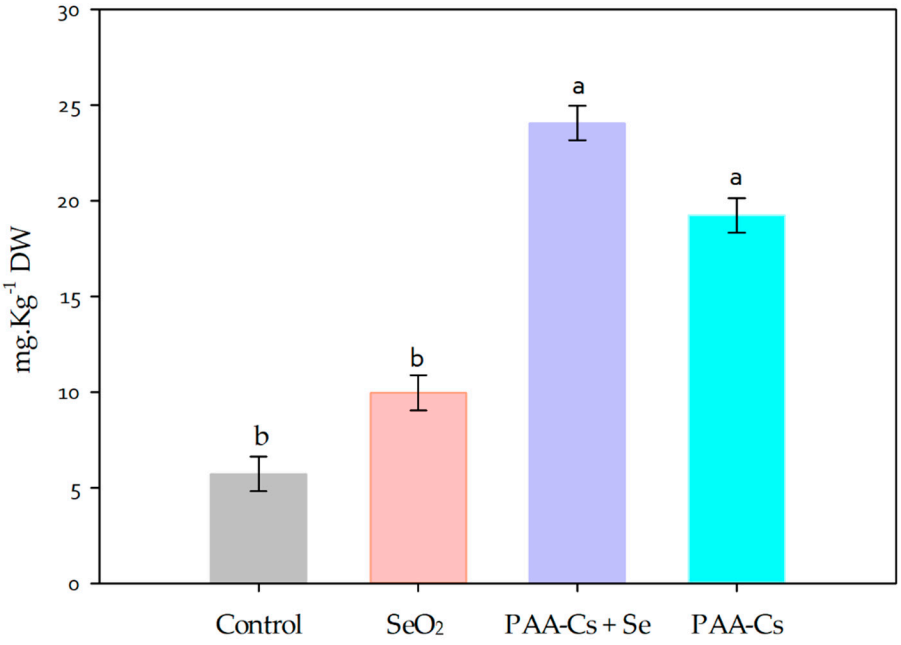

Figure 2. Mean values of the Se accumulation in lettuce plants. Means with the same letter are statistically equal (LSD, $p \leq 0.05$).

The results of the biofortification techniques of Se have a variable behavior according to the plant species, the form of application, dose and concentration of Se, and the production system implemented. For instance, in a soilless crop of lettuce[23] with applications of SeO_3 and SeO_4 in concentrations from 2 to 64 µmol/L in the nutrient solution, the authors reported that at the highest concentrations Se accumulation reached up to ~22 mg/Kg DW however it also resulted in Se toxicity with a decrease in biomass. Similarly, in lettuce seedlings [12] with H_2SeO_4 to the soil from 0.1 to 1 mg/Kg there was an increase in Se accumulation but it was toxic at the highest concentration; it accumulated up to 270 mg/Kg DW in the tissues but reducing the biomass up to 66% compared to the control. On the other hand, other studies[24] suggests the application of SeO_4^{2-} in conjunction with IO_3^- in order to achieve a double biofortification of lettuce 'Melodion' cv. in hydroponic cultivation, there is a synergistic interaction between both compounds with foliar applications, suggesting a transport through the phloem tissues. The values of selenium concentration obtained in this study seem adequate from the perspective of biofortification, since with the application of Cs-PAA + SeO_2 it was possible to biofortify Great Lakes lettuce, up to 24 mg Kg^{-1} DW, considering a portion of lettuce of 100 g FW, this adds up to 165 µg Se per serving of lettuce, which is an adequate amount for daily consumption since it exceeds the minimum requirement, but does not reach the doses that, under certain circumstances, could become harmful to the consumer. The recommended daily intake of Se in the human diet varies by region, in addition to the consumer particular health status. According to U.S. Food and Nutrition Board, the recommended intake is 60 to 75 µg Se daily[25]. However, in order to obtain the benefits that result from the intake of Se, such as reducing cancer risk, enhancing male fertility, and generally improving immune responses, it is necessary to increase this daily dose up to 200-300 µg daily[26]. Nevertheless, there are mixed reports about the recommended intake since according to the Nutritional Prevention of Cancer (NPC), they reported that the consumption of 200 µg a day in yeast supplements decreases the incidence only in people with low levels of Se in plasma, causing the opposite effect in people with adequate levels of Se[27]. Based on this fact, special attention should be paid to the amounts of accumulated Se by biofortified crops due to the small threshold of appropriate dose.

4 Conclusion

Our results indicate that Cs-PAA complexes can be beneficial in biofortification processes due to their tendency to increase the absorption of Se, as well as generating an increase in the activity of the GPX antioxidant enzyme, without affecting the development of the crop. Based on what has been observed, Se and the Cs-PAA complexes were effective to enhance plant resistances as well as the nutraceutical quality of Great Lakes lettuce.

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