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Comparison of Iodide, Iodate, and Iodine-Chitosan Complexes for the Biofortification of Lettuce

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Featured Application: Use of Cs-I complexes as material to biofortify soilless crops with iodine. under conditions of protected agriculture.

Abstract: Iodine is an essential trace nutrient for humans; its deficit can affect motor and cognitive development. Biofortifying crops with iodine is a way of promoting the adequate intake of this element. The uses of chitosan-iodine complexes for crop biofortification have not been previously studied. The present work evaluated the effects of KIO_3 and KI salts, chitosan-KIO_3 complex (Cs-KIO_3), and chitosan-KI complex (Cs-KI) application on lettuce, with a chitosan-only treatment as a control and water as the absolute control. Each treatment involved the application of 0, 5, and 25 mg I kg^{-1} soil applied before transplanting or 25 mg I kg^{-1} soil applied as split doses of 12.5 mg kg^{-1}, once immediately before transplanting and the second application 15 days later. Single application of Cs-KIO_3 at 5 and 25 mg I kg^{-1} increased lettuce biomass while the split-dose application (SDA) of Cs-KI (25 mg I kg^{-1}) led to a decrease in biomass. Maximum accumulation of iodine in lettuce was observed after the application of KIO_3 (25 mg I kg^{-1}) in two parts. This study shows that the use of chitosan complexes, especially Cs-KIO_3, may be a viable alternative for crop biofortification with iodine without affecting crop yields.

Keywords: trace element; biopolymer; iodine; complex; biofortification

1. Introduction

Iodine is an essential trace element for humans. Iodine deficit can cause iodine deficiency disorders (IDD), which, according to some estimates, affect around two billion people [1]. Recommended daily iodine intake levels are 90 µg for preschool children (0 to 59 months), 120 µg for children 6 to 12 years old, 150 µg for adults over 12 years old, and 200 µg for pregnant and nursing women, according to the World Health Organization (WHO), the United Nations Children's Fund (UNICEF), and the

International Council for Control of Iodine Deficiency Disorders (ICCIDD) [2]. Some experts believe that doses of 1–2 mg per day are probably safe for most people, although not all [3,4]. A worldwide initiative in 1920 led to the universal iodization of table salt, which led to a significant increase in iodine intake. Unfortunately, inorganic iodine salts have a disadvantage in that up to 20% can be lost through volatilization, on top of the percentages that are lost depending on the type of cooking used [5–7]. The WHO recommends that salt consumption not surpass more than 5 g per day, equivalent to >2 g of sodium per day, as high salt intake combined with insufficient potassium intake (<3.5 g per day) can provoke high blood pressure. Because of that, the WHO aims to reduce salt intake by 30% in the worlds' population by 2025 [8]. Thus, alternative options for adequate iodine intake while reducing salt consumption are necessary.

The iodine supplementation of foods or the biofortification of plant-based foods would be one alternative for maintaining iodine intake while reducing table salt consumption. The advantage of biofortification is that the iodine is found in organic forms that have greater bioavailability and stability against volatilization than the inorganic salt forms. Another advantage of biofortification is that it can be applied to frequently consumed or traditional crops in certain regions [9].

Iodine is not considered an essential nutrient for terrestrial plants; however, it plays an important part of algae metabolism [9]. The chemical forms of iodine most commonly used for crop biofortification are potassium iodide and potassium iodate. In lettuce, biofortification with different doses of iodide (I^-) and iodate (IO_3^-) cause changes in biomass, enzymatic activity, and antioxidant activity, suggesting they might cause toxicity in plants [10–14]. The type of iodine application, either sprayed on the foliage or applied directly to the growth substrate or soil, can affect the final concentrations of iodine in the edible parts of the plants. Some studies have found foliar iodine applications to be more effective than soil applications [11,15]. However, foliar applications are reportedly less effective for biofortifying fruits or seeds, supposedly due to lower iodine mobility through phloem when applied via foliar spray [9,16,17]. When iodine is applied to soil or substrate, its bioavailability depends on the volatilization rate and its interaction with other soil components like metal oxides or hydroxides. For example, $Fe(OH)_3$, $Al(OH)_3$, and MnO_2, can play an important role in the determination of iodine behavior in soil, both through inorganic iodine adsorption and iodine oxidation [18–20].

Chitosan (Cs) is a biodegradable, biological polymer that functions as a metal and trace metal complexing agent. Chitosan also possesses a wide variety of properties: it is a plant elicitor as it induces phytoalexin production; it protects plants from pathogen diseases as it inhibits microbial growth; it activates MAP-kinases and provokes chromatin alterations; and it participates in the synthesis of alkaloids and plant growth regulators. It is also biodegradable, biocompatible, induces several plant defense genes, and induces proteinase inhibitors. Responses to its application will depend on the concentration, plant species, and growth phase [21–24].

The electrostatic interactions between the amino group (positively charged; $-NH_2$) and iodate (negatively charged; $-IO_3$) are quite pronounced [7,25]. An increase in the degree of deacetylation of chitin leads to a greater complexing capacity, as that ability is related to the content of NH_2 [26]. To date, the exact mechanism by which the rhizosphere operates with the chitosan-iodine complexes is unknown. Iodide or iodate ions can be absorbed by the roots because they are bound to Cs only by electrostatic interactions that are weak [27]. It is possible that exchange reactions occur between the natural chelates of the roots (organic acids) and the chitosan-iodine complexes, this is due to the high molecular weight of the Cs that may not be absorbed. A study in sunflower with iron chelated by humic acids of low and high molecular weight refers to this [28]. On the other hand, it has been shown that Cs and Cs nanoparticles can be in the cell walls of the subsidiary cells of the lower epidermis [29]. However, no reports were found that indicated the similar effect on roots.

The present study was undertaken with the hypothesis that it would be possible to increase the absorption of iodine applied to substrate if it was applied as an iodine-chitosan complex. The objective was to determine if potassium iodide or iodate complexed with chitosan led to more effective lettuce biofortification than either salt in their uncomplexed forms.

2. Materials and Methods

2.1. Preparation of Cs-KI, Cs-KIO_3 Complexes and Iodine Salts

The Cs-KI and Cs-KIO_3 complexes were prepared in the Center for Applied Chemistry Research (CIQA). The chitosan used had a viscometric molecular weight of 200,000 g/mol and a 98% degree of deacetylation. First, a 1% Cs solution in 1% acetic acid (AcOH) was prepared by adding the Cs little by little over 3 h, with stirring at 300 rpm until completely dissolved, at a temperature of 60–65 °C. The resulting solution was filtered and adjusted to 1 L, for later use as the Cs controls.

For the iodine complexes, solutions of 0.1 mol dm^{-3} potassium iodine (KI) and 0.1 mol dm^{-3} potassium iodate (KIO_3) were dissolved in 1% Cs solution and adjusted to obtain complexes with Cs:I molar ratios of 5. Thus, complexes with 1.06 mg I per milliliter of complex solution were obtained.

For iodine salt-only treatments, solutions of 0.025 mol dm^{-3} KI and 0.025 mol dm^{-3} KIO_3 were prepared in 1 L deionized water. Each solution contained 3.17 mg I per milliliter.

2.2. Vegetable Material and Applied Treatments

The experimental work was performed in August 2017, in a greenhouse belonging to the Department of Horticulture, Agrarian Autonomous University Antonio Narro, in Saltillo, Mexico. Lettuce (*Lactuca sativa*), cv "Great Lakes," seeds were sown in 200-cell polystyrene trays filled with peat moss/perlite (1:1 *v*/*v*) mix. When the seedlings reached a size of approximately 15 cm, they were transplanted to 4 L, black polyethylene pots filled with peat moss and perlite mix (1:1 *v*/*v*). Before applying any treatments, a field capacity test was performed to evaluate the weight of the substrate. On average, a weight of 3 kg for substrate moistened to field capacity was obtained for each pot. The iodine concentrations used were 0, 5, and 25 mg kg^{-1} wet substrate, applied once, as well as 25 mg I kg^{-1} wet substrate, applied as two doses of 12.5 mg kg^{-1}: before and 15 days after transplanting (d.a.t).

2.2.1. Treatments Applied before Transplant

For the treatments applied prior to transplanting, the total volume of substrate per pot was divided into three parts, while the iodine salt, Cs-KI, and Cs-KIO_3 treatments were split into two parts. The first part of each treatment was applied over the first third of substrate in the pot. Another third of substrate was added on top and the remaining half of the treatment was applied over the top. Afterwards, the remaining third of substrate was added to fill the pot. The lettuce seedlings were transplanted to those pots. We assume this procedure allows the plant roots to have I^- and IO_3^- as salts or Cs complexes available as they grow.

Absolute controls (AC) only had water applied during the pot-filling process. Cs controls (CsC) had a total of 70.3 mL 1% Cs solution applied, to reach 0.2 g Cs per kg of substrate. Each treatment received the same final volume of Cs.

For the 5 mg I kg^{-1} substrate iodine salt treatments, a total of 4.7 mL of 0.025 mol dm^{-3} KIO_3 and 0.025 mol dm^{-3} KI, respectively, were added per pot. The 25 mg I treatments required the addition of 23.6 mL of each respective solution per pot.

A total of 14.07 mL of 0.1 mol dm^{-3} Cs-KI and 0.1 mol dm^{-3} Cs-KIO_3 solutions were added to the respective pots for the 5 mg I kg^{-1} treatments. To this, 56.2 mL 1% Cs solution per pot were also added in order to have the same Cs volume as in the controls. For the 25 mg I treatments, 70.34 mL of 0.1 mol dm^{-3} Cs-KI or 0.1 mol dm^{-3} Cs-KIO_3 was added per pot.

2.2.2. Treatments Applied before and after Transplant

For each split dose application-SDA (12.5 and 12.5 mg kg^{-1}) treatment, the same pot-filling procedure described above was used. All treatments received concentrations of 25 mg I kg^{-1} substrate. The treatment volumes were divided in two, with each half applied while filling the pots by thirds. The last half of the treatment was applied after adding the second third of substrate, then the pot

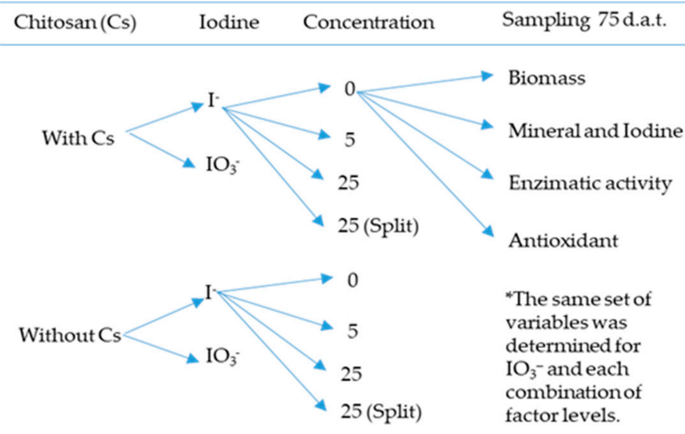
was filled by adding the last third of substrate. Afterwards, transplanting of the lettuce seedlings was performed as normal. The second application of the different treatments was applied by drench directly to the substrate at 15 d.a.t.

The experimental factors were arranged into a 2 × 2 × 4 factorial array (iodine species × Cs polymer × concentration) and a totally randomized experimental design was utilized. The treatments thus evaluated were: (1) Control-Cs (CsC); (2) absolute control (AC); (3) KIO_3 5 mg I kg^{-1} with Cs; (4) KIO_3 25 mg I kg^{-1} with Cs; (5) KIO_3 25 mg I kg^{-1} with Cs, split-dose application; (6) KIO_3 5 mg I kg^{-1} without Cs; (7) KIO_3 25 mg I kg^{-1} without Cs; (8) KIO_3 25 mg I kg^{-1} without Cs, split-dose application; (9) KI 5 mg I kg^{-1} with Cs; (10) KI 25 mg I kg^{-1} with Cs; (11) KI 25 mg I kg^{-1} with Cs, split-dose application; (12) KI 5 mg I kg^{-1} without Cs; (13) KI 25 mg I kg^{-1} without Cs; and, (14) KI 25 mg I kg^{-1} without Cs, split-dose application. Two Cs controls and two absolute controls were also included, for 16 total treatments. The control groups were included in order to evaluate the potential effects that location within the greenhouse, where the rest of the treatments where distributed, might have. There were 12 experimental units per treatment, from which 6 plants were chosen at random for evaluation. A total of 96 plants were used for experimental evaluations.

An automated, single step drip irrigation system was used throughout the experiment. Steiner nutrient solution (25%) was used for irrigation. A volume of 0.5 L solution was applied two times a day per pot. The solution pH was adjusted to 5.5–6.5 with phosphoric acid. Throughout the cultivation cycle, the electrical conductivity (EC) of the nutrient solution was maintained at approximately 1.40 mS cm^{-1}. Preventative treatment for whitefly was also applied. Within the greenhouse, the minimum temperature reached 8.3 °C, while the average was 17.7 °C, and the high was 32.4 °C. The minimum relative humidity was 30.3%, while the average and high were 54.8% and 75.5%, respectively. The mean photosynthetically active radiation (PAR) was 432.3 µmol m^{-2} s^{-1} while the maximum was 864.7 µmol m^{-2} s^{-1}, as measured with a WatchDog 1000 Series Micro Station, Spectrum technologies Inc., Aurora, IL, USA).

2.3. Sampling

The lettuces were harvested at 75 d.a.t. The 6 randomly chosen plants from each treatment were measured and weighed for their evaluation of biomass, mineral content, and iodine content (see Scheme 1). From those same plants, small samples from the central part of the heads were collected for the biochemical analyses. These lettuce head samples were kept in deep freeze and lyophilized.



Scheme 1. Treatments and general variables evaluated.

2.4. Biomass Production and Yields

Lettuce heads and leaves were weighed on an precision balance (OHAUS). The values were recorded as the biomass fresh weight (FW). A small part from the central part of each head (~10 g) was taken and stored at −86 °C for later determination of antioxidant and enzymatic activity. The heads'

remaining fresh matter was placed in paper bags. The leaves were separated and also placed in paper bags. Both head stems and leaves were dried in dehydrating ovens for 72 h at 75 ± 5 °C. Afterwards, their dry weights (DW) were recorded. Finally, mixed samples were prepared by combining the dry leaves and heads from each replica for each treatment. The mixed samples were placed in hermetic plastic bags for mineral analysis.

2.5. Mineral Content

Determination

Mineral content was determined from mixed samples of leaves and heads from 6 randomly chosen plants per treatment. Total nitrogen (N) content was determined according to the Kjeldahl method [30]. A sample of dry matter (0.05 g) was weighed out and then acid digested in 4 mL of digest mix (1 L of concentrated sulfuric acid with 25 g of potassium sulfate, 10 g of mercury red oxide, and 25 mL of saturated copper sulfate solution). The acid digest was then distilled along with 25 mL 50% sodium hydroxide. From the resulting distillate, 30 mL were placed in a beaker containing 2.2% boric acid and 4 drops of bromocresol green and methyl red. The samples were titrated with 0.025 N sulfuric acid. N content was calculated from the volume of sulfuric acid consumed.

Phosphorus content was determined through spectrophotometry [30]. Readings were performed on a Genesys 10S ultraviolet–visible (UV–Vis) spectrophotometer (Thermo Scientific, Waltham, MA, USA) at 640 nm. The contents of K, Mg, Ca, Na, Mn, Zn, and Fe were determined by atomic absorption spectrometry following wet digestion [31]. One gram of dry matter was digested with nitric acid at 100 °C. Afterwards, the solution was filtered through Whatman, Buckinghamshire, United Kingdom (No. 42 ash-free) paper filters, diluted accordingly, and measured in a Varian AA-1275 flame atomic absorption spectroscope (Palo alto, CA, USA).

2.6. Iodine Content Determination

The alkaline ash technique [32,33] was used to determine iodine content. A sample of dried, ground leaves and heads (0.5 g) was weighed out. Six replicas per treatment were evaluated. The sample was placed in crucible of known, constant weight and 2 mL 2 mol dm^{-3} KOH and 1 mL 2 mol dm^{-3} KNO_3 were added. After adding the reagents, pre-digestion took place while incubating in a stove at 100 °C for 2 h. The crucibles were placed in a muffle furnace at 580 °C for 3 h. After cooling back down to room temperature, the ashes were transferred to a conical tube for extraction with 2 mL 2 mM KOH. The samples were centrifuged at 12,000 rpm for 15 min. Finally, 1 mL of supernatant was decanted, and the volume adjusted to 10 mL with 2 mol dm^{-3} KOH. Quantification was performed with an Agilent 725 ICP-OES (Inductively coupled plasma-optical emission spectrometry, Santa Clara, CA, USA).

2.7. Chlorophyll Content Determination

The contents of chlorophyll a (Chl a), chlorophyll b (Chl b), and total chlorophyll were determined according to the technique described in Munira et al. [34]. A sample (1 g) of fresh plant material was mixed with 5 mL 90% acetone. A pinch of magnesium carbonate was added to protect and stabilize the chlorophylls. From the homogenized mixtures, 2 mL were taken, placed in a 2 mL tube, and centrifuged for 5 min. at 10,000 rpm and 4 °C. The supernatants were decanted and the absorbances of Chl a and Chl b were read at 663 nm and 645 nm, respectively. The total chlorophyll, Chl a, and Chl b content were expressed in mg g^{-1}, and were calculated according to the following formulas:

$$\text{Chlorophyll a } (\text{mg}\cdot\text{g}^{-1}) = 25.38 \times A_{663} + 3.64 \times A_{645} \quad (1)$$

$$\text{Chlorophyll b } (\text{mg}\cdot\text{g}^{-1}) = 30.38 \times A_{645} - 6.58 \times A_{663} \quad (2)$$

$$\text{Chlorophyll, total } (\text{mg}\cdot\text{g}^{-1}) = 18.8 \times A_{663} + 34.02 \times A_{645} \quad (3)$$

2.8. Biomolecule Extraction

Lyophilized leaves ground into fine powder with a pestle and mortar were used for biomolecule extraction. From each treatment, 200 mg samples of lyophilized leaves were placed in polypropylene microtubes. Polyvinylpyrrolidone (20 mg) and 1.5 mL 0.1 mol dm^{-3} phosphate buffer (pH 7–7.2) were added, the samples were sonicated for 5 min. and afterwards, centrifuged at 12,500 rpm for 10 min. at 4 °C. The supernatants were decanted and filtered through a nylon membrane. The filtrates were diluted 1:15 with phosphate buffer [35].

2.9. Total Protein (TP) Content Determination

The technique described by Bradford [36] was used for total protein content determination. From the biomolecule extracts, 100 µL of extract were taken, placed in a test tube, and 1 mL of Bradford (Hercules, CA, USA) reagent was added. The samples with Bradford reagent were left to incubate for 5 min. Afterwards, the absorbance was read at 595 nm in a Genesys 10S UV–Vis spectrophotometer (Thermo Scientific). The results were recorded, and concentrations were extrapolated from a calibration curve prepared with bovine serum albumin (BSA). The protein concentrations were reported as mg g^{-1}.

2.10. Free and Cell Wall-Bound Phenol Determination

Phenolic compound content was determined according to the technique described by Gurr et al. [37]. Extraction was performed on 6 dried samples from each treatment. Methanol (1 mL) was added to each sample, the samples were vortexed, then centrifuged at 13,500 rpm for 15 min. The supernatants were decanted into vials and stored for the determination of free phenols. The remaining pellets were incubated with 0.25 mL 2 mol dm^{-3} NaOH for 16 h at 70 °C. Then, 0.25 mL 2 N hydrochloric acid was added, and the samples were centrifuged at 13,500 rpm for 15 min. The resulting pellets were discarded, and the supernatants saved, as they were considered to contain the cell wall-bound phenols. To quantify the free and cell wall-bound phenols, 20 µL of the corresponding supernatant were taken and mixed with 980 µL distilled water, then 100 µL Folin–Ciocalteau reagent was added, and the mixture left to incubate for 5 min. Subsequently, 600 µL of sodium bicarbonate solution saturated with 0.1 mol dm^{-3} NaOH were added. The samples were left to incubate for 2.5 h. The sample absorbance was read at 725 nm and the results were expressed as mg of gallic acid equivalents per g^{-1} dry weight (DW).

2.11. Superoxide Dismutase (SOD) Determination

The biomolecule extract was assayed for superoxide dismutase (SOD) activity using the CAYMAN®SOD, assay kit (Ann Arbor, MI, USA). SOD activity can be quantified spectrophotometrically by measuring the oxidation of WST (water soluble tetrazolium salt) to WST-formazan by superoxide ions created by xanthine (X)/xanthine oxidase complexes. The inhibition of WST oxidation is attributed to the neutralization of superoxide radicals by SOD. The units of SOD activity were expressed in U/mL. The microwell assay plates were read at 450 nm in an Elx808 plate reader (BioTek, Winooski, VT, USA).

2.12. Catalase Activity Determination

Catalase activity was quantified by spectrophotometry. Two reaction times, T0 and T1, were recorded for this technique. The blank solution was prepared by mixing 0.1 mL biomolecule extract, 1 mL phosphate buffer (pH 7.2), and 0.4 mL of 5% H_2SO_4. For measuring the reactions at T0, 0.1 mL of biomolecule extract was mixed with 1 mL 100 mM H_2O_2 and immediately after, 0.5 mL 5% H_2SO_4. For the T1 samples, the same mix was prepared except that the 0.5 mL 5% H_2SO_4 was added after 1 min. The reaction between extract and peroxide occurred at 20 °C with constant agitation. Finally, the consumption of H_2O_2 was determined by reading the absorbance at 270 nm in a

UV–Vis spectrophotometer. The catalase activity units were expressed in mM H_2O_2 per total protein content [38].

2.13. Glutathione Peroxidase (GPX) Quantification

Quantification of glutathione peroxidase was performed using the method described by Xue et al. [39], using H_2O_2 as a substrate. Biomolecule extract (0.2 mL), 0.1 mol dm^{-3} reduced glutathione (0.4 mL), and 0.067 mol dm^{-3} Na_2HPO_4 (0.2 mL) were placed in a test tube. The mixture was heated in a 25 °C water bath for 5 min., then 1.3 mM H_2O_2 (0.2 mL) was added to start the catalytic reaction. The mixture was allowed to react for 10 min. and then was stopped with the addition of 1 mL 1% trichloroacetic acid. The mixture was left in an ice bath for 30 min. Afterwards, it was centrifuged at 3000 rpm for 10 min. A sample of supernatant (0.48 mL) was placed in a test tube, along with 2.2 mL of 0.32 mol dm^{-3} Na_2HPO_4 and 0.32 mL of 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The absorbance of the reaction mixture was read at 412 nm in a UV–Vis spectrophotometer. The catalytic activity of glutathione peroxidase was expressed as mM glutathione per minute per total protein content.

2.14. Glutathione (GSH) Quantification

Glutathione (GSH) content was quantified according to the spectrophotometric technique established by Xue et al. [39], utilizing the reaction with 5,5′-DTNB. Biomolecule extract (0.48 mL), 0.067 mol dm^{-3} Na_2HPO_4 (2.2 mL), and 1 mM DTNB dye (0.32 mL) were placed in a test tube and mixed. The absorbance of the samples was read at 412 nm in a UV–Vis spectrophotometer. Values were reported as mg L^{-1}.

2.15. Antioxidant Capacity Determination by ABTS $^{\bullet+}$

Total antioxidant content was determined using the CAYMAN®Antioxidant assay kit. The assay determines antioxidant content based on a sample's ability to inhibit the oxidation of ABTS$^{\bullet+}$ (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]). The quantity of ABTS radicals produced was determined by reading the microwell plate at 405 nm. The reported values were expressed in mM.

2.16. Statistical Analyses

A completely randomized experimental design was used, with 6 repetitions per treatment. A single pot with one plant was considered an experimental unit. The collected data was subjected to analysis of variance (ANOVA) and to separation of means using Fisher's least significant difference (LSD, p = 0.05) tests, using the 2018 InfoStat statistical analysis software package (InfoStat Group, Córdoba, Argentina).

3. Results

3.1. Biomass Production and Yields

According to the ANOVA presented in Table 1, the concentration of iodine (C) only had no impact on the leaf dry weight (LDW) of lettuce. On average, there were positive effects on biomass at iodine concentrations of 5 mg kg^{-1} observed. No interactions between C and the presence of Cs or the different iodine species were observed. Also, there were no treatments that affected all the biomass variables evaluated. For example, some treatments, such as Cs-KIO_3 (5 mg kg^{-1}), significantly increased leaf dry weight (LDW) while others (doubly applied Cs-KI, 25 mg kg^{-1}) significantly decreased the lettuce head fresh weight (HFW). It was only for the leaf fresh weight (LFW) that an interaction between iodine and Cs could be observed, as that value increased following application of Cs-KIO_3 (5 mg kg^{-1}). The two factors together appeared to play an important role in biomass increase. However, in general, for the evaluated biomass parameters, the source of iodine was an important determining factor, since there were significant differences associated with this variable, except for the head dry weight parameter.

Table 1. Comparison of lettuce biomass means.

Treatments	Concentration (mg I kg^{-1} Substrate)	g plant^{-1} (n = 6)			
		LFW	HFW	LDW	HDW
CsC	0	212.00 b–d	667.63 ab	17.62 c–e	28.48 a–d
Cs-KIO_3	5	294.32 a	695.52 a	24.27 a	34.05 a
Cs-KIO_3	25	264.95 ab	634.78 ab	22.50 ab	33.80 a
Cs-KIO_3 SDA	25	235.85 a–c	582.67 ab	19.58 a–c	29.28 a–c
AC	0	146.72 e–f	534.50 ab	14.63 de	22.37 d–e
KIO_3	5	201.42 c–f	579.57 ab	18.50 b–d	19.13 ef
KIO_3	25	201.17 c–f	595.68 ab	16.97 c–e	24.65 b–e
KIO_3 SDA	25	178.15 c–f	499.05 b	16.65 c–e	19.95 ef
CsC	0	196.97 c–f	596.07 ab	17.57 c–e	24.20 b–e
Cs-KI	5	190.12 c–f	577.57 ab	16.47 c–e	24.92 b–e
Cs-KI	25	155.77 d–f	526.18 ab	13.67 e	22.70 c–e
Cs-KI SDA	25	143.04 f	280.17 c	14.73 de	15.03 f
AC	0	194.55 c–f	499.48 b	17.37 c–e	25.87 b–e
KI	5	214.85 b–d	587.67 ab	19.13 b–d	29.57 ab
KI	25	205.10 b–e	581.43 ab	18.90 b–d	27.48 a–d
KI SDA	25	177.78 c–f	495.32 b	17.43 c–e	24.95 b–e
Concentration (C)		0.0311	0.0077	ns	0.0198
Chitosan (Cs)		0.0494	ns	ns	ns
Chemical species (CSp)		0.0041	0.0116	0.0229	ns
C × Cs		ns	ns	ns	ns
C × CSp		ns	ns	ns	ns
Cs × CSp		<0.0001	ns	0.0001	<0.0001
C × Cs × CSp		ns	ns	ns	ns

Means with the same letter are statistically identical (least significant difference (LSD), $p \leq 0.05$); sample size, 6 (n = 6), ns, not significant. SDA, split dose application; LFW, leaf fresh weight; HFW, head fresh weight; LDW, leaf dry weight; HDW, head dry weight.

3.2. Mineral Content

3.2.1. Macronutrient Content

Neither iodine source, be it I^- or IO_3^-, generally appeared to have antagonistic effects on the macroelements evaluated. The iodine species used, their concentrations, and the presence of Cs polymer had effects on various elements, when compared to other treatments, although there were hardly any differences between treatments and their respective controls with and without Cs (Table 2). Only magnesium concentrations increased significantly after application of 5 mg I kg^{-1} Cs-KI. There were significant differences among the Cs controls, although this was probably due to their location within the greenhouse. This is why the increases in Ca and Na after application of Cs-KI (5 mg kg^{-1}) cannot be considered significant, because even though their closest, neighboring Cs controls had lower contents of those elements, comparison with the other chitosan controls showed no differences. The interaction between the effects of concentration and iodine species were statistically significant for the concentrations of almost all the elements evaluated, except for K. On average, application of IO_3^- salts led to greater accumulation of macroelements than their I^- counterparts. However, these differences were not significant with respect to the controls.

3.2.2. Micronutrient Content

The source of iodine (I^- or IO_3^-) had a significant effect on the concentrations of microelements in lettuce. As observed with the macroelements, there were no antagonistic effects between the application of iodine and the concentrations of microelements (Table 3). Apart from that, there was a significant increase in Mn when Cs-KI is applied (single application, 25 mg I kg^{-1}). On the other hand, it appears that the interaction between iodine concentrations and Cs did not exert any significant effects on microelement concentrations.

Table 2. Macroelement contents in mixed samples of lettuce leaves and heads.

Treatments	Concentration (mg I kg^{-1} Substrate)	% Dry Weight (n = 6)					
		N	P	K	Ca	Mg	Na
CsC	0	0.82 b	0.43 a	13.27 a	4.20 ab	5.78 b	13.97 a
Cs-KIO_3	5	1.29 ab	0.40 a	12.01 ab	2.25 cd	3.91 c	7.57 b
Cs-KIO_3	25	1.48 a	0.24 b–e	10.81 a–c	2.08 c–e	3.62 cd	3.91 bc
Cs-KIO_3 SDA	25	1.40 ab	0.25 b–d	8.06 b–d	1.00 d–f	2.20 de	3.24 c
AC	0	1.33 ab	0.26 b–d	8.15 b–d	1.71 c–f	1.91 e	3.16 c
KIO_3	5	1.21 ab	0.20 c–g	7.73 b–d	1.87c–f	2.25 c–e	3.41 c
KIO_3	25	1.55 a	0.22 b–f	8.06 b–d	2.95 bc	3.08 c–e	6.03 bc
KIO_3 SDA	25	1.36 ab	0.29 b	6.61 cd	1.50 d–f	2.70 c–e	4.32 bc
CsC	0	1.21 ab	0.20 c–g	6.78 cd	1.41 d–f	2.33 c–e	3.78 bc
Cs-KI	5	1.43 ab	0.28 bc	7.48 b–d	5.24 a	7.90 a	14.26 a
Cs-KI	25	1.19 ab	0.24 b–d	6.91 cd	1.29 d–f	2.33 c–e	3.25 c
Cs-KI SDA	25	1.01 ab	0.12 g	5.49 d	1.21 d–f	2.20 de	3.29 c
AC	0	1.13 ab	0.15 e–g	5.53 d	0.75 ef	2.04 de	2.62 c
KI	5	1.18 ab	0.13 g	8.02 b–d	0.96 d–f	2.37 c–e	5.41 bc
KI	25	0.93 ab	0.14 fg	6.15 cd	1.08 d–f	1.91 e	2.95 c
KI SDA	25	1.07 ab	0.19 d–g	4.16 d	0.67 f	1.54 e	2.53 c
Concentration (C)		ns	0.0001	0.0004	<0.0001	<0.0001	ns
Chitosan (Cs)		ns	<0.0001	<0.0001	<0.0001	<0.0001	0.0107
Chemical Species (CSp)		0.0147	<0.0001	<0.0001	<0.0001	0.0368	ns
C × Cs		ns	<0.0001	ns	<0.0001	<0.0001	ns
C × CSp		0.006	<0.0001	ns	<0.0001	<0.0001	0.0061
Cs × CSp		ns	ns	0.0049	0.0002	ns	ns
C × Cs × CSp		ns	0.0006	ns	<0.0001	<0.0001	ns

Means with the same letter are statistically identical (LSD, $p \leq 0.05$); sample size, 6 (n = 6), ns, not significant.

Table 3. Microelement content in mixed samples of lettuce leaves and heads.

Treatments	Concentration (mg I kg^{-1} Substrate)	mg kg^{-1} Dry Weight (n = 6)			
		Zn	Fe	Mn	Cu
CsC	0	49.24 a	166.17 a–c	86.49 de	3.82 ab
Cs-KIO_3	5	45.89 ab	156.47 a–d	79.15 e	4.16 a
Cs-KIO_3	25	37.77 b	157.72 a–d	88.35 de	3.83 ab
Cs-KIO_3 SDA	25	40.05 ab	158.38 a–d	90.40 c–e	3.99 a
AC	0	39.24 ab	171.09 ab	97.76 b–e	2.99 ab
KIO_3	5	41.25 ab	181.96 a	96.31 b–e	2.99 ab
KIO_3	25	42.56 ab	132.33 b–e	106.89 b–e	3.49 ab
KIO_3 SDA	25	42.72 ab	143.96 a–e	125.02 a–c	3.66 ab
CsC	0	39.56 ab	133.65 b–d	98.74 b–e	2.83 ab
Cs-KI	5	39.91 ab	133.55 b–e	131.72 ab	3.66 ab
Cs-KI	25	41.75 ab	137.41 b–e	150.55 a	3.16 ab
Cs-KI SDA	25	41.89 ab	138.14 b–e	116.86 a–d	3.49 ab
AC	0	36.09 b	120.26 de	85.00 de	3.00 ab
KI	5	37.59 b	100.78 e	107.77 b–e	2.99 ab
KI	25	37.91 ab	125.86 c–e	120.70 a–d	3.33 ab
KI SDA	25	36.56 b	129.45 b–e	108.19 b–e	2.49 b
Concentration (C)		ns	ns	0.0001	ns
Chitosan (Cs)		0.0177	0.0333	ns	0.0012
Chemical Species (CSp)		0.0036	<0.0001	<0.0001	0.0013
C × Cs		ns	ns	ns	ns
C × CSp		ns	0.0051	0.0003	ns
Cs × CSp		ns	ns	<0.0001	ns
C × Cs × CSp		0.0492	0.0211	ns	ns

Means with the same letter are statistically identical (LSD, $p \leq 0.05$); sample size, 6 (n = 6), ns, not significant.

3.2.3. Iodine and Minerals Correlation Analysis

A general analysis of correlation between iodine and each mineral element was carried out (Table 4). Of the elements evaluated, manganese, potassium, and calcium demonstrated significant correlations. Manganese had a positive correlation while for the other two, the correlation was negative. Regardless, the correlation values of each element with iodine are low. In that case, biofortification with iodine is not expected to interfere with the assimilation or accumulation of other mineral elements. There are various scales used to score Spearman correlation coefficients [40]. For this research, scale 2 was chosen. That scale indicates that a Spearman coefficient greater than |0.5| corresponds to a moderately strong correlation.

Table 4. Correlation analysis of iodine and minerals.

Treatments	Variable 1	Variable 2	Spearman	*p*-value
General treatment analysis	Iodine	Zinc	−0.04	0.7056
		Manganese	0.4	0.0001
		Iron	−0.06	0.5316
		Copper	−0.03	0.7894
		Sodium	−0.16	0.1275
		Potassium	−0.31	0.0018
		Nitrogen	0.05	0.6013
		Magnesium	−0.18	0.0827
		Phosphorus	−0.17	0.0951
		Calcium	−0.22	0.0301

Values of $\alpha \leq 0.05$ are significant. Spearman correlation coefficients greater than +0.5 indicate positive, moderately strong correlations or less than −0.5 indicate negative, moderately strong correlations, according to a previously described scale [40].

3.3. Iodine Content in Lettuce

The highest concentrations of iodine were seen in lettuce subjected to the SDA treatments (25 mg I kg^{-1} KI or KIO_3), regardless of Cs (Figure 1). SDA of KI and Cs-KI increased lettuce iodine concentrations to levels 77.9- and 63.7-times, respectively, above the corresponding controls. Similarly, SDA of KIO_3 and Cs-KIO_3 increased concentrations by factors of 97.4 and 36.4, respectively. The highest recorded concentration was 103.16 mg I kg^{-1} dry weight (DW), following SDA of KIO_3 (25 mg I kg^{-1}). Apart from those results, single applications of Cs- KIO_3 and KIO_3 (25 mg I kg^{-1}) also led to improvements in iodine concentrations (17.4- and 18.2-times above control concentrations, respectively). Single applications of KI and Cs-KI (25 mg I kg^{-1}) increased iodine concentrations by factors of 14.4 and 24.4, respectively. However, these improvements were not significant. According to the analysis of variance the iodine species did not have a significant impact on the results, rather the concentrations applied, and the presence of chitosan were the statistically important factors. The only interaction with significant differences that the iodine species factor had was with the chitosan factor (Figure 1).

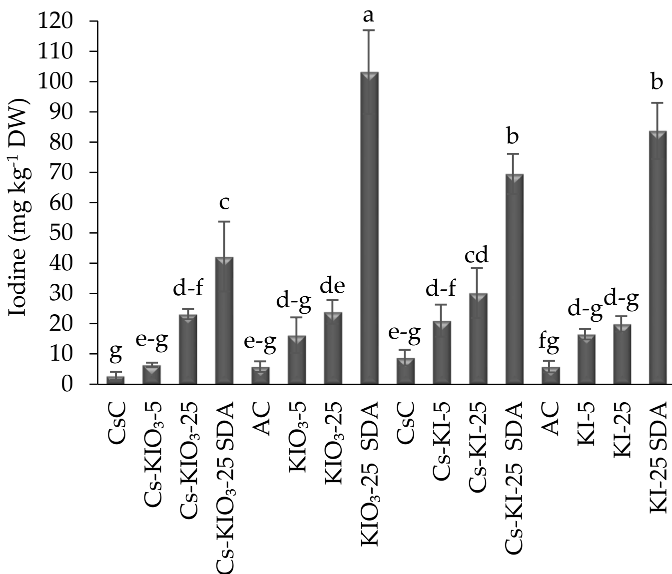


Figure 1. Iodine concentrations in mixed samples of lettuce leaves and heads following iodine salt treatments with and without chitosan. Means with the same letter are statistically identical (LSD, $p \leq 0.05$); the bars represent the standard error (n = 6).

3.4. Cell Wall-Bound and Free Phenol Content

Only KIO_3 at high concentrations (25 mg I kg^{-1}), both singly and doubly applied, led to increases in wall-bound phenols compared to the absolute controls (Figure 2). None of the factors alone had significant effects on phenol concentrations. However, the interaction between the iodine species and chitosan factors led to significant differences between treatments, as did the interaction between all three experimental factors. Conversely, there was neither a positive nor negative effect on the concentration of free phenols. Overall, the presence or absence of Cs, along with either iodine salt, had little to no effect on phenol content.

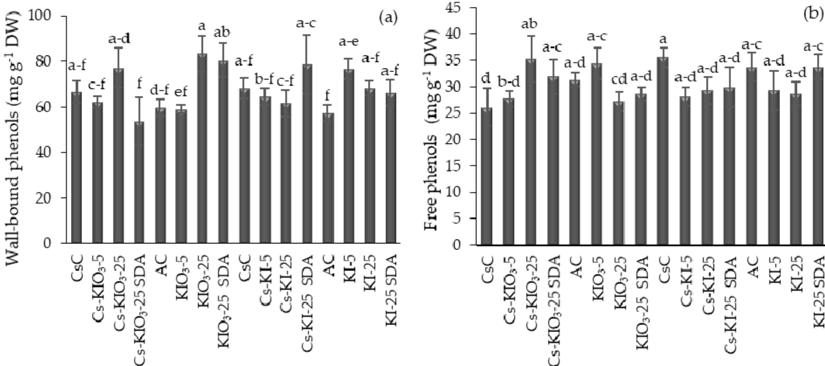


Figure 2. (**a**) Content of cell wall-bound phenols. (**b**) Content of free phenols measured in dry lettuce samples. Means with the same letter are statistically identical (LSD, $p \leq 0.05$); the bars represent the standard error (n = 6).

3.5. Chlorophyll Content

According to the analysis of variance, only the interaction between chitosan and the different iodine species had an effect on Chl a and total chlorophyll (Ct). However, with respect to the controls, there were no significant differences (Table 5). The Chl b concentration did not change after any treatment. In general, the application of I^- or IO_3^- did not exert significant changes in chlorophyll content, either with or without chitosan.

Table 5. Content of chlorophyll a, chlorophyll b, and total chlorophyll.

Treatments	Concentration (mg I kg^{-1} Substrate)	mg g^{-1} Dry Weight (n = 6)		
		Chl a	Chl b	Ct
CsC	0	0.10 b	0.05 b	0.15 c
Cs-KIO_3	5	0.11 b	0.09 ab	0.19 bc
Cs-KIO_3	25	0.10 b	0.09 ab	0.19 bc
Cs-KIO_3 SDA	25	0.19 ab	0.12 ab	0.31 a–c
AC	0	0.18 ab	0.18 ab	0.36 a–c
KIO_3	5	0.28 a	0.19 ab	0.47 a–c
KIO_3	25	0.18 ab	0.33 a	0.51 a–c
KIO_3 SDA	25	0.32 a	0.24 ab	0.56 ab
CsC	0	0.17 ab	0.12 ab	0.30 a–c
Cs-KI	5	0.25 ab	0.12 ab	0.36 a–c
Cs-KI	25	0.28 a	0.12 ab	0.39 a–c
Cs-KI SDA	25	0.28 a	0.31 ab	0.59 a
AC	0	0.17 ab	0.17 ab	0.35 a–c
KI	5	0.11 b	0.08 ab	0.19 bc
KI	25	0.18 ab	0.09 ab	0.27 a–c
KI SDA	25	0.30 a	0.12 ab	0.42 a–c
Concentration (C)		0.038	ns	ns
Chitosan (Cs)		ns	ns	ns
Chemical Species (CSp)		ns	ns	ns
C × Cs		ns	ns	ns
C × CSp		ns	ns	ns
Cs × CSp		0.0035	ns	0.0089
C × Cs × CSp		ns	ns	ns

Means with the same letter are statistically identical (LSD, $p \leq 0.05$); sample size, 6 (n = 6), ns, not significant. Chl a, chlorophyll a; Chl b, chlorophyll b; Ct, chlorophyll total.

3.6. Enzymatic Activity, Antioxidants, and Proteins

No experimental treatment produced significant differences, compared to the chitosan and absolute controls, for superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPX), nor total antioxidant activity (Figure 3). It appears that iodine treatments did not directly either contribute to an increase or decrease in catalytic and antioxidant activity, presumably because there was no plant stress generated. However, total protein content (TP) increased considerably after KI treatments, both at low (5 mg I kg^{-1}) and high (25 mg I kg^{-1}) doses applied either once or as split dose.

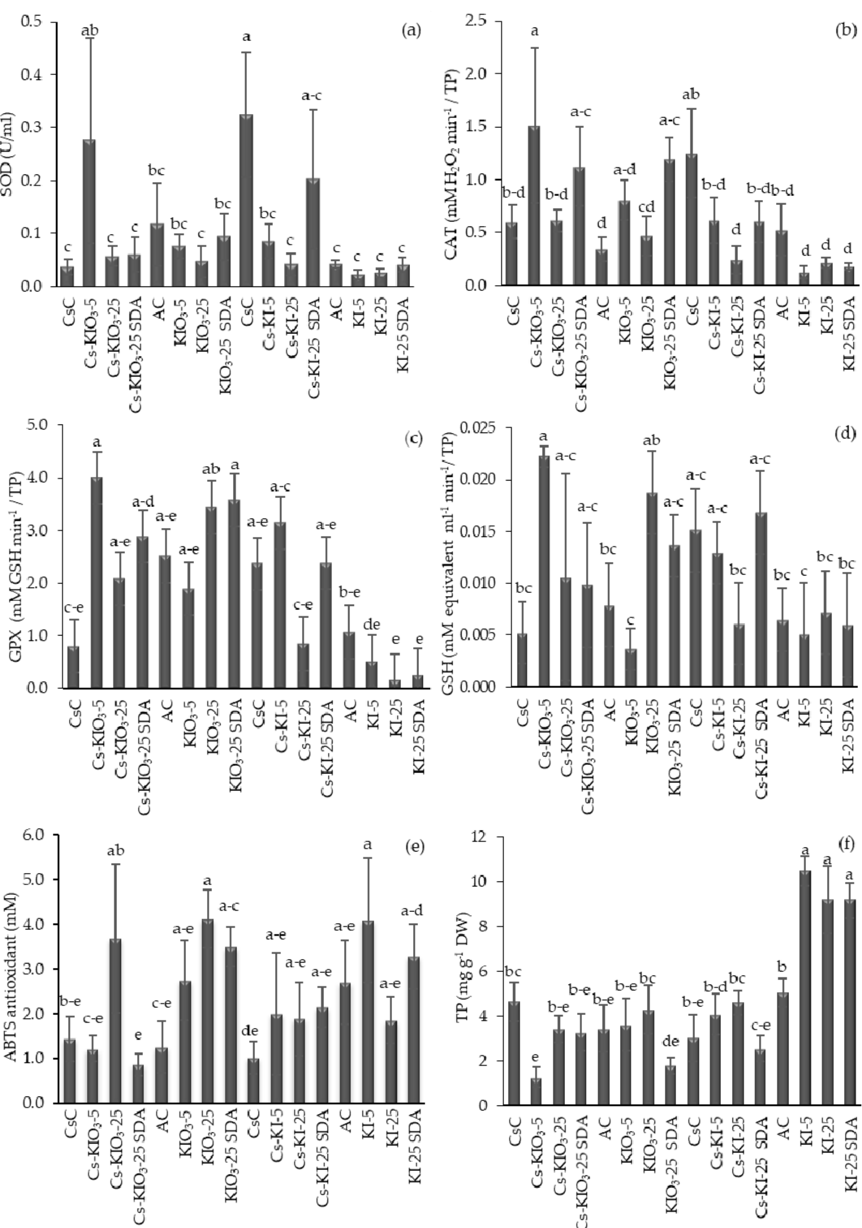


Figure 3. Changes in enzymatic, antioxidant, and protein content of lettuce after treatment with iodine and chitosan. (**a**) Superoxide dismutase, SOD; (**b**) catalase, CAT; (**c**) glutathione peroxidase, GPX; (**d**) reduced glutathione, GSH; (**e**) total antioxidant content; (**f**) total protein, TP. Means with the same letter are statistically identical (LSD, $p \leq 0.05$); sample size, 6 (n = 6); the bars represent the standard error. Sample size was 6 (n = 6) for all measurements except SOD (n = 5).

Despite the fact that there are differences in several enzymes in one factor or between factors detected in ANOVA, there is no enzyme activity that has been increased by any treatment.

4. Discussion

Four possible outcomes for iodine biofortification have been described: (1) it has a wholly negative impact on plant growth and other physiological and biochemical variables; (2) it has no effect on plants; (3) it has a wholly positive impact on plant growth and other physiological and biochemical variables; or (4) it has mixed positive and negative effects on plants [9,41–43]. With the exception of three specific treatment combinations, there were no changes in lettuce biomass. Something similar happened with the concentrations of mineral nutrients, which potentially indicates an ideal scenario where increases in iodine content do not antagonize the accumulation of other nutrients such as iron and zinc. Biofortification with iodine was achieved and we found greater concentrations after iodine treatments applied as two doses. The working hypothesis was that the presence of chitosan would augment the absorption of iodine in lettuce plants, but the results did not show any appreciable changes for treatments that included chitosan. There have only been two similar studies that investigate the uses of chitosan biopolymers with iodine, applied as thin films. One involved the application of Cs-KI films on tomato plants and found that it did not affect the antioxidant activity of the fruit [42]. The other applied edible films of Cs-KIO_3 to hot pepper and found that the iodine ion does not leach away due to the strong ionic interactions between the chitosan cationic groups and IO_3^- [7].

4.1. Biomass Production and Yields

With the exception of three treatment combinations, no impact on plant growth was found after iodine application. There were increases in LFW and LDW biomass following a single application of Cs-KIO_3, which were consistent with results from previous lettuce biofortification studies using KIO_3 [10,15,44]. Conversely, there were decreases in HFW and HDW biomass after SDA of Cs-KI, potentially due to the second application direct to the substrate at 15 d.a.t. and subsequent rapid absorption by the plant roots. In addition, there is a high correlation between LFW and LDW ($R = 0.9$).

The I^- anion is known to have greater solubility in soil and better absorption by roots than its IO_3^- counterpart [44]. Reports of greater plant sensitivity to high doses of KI than to KIO_3 applied to the substrate have been published [15,45]. At low doses (0.013–0.129 mg L^{-1}), there are no toxic effects reported for either iodine species [45]. In hydroponic systems, lettuce has demonstrated greater sensitivity to I^-. Phytotoxic effects from excessive iodine accumulation were seen at I^- doses of 80 µM, while IO_3^- did not affect biomass at doses up to 240 µM [10].

Considering those previous reports, the iodine concentrations utilized in the present work were well within the ranges used for lettuce cultivation. The best iodine species for biofortification is reportedly IO_3^- [12,13,43]. Our study found that Cs-KIO_3 complexes (at 5 and 25 mg I kg^{-1}) applied to the substrate led to biomass increases. Compared to KI, that iodine salt is less phytotoxic. Additionally, appropriate doses of chitosan have been found to stimulate plant growth, development, and yields [24,46]

The largest improvements to LFW, LDW, and HDW were seen after single applications of Cs-KIO_3 (at 5 and 25 mg I kg^{-1}), rather than either Cs-KI or KIO_3. Based on those results, we believe that the complex of Cs and IO_3^- favor improvements in biomass, as there are also other studies that show that Cs and IO_3^- alone have positive effects on plant biomass [9,22,47]. Cs by itself is a good source of C, H, O and N for plants, it also works as a chelating agent for metals, which favors chelated elements that are available in assimilable forms for plants, and it has also been related for exercising its function as an elicitor, these and other Cs functions can contribute to plant growth [23,47]. On the other hand, IO_3^- is less phytotoxic than I^-, as it must first be reduced to I^- before it can be assimilated. Perhaps this step is a checkpoint for the plant's signaling processes. IO_3^- could also be acting as an elicitor. On the other hand, I^- is rapidly assimilated by the plant, probably leading to greater accumulation in the chloroplasts while exerting its phytotoxic effects, which have been noted as chlorosis or biomass

reductions [10,15]. However, the mechanisms of iodide and iodate root absorption have not yet been fully elucidated and little is known for sure about the subject [48,49].

4.2. Mineral Content

There were significant changes between treatments in the concentrations of N, P, K, Ca, Na, Cu, Fe and Zn. However, those changes were within the ranges seen for the absolute and chitosan controls and no other antagonistic effects between iodine and the mentioned minerals could be verified. Magnesium and manganese concentrations went up after single applications of Cs-KI (5 and 25 mg I kg^{-1}, respectively). In this study, we find significant positive correlations ($R \geq 0.8$) between Ca-Mg, Mg-Na and Ca-Na.

Soil application of fertilizers containing KI (0.5, 1.0, and 2.0 kg I ha^{-1}) reportedly led to increases in Ca, Mg, Mn, and Cd levels in lettuce [11]. Similarly, levels of Mg, Mn, and Cu in tomato leaves and stems increased with application of KI solutions (4–100 mg I kg^{-1}), although iron levels fell [50]. The authors attribute those results to the changes in redox equilibrium causes by iodine during cultivation. Increases in Mn and Cu concentrations in prickly pear cactus have been seen following KI (10^{-4} mol dm^{-3}) application, as well as P and Mg increases after KIO_3 (10^{-4} mol dm^{-3}) application via fertigation [51]. Conversely, negative correlations have been seen for K, Mg, Ca, S, Na, B, Cu, Fe, Mn, Zn, Cd and Pb content after fertilization with either KI or KIO_3 [14]. In the present study, there were no negative correlations seen in the evaluated minerals after I^- or IO_3^- application, either with or without chitosan.

4.3. Iodine Content in Lettuce

Even though iodine is not considered an essential nutrient for plants, several investigations have shown that the right doses of iodine can improve yields and that it is translocated to diverse tissues within the plant. Maximum concentrations of iodine were seen following application of KIO_3 (25 mg I kg^{-1}) as a split dose. Possibly, iodine was rapidly absorbed by the roots due to the direct application to the substrate. Some authors have suggested that biofortification is most successfully with IO_3^- as it does not have phytotoxic effects and the biomass either increases or is not negatively affected [12,47,52].

Regarding the original question of whether Cs-KI or Cs-KIO_3 complexes would increase iodine absorption, neither complex had an outstanding impact on iodine content when compared to the treatments without chitosan. For example, for I^-, the plant response was similar after single application treatments with and without Cs. The greatest concentrations were after SDA of KI treatments, even those changes were not significant. In contrast, KIO_3 and its Cs complexed form induced significant differences in iodine concentrations after two applications. Of the two forms, the KIO_3 alone led to a greater increase in iodine concentration. There are two potential explanations for these results. One, in order to be absorbed IO_3^- must first be reduced to I^-, a process that is carried out by iodate reductase enzymes [53]. So, when IO_3^- is complexed with Cs, the reduction process takes longer than when the free salt is applied and thus it becomes bioavailable slower. Two, the Cs-KIO_3 may become fixed in the ground, not volatilizing, which means crops with short cultivation cycles, like lettuce, would be unable to fully take advantage of its presence. Both I^- and IO_3^- applied to soil are converted to organic iodine after only 14 days, and in that form become fixed in the ground and less readily soluble in water [54]. This could explain why there was no significant difference between I^- and IO_3^- applied in a single treatment, with and without Cs, at either concentration (5 or 25 mg I kg^{-1}).

Several authors have reported high concentrations of iodine after biofortification with KIO_3 [15,55]. Conversely, iodine content in lettuce after biofortification with high doses of iodide during two trials (summer and winter) was up to five times higher than with iodate. The iodine concentrations of inner and outer leaves reached 653 and 764 μg I kg^{-1}, respectively [45]. Doses of iodine below 2.5 mg I kg^{-1} have no effect on biomass [55,56], while at times doses between 5 and 25 mg I kg^{-1} applied to soil have been found to not affect yields [57,58]. Those previously reported results are consistent with the

findings of the present study, with the exception of the SDA of Cs-KI (25 mg I kg^{-1}) which ended up reducing biomass.

4.4. Phenol Content

This study found an increase in cell wall-bound phenols after application of IO_3^- (25 mg I kg^{-1}), either in single dose or SDA, when compared to the absolute controls. The lack of changes in phenol concentrations may indicate that no oxidative stress was induced in the plants following treatment, except for the case of the treatments mentioned. In those cases, the action of cell wall-bound phenols could have potentially impeded iodine oxidation, which could be why no increases in free phenols were seen. Substrate and foliar applications of I^- or IO_3^-, either at 1 μM daily or 100 μM every two weeks, were not found to significantly affect phenol concentrations in tomato seedlings (var. Rio Grande) [35]. Similarly, in chili pepper covered with edible films of Cs-IO_3, no changes in total phenol content were seen for either the controls or the treated peppers [7]. On the other hand, tomatoes coated with chitosan-iodine films demonstrated greater antioxidant activity and total phenol content than uncoated fruit [42].

4.5. Chlorophyll Content

Little is known about the mechanisms of iodine toxicity or transport within plants. What is known, however, is that I^- toxicity is a consequence of intracellular oxidation or electron loss turning it into molecular iodine, which can bind to cellular components including chlorophyll [58]. In this study, the applied doses of iodide and iodate did not cause changes in chlorophyll content compared to the controls. Total chlorophyll has a high correlation with Chl a and Chl b and likewise Chl a and Chl b ($R \geq 0.7$). Similarly, other authors have reported insignificant changes in the contents of photosynthetic pigments [59,60]. Chitosan, on the other hand, applied in concentrations of 0.10%, 0.15%, 0.20%, and 0.30% has been observed to increase the index of chlorophyll content in lettuce leaves from 29.8 to 34.4, 35.5, 37.5, and 41.4, respectively [24]. Iodide does not inhibit chlorophyll synthesis, but it is known to reduce photosynthetic activity [59]. Nonetheless, chloroplasts are known major sites of ROS production [60]. It would appear that at the doses used in this study, iodide and iodate did not significantly affect the chlorophyll content of lettuce.

4.6. Total Protein Content

Treatments with KI were the only treatments that led to increases in total protein content. Those increases may be due to the fact that KI is more readily assimilated, potentially leading to the production of secondary metabolites and the concomitant production of the proteins necessary for that metabolism. Moreover, the amount of total proteins has a highly significant negative correlation ($R \geq 0.8$) with the enzymatic activity of GSH and GPX. Previous works have also observed significant increases in total protein content, although those treatments used IO_3^-, with maximum protein concentrations reached at doses of 80 μM iodine [61].

4.7. Enzymatic and Antioxidant Activity

There were no significant differences observed in enzymatic activity after any treatments. Once again, it was demonstrated that the applied doses of iodine did not induce a defensive response in lettuce. There are high positive correlations ($R \geq 0.5$) between enzymatic activities of GSH-GPX, CAT-GPX, and CAT-GSH that we believe have to do with maintaining a balance at the cellular level. There are no high and significant correlations between enzyme and antioxidant activities, with the exception of SOD and wall-linked phenols where there is a low but significant correlation. The latter we can attribute it to the fact that SOD heads the first line of defense of enzymatic activity and its correlation with phenols linked to the wall, we perceive it as a redox or neutralization mechanism against high concentration IO_3^- salts. Conversely, some authors have reported increases in enzymatic activity following biofortification with iodine. During soilless cultivation of lettuce, SOD was reduced

after application of I^- at 20, 40, and 80 μM, while IO_3^- led to its increase in doses greater than 40 μM. Ascorbate peroxidase (APX) activity increased with IO_3^- application at any dose, while CAT activity also increased with IO_3^- as well as I^- application, reaching its maximum value at IO_3^- doses ≥80 μM [43]. Similarly, hydroponic lettuce grown under salt stress and biofortified with IO_3^- had increased activities of SOD, APX, Dehydroascorbate reductase (DHAR), and Glutathione reductase (GR) [12]. Soybeans grown under Cd^{2+} stress and biofortified with IO_3^- at various concentrations (20, 40, and 80 μM) demonstrated increased SOD, APX, and GR activity [62]. Tomato biofortified with I^- or IO_3^-, applied to soil or foliage, either daily (1 μM) or biweekly (100 μM), had reduced SOD activity following the biweekly I^- treatments and the daily, foliar treatments of either iodine salt. However, the daily application of foliar I^- led to increases in the concentrations of non-enzymatic antioxidants, namely ascorbate (22%) and glutathione (85%) [35].

Plants have evolved several antioxidant systems to help them avoid damage from oxidative stress. One of those systems involves the enzymes SOD, CAT, and APX, acting in that order. SOD reduces O_2^- to H_2O_2, which in turn is a substrate for CAT, APX, and other enzymes [60]. Iodide is considered an inorganic antioxidant that reacts with ROS (ozone, singlet oxygen, and superoxide) at rates 12 to 500 times those of ascorbate and glutathione [63]. In aquatic plants of the genus *Laminaria*, the enzyme haloperoxidase catalyzes the oxidation of iodide to hypoiodous acid and molecular iodine in the presence of H_2O_2 [64].

The results of the present work differed from those previously reported, although that could be attributed to a number of factors. For example, in this study, cultivation was substrate-based while previous reports have used hydroponic systems [43]. Additionally, the accumulation of iodine and other biomolecules can depend on factors such as the dose and species of iodine applied, the plant species, and the type of application, be it foliar or directly to the substrate, all of which can lead to the differential accumulation of iodine [15].

5. Conclusions

The biofortification of lettuce using complexes of chitosan with KI or KIO_3 (Cs-KI and Cs-KIO_3) did not induce negative effects on the mineral content, chlorophyll content, enzymatic activity, antioxidant content, nor the phenol content of lettuce. Moreover, Cs-KIO_3, either at 5 mg I kg^{-1} or 25 mg I kg^{-1}, applied prior to transplanting increased plant biomass and led to iodine levels of 6.4 and 23.1 mg I kg^{-1} dry weight, respectively. Plant biomass was reduced after split-dose application (SDA) of Cs-KI before and after transplanting. The manner of treatment application as a split dose before and after transplant was a determining factor for greater iodine accumulation. Application before and after transplanting of KIO_3 led to the highest iodine concentrations (103.2 mg I kg^{-1} dry weight), although biomass was considerably reduced compared to single applications of Cs-KIO_3 at either concentration (5 or 25 mg I kg^{-1}). Although Cs-KI and Cs-KIO_3 did not increase iodine absorption compared to the free salts, in some cases they caused increases in biomass while maintaining levels of iodine accumulation in lettuce leaves. As far as we know, there are no similar reports on crop biofortification with chitosan-iodine complexes applied to either soil or substrate. Further biofortification studies with chitosan-iodine complexes in crop species other than lettuce are required in order to evaluate the effects they may have when treatment times are longer.

Author Contributions: I.E.D.R., experimentation, chemical analysis, analysis of data and draft writing; H.O.O., design of experiments and synthesis of Cs complexes; L.I.T.T., design of experiments and chemical analysis; A.J.M., methodology, experimentation; S.G.M., methodology, experimentation; B.C.G., chemical analysis; M.C.D.l.F., data analysis; A.B.M., conceptualization, methodology, data analysis. All authors were responsible for review and editing. All authors have read and agreed to the published version of the manuscript.

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