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Combined bio fortification of spinach plant through foliar spraying with iodine and selenium elements

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The aim of this study was to evaluate the impact of combined biofortification of spinach with iodine (I) and selenium (Se). For this purpose, the spinach plant was cultivated in an open field (Dehdasht, Iran), biofortified with potassium iodide (KI) and sodium selenate (Na_2SeO_4) at different concentrations (Se 1 mg/L-I 1 mg/l; Se1-I1, Se2.5-I1, Se5-I1, Se1-I5, Se2.5-I5, and Se5-I5) through spraying the leaves twice during the growth season. Results indicated that while iodine did not have any effect on plant yield, selenium fortification at 2.5 mg/L significantly increased production (60.05 t/ha). However, both elements were successfully accumulated in the leaves of the plant. Therefore, the highest accumulation for both elements was noted by Se5-I5 sample. Meanwhile, the joint biofortification of spinach improved the activity of antioxidant enzymes, macro/microelements content, photosynthetic pigments, nitrate reductase activity, ascorbic acid, total phenol content, carotenoid compounds, total soluble solids, and dry matter percentage, while decreasing the nitrate and malondialdehyde contents in the leaves, resulting in a plant with improved dietary properties and yield production. In this regard, treatment Se2.5-I5 was the best treatment in relation to various tests conducted.

Keywords Biofortification, Selenium, Iodine, Spinach, Foliar spraying

Food safety is a priority for the improvement of the health status of the world's population. Two billion people (about 1/3 of the society) are affected by different micronutrient deficiencies. Microelements usually have vital role in biological processes as well as in humans, animals, and plants growth. Iodine and selenium are two trace elements with high essentiality for animal and human health. These elements are key factors in metabolic pathways of mammalian cells[1].

Diseases like mental retardation, goiter, reproductive failure, and hyperthyroidism comes from iodine deficiency. Proper iodine intake is critical during pregnancy and early childhood due to the direct role of thyroid hormones on brain growth and development. Although sever deficiency in iodine currently is less observed due to the table salt iodization in many countries; however, moderate iodine deficiency and its adverse impacts on health is still seen in many regions, especially regarding this subject that the salt consumption has been reduced in recent years due to the direct effect of salt on hypertension and cardiovascular diseases[2].

On the other hand, selenium deficiency has affected approximately 800 million of the world's population (15%), putting them at risk of heart disease, cancer, impaired immunity, and thyroid disorders[3]. The entrance of selenium into the human's diet occurs through the accumulation of this element from soil in the edible parts of the plants. Selenium is taken up as selenite through the plant roots. Besides, the beneficial effects of selenium in alleviating physiological stress by increasing crop production and improving tolerance levels have been suggested in some studies[4,5].

Regarding the deficiency of selenium and iodine and the related health hazards, alternative strategies are needed to mend these elements supply, principally in areas where deficiency in micronutrients are existed in the soil and therefore, in the food crops. In this regard, plants micronutrients biofortification programs are greatly desirable[6].

Biofortification of fodder crops and vegetables can be an effective strategy for justifying dietary requirements of iodine and selenium in animals and humans. For this purpose, leafy vegetables are good candidates as it has

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been indicated that selenium/iodine accumulates properly in shoot organs of plants with high transpiration[7]. Lawson et al.[8] reported that fortification through spraying iodine resulted in higher accumulation rates in butterhead lettuce compared to the soil application. In addition, iodine uptake in nectarine, plum, and tomato fruits increased significantly only after foliar spray treatments[9]. Similar results were obtained for other leafy vegetables, too[10]. Oliveira et al.[11] improved the selenium and carotenoids content of the carrot shoots and roots, respectively, by selenite foliar application. Other studies have confirmed that vegetables have good prospects for selenium/iodine biofortification. Lawson et al.[8] and Cakmak et al.[12] declared that foliar sprays of vegetables and cereals induce better results than soil drenches.

To the best of our knowledge, there is currently no information available on the simultaneous enrichment of spinach with iodine and selenium. The significant innovation of this study lies in its comparative analysis of the simultaneous enrichment of spinach with iodine and selenium under field conditions. Our research hypothesis posits that the spraying spinach leaves with iodine and selenium during growth can be effectively enriched, thereby enhancing the contribution of spinach to meet the Recommended Daily Allowance (RDA) for both elements. Therefore, this study aimed to evaluate the biofortifying potential of spinach as a leafy vegetable by spraying the leaves with iodine and selenium. Spinach is a popular vegetable in the dietary program of people in most parts of the word, but it has not been studied as a high potential plant for micronutrient fortification. Beside the iodine and selenium accumulation, plant dietary properties were measured to clarify the effect of biofortification.

Materials and methods

Materials

Potassium iodide, sodium selenite, tetramethylammonium hydroxide, nitric acid, hydrogen peroxide, ascorbate, EDTA, polyvinylpyrrolidone, Na-phosphate buffer, EDTA, hydrogen peroxide, t-butyl hydroperoxide, glutathione reductase, Tris–Ca–codylic sodium salt, nitroblue tetrazolium, Triton X-100, pyrogallol, sulfanilic acid, hydrochloric acid, naphthyl ethylenediamide, thiobarbituric acid, trichloroacetic acid, and perchloric acid, all were purchased from Merck (Darmstadt, Germany).

Preparation of plant treatments

In year 2022, an experimental open field, located in Dehdasht (Iran), was used for growing the *Sirius* variety of spinach. This spinach was cultivated in sod-podzol soil characterized according to Table 1. The planting density was established at 14 plants per square meter, with a spacing of 15 cm between individual plants and 50 cm between rows. The plant leaves were sprayed with solutions containing selenium + iodine in a two-step approach. The first step was performed three weeks after planting, while the second stage was performed five weeks post-planting. Sampling was performed at the end of the vegetative growth phase. Each plot measured 4.0 m^2 (2.0 × 2.0 m), whereas the experiment was replicated thrice. To prepare the selenium + iodine solutions, potassium iodide and sodium selenate were used at different concentrations (selenium 1 mg/L-iodine 1 mg/L; Se1-I1, selenium 2.5 mg/L-iodine 1 mg/L; Se2.5-I1, selenium 5 mg/L-iodine 1 mg/L; Se5-I1, selenium 1 mg/L-iodine 5 mg/L; Se1-I5, selenium 2.5 mg/L-iodine 5 mg/L; Se2.5-I5, and selenium 5 mg/L-iodine 5 mg/L; Se5-I5). Therefore, six treatments were considered for the experiment as well as a control sample which no spraying was performed on the leaves.

Iodine content

To measure the plant leaves iodine content, plant material was dried (at 50 °C), ground, and mixed (0.5 g) with 10 mL double-distilled water. After the addition of 1 mL of tetramethylammonium hydroxide solution (25%), the mixture was incubated (3 h, 70 °C), cooled, and made up to 30 mL with double-distilled water. Then, the mixture was centrifuged (4,500 rpm, 15 min, at 5 °C) and the iodine content of the samples was measured using an inductively coupled plasma optical emission spectrometry instrument (ICP-OES, AVIO 200, Perkin Elmer, USA)[13].

Selenium content

A method adapted from Montes-Bayón et al.[14] was used to measure the selenium concentration in the plant leaves. In summary, 0.25 g of dried tissue was digested using microwave technique with nitric acid (8 ml) and hydrogen peroxide (2 mL) at 180 °C until a colorless extract was formed. The resulting extract solution was then diluted with Milli-Q water to a final volume of 50 mL. The total selenium concentration was determined using inductively coupled plasma mass spectrometry (ICP-MS, NexION®, Perkin Elmer, USA) using an external calibration and internal standards (gallium, Ga, at 5 ng mL^{-1}).

Total soluble solids, dry matter, and yield determination

Total soluble solids, as a sweetness factor, were measured using a digital refractometer (Atago PR-101, Japan) at 20 °C. The results were expressed as percentage. To measure the dry matter (%) of samples, the precise weighted

pH	EC (ds/m)	O.C (%)	Silt (%)	Clay (%)	Sand (%)	*N* %	*P* (Av.) (mg/kg)	K (Av.) (mg/kg)	Fe ppm	Zn ppm	Mn ppm	Cu ppm	I (mg/kg)	Se (mg/kg)
7.79	0.81	1.09	55.8	28	15.2	0.076	3.8	466	0.40	0.68	0.80	2.94	0.87	0.2

Table 1. The physico-chemical analysis of soil at a depth of 0–30 cm.

samples were dried in an oven at 105 °C for 24 h until a constant weight reached. The spinach plant total mass and the mass of green parts of a plot were used to measure the yield of the production[15].

Enzyme assays

Preparation of enzyme extract

To perform enzyme assays, the enzyme extraction was first prepared. For this purpose, the leaves (0.3 g) were pulverized with ice-cold Hepes buffer (3 mL, 25 mM, pH 7.8) containing reduced ascorbate (2 mM), EDTA (0.2 mM), and polyvinylpyrrolidone (2%). The homogenate was then subjected to centrifugation (4 °C, 20 min, 12,000 rpm) after which the supernatants were utilized to determine enzyme activity[16].

Assay of ascorbate peroxidase (AP) activity

Nakano and Asada[16] method was used to measure AP activity. The assay relies on the reduction in absorbance at 290 nm along with the ascorbic acid oxidation. The 2 mL reaction mixture consisted of ascorbate (0.5 mM), Na-phosphate buffer (25 mM, pH 7.0), EDTA (0.1 mM), hydrogen peroxide (0.1 mM), and supernatant (0.1 mM). The reaction was initiated with the addition of hydrogen peroxide.

Assay of catalase activity

Catalase activity was measured by monitoring the decrease in absorbance (240 nm, for 30 s) after addition of hydrogen peroxide as described by Aebi[17]. The molar extinction coefficient of hydrogen peroxide at 240 nm is 0.0394 mM^{-1} cm^{-1}. The assay mixture consisted of hydrogen peroxide (15 mM), leaf extract (50 µL), and potassium phosphate buffer (100 mM, pH 7.0) in a total volume of 3 mL. One unit of catalase activity was defined as the amount of hydrogen peroxide that was decomposed (nmol) per min[16].

Assay of glutathione peroxidase (GPX) activity

The activity of GSH-PX was determined using the method described by Hopkins and Tudhope[18]. In this method, t-butyl hydroperoxide is used as the substrate. The assay mixture contained potassium phosphate buffer (50 mM, pH 7.0), NADPH (0.28 mM), EDTA (2 mM), reduced glutathione (GSH, 0.13 mM), glutathione reductase (GR, 0.16 U), enzyme extract (50 µg protein), and t-butyl hydroperoxide (0.073 mM). One unit of GPX activity was defined as the amount of enzyme that catalyzes the oxidation of NADPH per min per g of protein (IU g^{-1} protein).

Assay of superoxide dismutase (SOD) activity

Masayasu and Hiroshi[19] method was used to measure the activity of SOD using Tris–Ca–codylic sodium salt buffer (pH 8.2, containing 0.1 mM EDTA). The assay mixture consisted of enzyme extract (containing 50 mg protein), nitroblue tetrazolium (NBT, 0.055 mM), Triton X-100 (1.42%), and pyrogallol (16 mM). One unit of the enzyme is defined as the amount required to prevent the NBT reduction by 50% within a 1 min time period.

Assay of nitrate reductase activity

In order to measure nitrate reductase activity, 1 mL of 2-propanol (4%) was added to 5 mL of phosphate buffer solution (100, pH = 5.7) containing 30 mM potassium nitrate. After washing with distilled water, 300 mg of leaves were placed in a dark glass container and after 1 h of storage at 30 °C, 1 mL of sulfanilic acid was dissolved in 2 normal hydrochloric acid and then 1 mL of naphthyl ethylenediamide (0.02%) was added. The absorbance (UV2100, USA) was measured at 540 nm after 20 min with changing the color to pink[20].

Malondialdehyde (MDA) determination

Ohkawa et al.[21] method was used to measure MDA. For this purpose, 0.2 gram of plant tissue (leaf) was cut into small pieces and homogenized in 2 mL of 5% trichloroacetic acid solution on ice. The mixture was then centrifuged (12,000 rpm, 15 min), and the supernatant was collected. The supernatant (0.5 mL) was mixed with 0.5 mL of thiobarbituric acid solution and 20% trichloroacetic acid, and incubated at 96 °C for 25 min. The mixture was then centrifuged under cold conditions (10,000 rpm, 5 min). The supernatant absorbance was determined at 532 nm. The 20% trichloroacetic acid and thiobarbituric acid solution was used as the control. The amount of MDA was determined using a standard curve.

Calcium content assay

The Ca content was determined using the method described by Manganaris et al.[22]. The cell wall material (20 mg) was digested in a triacid solution consisting of sulfuric acid (H_2SO_4), nitric acid (HNO_3), and perchloric acid ($HClO_4$) in a ratio of 1:5:1 (v/v/v) at 80 °C until red vapor was observed. The temperature was then increased to 150 °C and maintained for more than 7 h until a clear residue was obtained. Finally, the Ca content was quantified using atomic absorption spectroscopy (Perkin-Elmer 5100, at 422.7 nm). The results were expressed as mg/100 g (dw).

Element composition

Nitrogen was measured using Kjeldahl method[23]. Phosphate was evaluated using colorimetry method with the help of molybdate and malachite green[24]. Potassium, Mg, and S were measured using flame photometry (Model 18, Perkin-Elmer, USA)[25].

Nitrate determination

The nitrate content of spinach leaves was determined spectrophotometrically using the method described by Bian et al. (2016). After grounding 0.2 g of each leaf treatment (in liquid nitrogen) and suspending in distilled

water (10 mL), the samples were boiled (100 °C, 30 min.), cooled, then filtered, and finally diluted (to 25 mL using distilled water). Then, 0.1 mL of the extract was mixed with salicylic acid-concentrated sulfuric acid solution (0.4 mL, 5% w/v). The mixture was allowed to react at ambient temperature for 20 min. The reaction was terminated by the addition of NaOH solution (9.5 mL, 8% w/v). The absorbance of the resulting solution was measured at 410 nm and then was used to calculate the nitrate content using its standard curve[26].

Ascorbic acid and total phenolic content (TPC) determination

For measuring ascorbic acid, the titration method using 2, 6-dichlorophenol-indophenol was used[27]. The total phenolic content was measured using a spectrophotometric method at 765 nm, with the use of Folin-Ciocalteu reagent (FCR). A calibration curve was also constructed by plotting different concentrations of gallic acid versus absorbance.

Total antioxidant capacity

The total antioxidant activity of spinach leaves was measured using the DPPH radical (2,2-diphenyl-1-picrylhydrazyl) scavenging capacity method. This assay quantifies the ability of antioxidants in the sample to quench the DPPH radical, which has a distinctive purple color with an absorption maximum at 517 nm. The DPPH radical percent inhibition was reported as the result[28].

Chlorophyll and carotenoids measurement

For this purpose, 0.2 g of leaves were homogenized in 80% acetone. After centrifugation at 2,500 rpm, the supernatant was collected, and its absorbance was measured at 647 nm for chlorophyll b, 663 nm for chlorophyll a, and 470 nm for carotenoids. The chlorophyll and carotenoid contents were calculated using the following formula[29]:

$$\text{Chl. a}\,(\text{mg/L}) = (12.25 \times \text{A}_{663}) - (2.79 \times \text{A}_{647})$$

$$\text{Chl. b}\,(\text{mg/L}) = (21.5 \times \text{A}_{647}) - (5.1 \times \text{A}_{663})$$

$$\text{Chl. a} + \text{b}\,(\text{mg/L}) = (7.15 \times \text{A}_{663}) - (18.71 \times \text{A}_{647})$$

$$\text{Carotenoid content}\,(\text{g/g}) = \text{A} \times \text{V}\,(\text{mL}) \times 104/\text{A}_{1\text{ cm}}^{1\%} \times \text{P}\,(\text{g})$$

where A is absornace, V is the extract volume; P = the weight of sample; $A_{1\text{cm}}^{1\%}$ = 2592 (β-carotene extinction coefficient in petroleum ether.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA), and the mean separations were conducted via the Duncan multiple range test, referencing a significance level of 0.05. This analysis was performed using SPSS software (21, IBM, Armonk, USA).

Results and discussion

The se and I content in the spinach leaves

The combined application of selenium and iodine resulted in a significant increase of selenium and iodine in the spinach leaves in comparison with the control sample (Table 2). Meanwhile, the increase in selenium/iodine concentration led to an increase in selenium/iodine content as a two-fold increase in selenium content was observed along with the increase in selenium concentration from 1 to 5 mg/L. However, this level of increase in selenium concentration was not observed for iodine. In other words, the increase in iodine content was much lower than the increase in selenium content. At the same time, as the concentration of consumed selenium (or consumed iodine) increased, the level of iodine (or selenium) in the leaves also increased, indicating the effect of the two microelements in increasing each other's absorption and even the effect of their concentration in improving the rate of absorption in the plant. This may be due to the synergistic interaction between selenium and iodine uptake by the plant, increasing their bioavailability to the plant or reducing any antagonistic effect when applying them together at optimized concentrations and in the right forms[30].

Treatments	Selenium (ng/g)	Iodine (mg/kg.dw**)	Dry matter (%)	Total soluble solids (%)	Yield (t/ha)
Control	26.17 ± 0.45 g*	10.44 ± 0.41e	7.22 ± 0.21d	5.31 ± 0.20d	40.80 ± 3.5c
Se1-I1	31.15 ± 0.52f	10.86 ± 0.32ed	7.45 ± 0.14d	5.88 ± 0.11c	40.86 ± 2.5c
Se2.5-I1	38.90 ± 0.87d	11.70 ± 0.33c	9.91 ± 0.24b	6.47 ± 0.23b	60.07 ± 1.4a
Se5-I1	58.22 ± 1.56b	12.55 ± 0.25b	7.78 ± 0.13c	6.24 ± 0.17b	50.17 ± 2.8b
Se1-I5	33.24 ± 0.43e	11.37 ± 0.30dc	9.49 ± 0.31b	5.68 ± 0.12c	50.80 ± 4.5b
Se2.5-I5	41.66 ± 0.92c	12.81 ± 0.26b	10.12 ± 0.23a	7.27 ± 0.20a	60.04 ± 3.2a
Se5-I5	63.57 ± 1.23a	13.69 ± 0.21a	9.17 ± 0.26b	6.93 ± 0.23a	50.50 ± 3.4b

Table 2. Effect of spinach biofortification with selenium + iodine on selenium, iodine, dry matter content, total soluble solids, and yield of the plant. *Different lowercase letters in each column indicate a significant difference between the treatments ($P \leq 0.05$). **Dry weight.

Numerous studies have shown that plant selenium and iodine content tends to increase with higher applied doses[30,31]. However, it is important to recognize that each plant species has a maximum selenium or iodine tolerable dose. Exceeding this threshold can lead to phytotoxic effects. These toxic effects may manifest as reduced yields, alterations in organoleptic properties, alterations in pigment content, or decreased enzyme activity in the plants. This phenomenon aligns with Shelford's law, which states that plant growth is constrained by environmental factors—such as selenium/iodine concentration—when they are present at excessively high or low levels[2]. Li et al. (2017) indicated that iodine biofortification influenced the pepper fruits quality. This study indicated that fertilizing with potassium iodide led to the accumulation of iodine in pepper fruits. Consequently, these biofortified peppers can fulfill the daily iodine intake recommended by WHO. Additionally, even low doses of iodine (0.25–1.0 mg/L) enhance the quality of the peppers by reducing fruit acidity and increasing vitamin C content[32]. Puccinelli et al.[33] declared that using sodium selenate and potassium iodide together was found to be effective for biofortifying lettuce with both selenium and iodine. Rakoczy-Lelek et al. (2021) confirmed that the Foliar application of iodine or selenium increased the iodine and selenium content of carrot. Smoleń et al.[34] indicated that the biofortification of lettuce with iodosalicylate resulted in a higher iodine content in the leaves of the plant compared to the control sample.

The leaves dry matter content, total soluble solids, and plant yield

According to the results (Table 2), the increasing in the concentration of iodine from 1 to 5 mg/L, increased the dry weight of the leaves significantly, while spraying the leaves with selenium, up to a concentration of 2.5 mg/L, increased the dry matter to 9.91% (for Se2.5-I1) and 9.49% (for Se2.5-I5) from 7.22% (for control). However, the further increase in selenium concentration up to 5 mg/L caused a decrease in the dry weight of the leaves.

The total soluble solids results confirmed the obtained results by the dry matter test as the total soluble solids of spinach leaves increased by spraying iodine on the leaves as well as by increasing the iodine concentration from 1 to 5 mg/L. This increase was also true for selenium up to a concentration of 2.5 mg/L (Table 2). However, a further increase in the dose of selenium concentration from 2.5 to 5 mg/L led to a decrease in total soluble solids values. Golubkina et al.[6] employed iodine combined with selenium for leaf biofortification of Indian mustard. The results showed a significant increase in the biomass of both underground and aboveground plant parts.

The present results demonstrated that the simultaneous application of selenium and iodine enhances the dry matter of the plant (Table 2). The growth-stimulating effect of selenium is attributed to improved water supply, increased chlorophyll biosynthesis, and enhanced assimilation of sulfur (S) and nitrogen (N), as would indicate in the next sections. Iodine encourages plant growth by improving N accumulation. Both microelements are responsible for robust antioxidant defense to the plants.

The plant yield (Table 2) increased significantly due to the biofortification with selenium + iodine and selenium concentration up to 2.5 mg/L. An increase in selenium concentration from 2.5 to 5 mg/L as well as iodine concentration from 1 to 5 mg/L did not have a significant effect on spinach yield. Therefore, the highest plant yield was obtained in Se2.5 + I1 and Se2.5 + I5 (60.055 t/ha) samples with no significant difference between them ($P > 0.05$). In the present research, the combined application of selenium and iodine was effective in enhancing yield. In contrast with our findings, the lack of plant growth-stimulating effect upon the combined application of selenium and iodine has been reported by Golubkina et al.[6] on Indian mustard and Smoleń et al.[1] on carrot.

Biochemical evaluations of spinach leaves

The application of selenium and iodine significantly influenced ($P \leq 0.05$) the activity of superoxide dismutase (SOD), glutathione peroxidase (GPX), ascorbate peroxidase (AP), and catalase in spinach leaves (Table 3). The activity of these enzymes increased with the application of selenium and iodine as well as with the increase in selenium/iodine concentration ($P \leq 0.05$), except for iodine concentration that did not affect the GPX activity ($P > 0.05$). This suggests that selenium and iodine supplementation can enhance the ability of spinach leaves to scavenge superoxide radicals. Meanwhile, increasing in catalase activity indicates that selenium/iodine biofortification can increase the leaves' ability to break down hydrogen peroxide[35].

It has been reported that selenium at proper concentrations is the activator of the antioxidant system of the plants[36]. On the other hand, the lower concentration of hydrogen peroxide in iodine treated plants has been reported before, which is an indicator of activating the detoxifying enzymes, responsible for protection of cells from oxidative damage[37]. When the plant placed under stress conditions characterized by an excess of reactive

Treatments	Catalase (IU/g)	SOD (IU/g)	GPX (IU/g)	AP (IU/g)	MDA (µmol/g)
Control	4.53 ± 0.11d*	5.30 ± 0.25e	4.04 ± 0.12c	3.12 ± 0.10d	30.3 ± 0.54 g
Se1-I1	4.86 ± 0.12c	6.16 ± 0.25d	5.57 ± 0.22b	3.24 ± 0.21d	23.5 ± 0.36e
Se2.5-I1	6.07 ± 0.15b	6.93 ± 0.11c	5.57 ± 0.23b	4.10 ± 0.24c	17.3 ± 0.33c
Se5-I1	5.59 ± 0.12c	6.49 ± 0.14d	6.63 ± 0.32a	4.77 ± 0.14ba	21.1 ± 0.14d
Se1-I5	5.34 ± 0.21c	5.76 ± 0.22e	4.30 ± 0.23c	3.76 ± 0.51a	26.7 ± 0.54f
Se2.5-I5	6.83 ± 0.14a	7.84 ± 0.13a	5.25 ± 0.14b	4.53 ± 0.22cb	12.6 ± 0.22a
Se5-I5	6.48 ± 0.24a	7.37 ± 0.15b	6.14 ± 0.24a	4.96 ± 0.32a	14.4 ± 0.13b

Table 3. Effect of spinach biofortification with selenium + iodine on the leaves enzymes activity as well as malondialdehyde (MDA) content. *Different lowercase letters in each column indicate a significant difference between the treatments ($P \leq 0.05$).

oxygen species (ROS), SOD is the first enzyme that is activated in the antioxidant defense system. Superoxide dismutase mitigates ROS and generates hydrogen peroxide[36]. Hydrogen peroxide acts as a signaling molecule involved in various biochemical and physiological processes. In normal cellular metabolism, hydrogen peroxide is produced at baseline levels, particularly during photorespiration and photosynthesis. However, hydrogen peroxide levels can increase significantly when the plant undergoes a variety of biotic and abiotic stresses. Under such situation, it can generate hydroxyl radicals, when reduced transition metals, like Fe^{2+}, are presented in the environment[37]. Consequently, hydrogen peroxide is a primary contributor to lipid peroxidation, which is then breaking down by peroxidase, catalase, and AP into oxygen and water[36]. It is well-established that a decrease in hydrogen peroxide concentration within plant cells is associated with the activity of the catalase and AP, which are crucial for removing this ROS produced during photorespiration and under oxidative stress conditions[38]. In this context, Ahmad et al.[39] highlighted the significant role of selenium in plants as it helps reduce oxidative stress by enhancing the activity of defense mechanisms against oxidative damage, including increased SOD, CAT, GPX, and AP activity. Therefore, the application of selenium significantly affected the levels of hydrogen peroxide. On the other hand, studies indicate that the application of iodine, particularly in the form of potassium iodide, leads to an increase in the activity of key antioxidant enzymes such as SOD, AP, and catalase. This enhancement helps mitigate oxidative damage caused by ROS in various plant species, including soybean and lettuce[40,41].

Malondialdehyde (MDA) content decreased significantly by the application of Selenium + iodine (Table 3). Meanwhile, with increasing the concentration of selenium to 2.5 mg/L and iodine to 5 mg/L, MDA concentration decreased significantly, too. However, the increase in selenium concentration from 2.5 to 5 mg/L resulted in an increase in MDA content. In this regard, Se2.5-I5 treatment indicated the lowest amount of MDA (12.6 µmol/g), while the control sample had the highest value (30.3 µmol/g). Therefore, as the dose of selenium increased a higher concentration of MDA was observed. This increase in MDA levels indicates the incidence of oxidative stress, leading to cell membrane damage and reduced productivity. Malondialdehyde is a byproduct of lipid peroxidation and has been widely used as a marker for identifying damage caused by ROS. Previous studies have consistently reported elevated MDA content in plants subjected to high selenium doses[42]. de Oliveira et al.[36] concluded that selenium decreased the MDA values up to 0.75 mg/kg and the higher concentration of selenium resulted in higher values of MDA in potato.

Elements composition

The levels of Ca, S, P, K, N, and Mg in spinach leaves increased with the spraying of selenium along with iodine on spinach leaves (Table 4). Additionally, the increase in the concentration of these two microelements also led to an increase in the concentration of the aforementioned elements. Thus, the Se5-I5 treatment showed the highest levels of Ca, S, P, K, N, and Mg, and the Se2.5-I5 treatment generally ranked after that. In contrast, the control sample exhibited the lowest levels of these elements. Similar results were also observed for N. However, it was noted that with a further increase in selenium concentration from 2.5 to 5 mg/L, a decrease in the concentration of N was observed. Thus, the Se2.5-I5 sample exhibited the highest level of N (2810 mg/100 g), followed by the Se5-I5 treatment (2735 mg/100 g), while the lowest level, as expected, was observed in the control sample. In this context, the beneficial impact of iodine and selenium on different macro/micronutrient elements accumulation may be linked to the increased presence of iodine/selenium in plant tissues, which promotes the activity of enzymatic systems that utilize these elements. On the other hand, selenium and iodine can influence the uptake and metabolism of other essential nutrients in plants. For instance, selenium has been shown to interact with macronutrients like N, P, and S which can enhance the overall nutrient profile of the plant. This interaction may lead to improved nutrient absorption and translocation within the plant, resulting in higher concentrations of beneficial elements in edible parts[43,44]. Meanwhile, biofortification with selenium and iodine has been linked to improved plant growth and resilience against abiotic stresses such as drought and salinity. This enhanced growth can lead to increased biomass and nutrient content, as healthier plants are generally more efficient at absorbing and utilizing nutrients[44]. According to Pazurkiewicz-Kocot et al.[45], changes in the plasma membrane's ion coefficient influence the absorption, transport, and accumulation of nutrients within plant cells.

Duborska et al.[46] and Bialowas et al.[47] emphasizes the benefits of simultaneous biofortification with both selenium and iodine, gives promising results in enhancing the overall nutrient profile of crops, including increased accumulation of Ca, S, P, K, and Mg. Golubkina et al.[6] reported that the joint application of selenium and iodine resulted in Al, B, Mn, Cd, and Sr accumulation[6]. Duborska et al.[46] demonstrated that foliar application

Treatments	Ca (mg/100 g)	S (mg/100 g)	*P* (mg/100 g)	K (mg/kg.dw**)	Mg (mg/g)	*N* (mg/100 g)
Control	104.4 ± 0.21 g*	87.40 ± 0.47f	317 ± 5 g	1120 ± 10 g	7.96 ± 0.11d	2241 ± 10 g
Se1-I1	118.5 ± 0.23f	91.26 ± 0.54e	343 ± 3f	1206 ± 9e	8.32 ± 0.21c	2350 ± 15e
Se2.5-I1	137.2 ± 0.31d	95.13 ± 0.67d	432 ± 2c	1314 ± 15d	8.84 ± 0.34c	2540 ± 11c
Se5-I1	152.0 ± 0.17b	98.41 ± 0.21c	414 ± 4d	1387 ± 13c	9.73 ± 0.44b	2466 ± 20d
Se1-I5	126.6 ± 0.11e	96.36 ± 0.77d	387 ± 3e	1174 ± 11f	9.50 ± 0.47b	2285 ± 9f
Se2.5-I5	143.5 ± 0.41c	103.17 ± 0.74b	467 ± 5b	1435 ± 14b	10.68 ± 0.51a	2810 ± 11a
Se5-I5	157.6 ± 0.14a	107.04 ± 0.57a	494 ± 6a	1471 ± 8a	11.15 ± 0.14a	2735 ± 10b

Table 4. Effect of spinach biofortification with selenium + iodine on the elements composition of the leaves. *Different lowercase letters in each column indicate a significant difference between the treatments ($P \leq 0.05$). **Dry weight.

of selenate has shown positive effects on the accumulation of Ca, Mg, and K in various crops, including wheat and rice[2,46].

Nitrate content and the nitrate reductase activity of spinach leaves

The nitrate content of spinach leaves and the activity of nitrate reductase were affected by spraying the plant with selenium + iodine (Table 5), resulting in decreasing the nitrate content of the leaves after the spinaches were sprayed with the enrichment solutions and increasing the nitrate reductase activity. With the increase of selenium and iodine concentration, the amount of nitrate content in spinach leaves also decreased and the activity of nitrate reductase increased. Therefore, the highest amount of nitrate (917 mg/Kg) and the lowest nitrate reductase activity (43.1 µmol NO_2/g.fw) were observed in the control sample. However, the proper selenium concentration for decreasing the nitrate content and increasing nitrate reductase activity was 2.5 mg/L, and increasing the selenium concentration up to 5 mg/L resulted in a decrease in nitrate reductase activity.

It is reported that selenium boosts the activity of nitrate reductase, resulting in lower nitrate levels in plants. Research indicates a strong interconnection between selenium and nitrogen metabolism[48]. This relationship is particularly evident in the increased activity of nitrate reductase when plants are enriched with selenium, leading to a reduction in nitrate concentrations. This phenomenon has been confirmed in various crops, including potatoes, sunflowers, and wheat. Additionally, a negative correlation has been observed between selenium levels and nitrate content in the circadian biorhythms of perennial onions and other crops[35].

Iodine has been shown to enhance the activity of nitrate reductase in plants. This leads to increased conversion of nitrate to nitrite, reducing nitrate levels. The impact of iodine on nitrate accumulation is more complex and depends on the iodine form and concentration. Iodine can limit nitrate accumulation by inhibiting nitrification (conversion of ammonium to nitrate) and denitrification (conversion of nitrate to nitrogen gases). However, at higher iodate levels (1 mg/L), nitrate content was generally lower compared to the lower iodine levels, indicating the positive effect of iodine concentration on nitrate content reduction. Therefore, Se2.5-I5 treatment resulted in the best result, regarding the lowest nitrate amount and highest nitrate reductase activity. It has been reported that iodine biofortification did not change nitrate content in lettuce[49]. In another study, a negative correlation was observed between selenium levels and nitrate content in perennial onions and other crops, suggesting a similar effect may occur with iodine[35].

Ascorbic acid, total phenol content (TPC), carotenoids, and antioxidant activity

Selenium plays a crucial role in the antioxidant system of plants, helping to protect them from various biotic and abiotic stressors such as salinization, drought, harmful insects, and flooding. Numerous scientific studies have highlighted its interaction with other natural antioxidants, demonstrating that enriching plants with this micronutrient leads to increased levels of polyphenols, ascorbic acid, carotenoids, flavonoids, and chlorophyll.

In the present study, the application of selenium and iodine enhanced the biosynthesis of ascorbic acid, total phenols, and carotenoids. Meanwhile, the increase in these microelements levels also induced an increase in the concentration of the ascorbic acid, total phenols, and carotenoids, except for carotenoids, in which the iodine concentration did not show any significant effect on its content.

The enhancement of ascorbic acid biosynthesis following selenium enrichment (Table 5) indicates a good relationship between plants selenium and ascorbic acid content[35].

The increase in ascorbic acid content is attributed to selenium's role in enhancing the plant's antioxidant capacity and influencing metabolic pathways associated with ascorbic acid production. Selenium may stimulate the expression of genes involved in ascorbic acid synthesis and improve the overall antioxidative metabolism of the plant, thereby promoting higher ascorbic acid levels[50]. Golob et al.[50] reported that in biofortified pear-jujube, the selenium treatment resulted in elevated levels of ascorbic acid, indicating a positive correlation between selenium supplementation and ascorbic acid biosynthesis[51].

The presence of iodine may stimulate the plant's defense mechanisms, resulting in higher accumulation of ascorbic acid as a response to stress conditions induced by iodine application. In addition, it has been confirmed that iodine can stimulate chlorophyll content, potentially leading to increased photosynthetic activity and, consequently, higher ascorbic acid production[52]. It has been reported that iodine biofortification can lead to increased ascorbic acid levels in cabbage and broccoli microgreens, particularly when combined with other

Treatments	Nitrate (mg/Kg)	Nitrate reductase (µmol NO_2/g.fw)	Ascorbic acid (mg/100 g)	Total phenol (mg Gal/g.fw**)	Carotenoid (mg/100 g)
Control	917 ± 11a*	43.1 ± 0.23 g	7.27 ± 0.12f	4.17 ± 0.21e	2.31 ± 0.12 g
Se1-I1	860 ± 9b	55.5 ± 0.47e	7.70 ± 0.13e	4.82 ± 0.14d	3.16 ± 0.05e
Se2.5-I1	793 ± 12d	74.3 ± 0.65c	9.55 ± 0.15c	5.74 ± 0.31c	4.13 ± 0.13d
Se5-I1	668 ± 13f	66.9 ± 0.44d	10.47 ± 0.14b	6.80 ± 0.15b	5.55 ± 0.15b
Se1-I5	835 ± 10c	47.4 ± 0.14f	8.11 ± 0.14d	5.27 ± 0.25c	2.74 ± 0.11f
Se2.5-I5	744 ± 15e	91.5 ± 0.34a	9.55 ± 0.20c	6.33 ± 0.32b	4.84 ± 0.12c
Se5-I5	616 ± 10 g	86.2 ± 0.54b	11.25 ± 0.13a	7.47 ± 0.24a	6.28 ± 0.04a

Table 5. Effect of spinach biofortification with selenium + iodine on nitrate, nitrate reductase, ascorbic acid, total phenolic content, and the carotenoid content of the leaves. *Different lowercase letters in each column indicate a significant difference between the treatments ($P \leq 0.05$). **Fresh weight.

Treatments	DPPH (%)	Chlorophyll a (mg/kg.dw)	Chlorophyll b (mg/100 g)	Total chlorophyll (mg/g.fw**)
Control	31.5 ± 0.22 g*	6.26 ± 0.12c	2.99 ± 0.10e	9.42 ± 0.41e
Se1-I1	36.6 ± 0.32e	6.42 ± 0.21c	3.44 ± 0.12d	10.22 ± 0.11d
Se2.5-I1	43.7 ± 0.68c	7.70 ± 0.56b	3.85 ± 0.14ba	11.59 ± 0.21ba
Se5-I1	40.2 ± 0.55d	7.11 ± 0.41b	3.52 ± 0.11dc	10.66 ± 0.23c
Se1-I5	33.4 ± 0.45f	6.52 ± 0.55c	3.23 ± 0.24e	9.60 ± 0.25e
Se2.5-I5	51.6 ± 0.98a	7.92 ± 0.74a	3.98 ± 0.15a	11.94 ± 0.14a
Se5-I5	46.2 ± 1.23b	7.42 ± 0.35a	3.70 ± 0.13cb	11.16 ± 0.32bc

Table 6. Effect of spinach biofortification with selenium + iodine on DPPH (%) and chlorophyll content of the leaves. *Different lowercase letters in each column indicate a significant difference between the treatments ($P \leq 0.05$). **Fresh weight.

nutrient treatments[53]. Blasco et al.[37] demonstrated that application of iodine could significantly increase the ascorbic acid content of lettuce.

On the other hand, increasing in selenium levels positively augmented the activity of phenyl alanine ammonia-lyase (data not shown). Phenylalanine ammonia-lyase is an enzyme crucial for polyphenol biosynthesis, leading to higher total polyphenol and flavonoid content[50,54].

It seems that the effect of iodine on TPC can vary by plant species. While some studies suggest that iodine can stimulate polyphenol accumulation, others indicate that it may not have a significant impact, as seen in certain varieties of mint where iodine application led to decreased polyphenol levels[50,54].

The increase in carotenoids upon selenium biofortification, in this study, can be attributed to: (1) the increasing in the nitrate reductase activity, which in turn leads to higher concentrations of amino acids, ureides, and proteins, and therefore, enhances carotenoid biosynthesis; (2) increasing in photosynthetic pigments concentration including carotenoids in the plant leaves; (3) activating the genes expression involved in the production of enzymes like dihydroflavonol reductase, chalcone synthase, and PAL. These enzymes are essential components in the biosynthesis of carotenoids and other pigments[55]. In line with our results, Ramezani et al.[55] obtained the highest carotenoid content in fenugreek after the application of 4 mgL^{-1} of selenium. However, in another study, the carotenoid and chlorophyll content of mint decreased due to iodine application, which was inconsistent with the findings of Ramezani et al.[55] studies. In our findings, iodine concentration seems to be ineffective on carotenoid content of the spinach treatments.

The biofortification of spinach with iodine and selenium resulted in increased antioxidant activity in the leaves (Table 6), meanwhile as the concentration of these micronutrients increased, antioxidant activity also rose. Therefore, the Se5-I5 treatment showed the highest antioxidant activity (46.2%), while the control exhibited the lowest (31.5%). Selenium and iodine, significantly boosted antioxidant activity. This enhancement supports the function of antioxidant enzymes, which work synergistically with other antioxidant compounds like ascorbic acid and polyphenols, thereby positively influencing photosynthesis. Overall, the impact of selenium and iodine on the accumulation of plant antioxidants is known to depend on the chemical forms of these elements, their concentration, and the method of application[56]. Golubkina et al.[6] declared that foliar biofortification of mustard significantly increased the plant antioxidant activity. Similar results were also reported by Medrano-Macías et al.[56].

Photosynthetic pigments

The elevated concentration of chlorophyll indicates an enhanced photosynthetic capacity, oxygen production, regulation of plant growth and development with improved antioxidant properties and potential health benefits. These, all, reflect an overall improvement in plant performance. Meanwhile, chlorophyll gives plants their characteristic green color. chlorophyll absorbs light most efficiently in the blue and red regions of the visible spectrum, while reflecting green light, which is the reason for plants appear green to our eyes. Biofortifying spinach with selenium + iodine led to increased accumulation of chlorophyll a, chlorophyll b, and total chlorophyll in the leaves. The findings of different research in literature highlights the role of selenium in chlorophyll biosynthesis[57], as well as the unclear effects of foliar iodide enrichment on chlorophyll levels in salads[58], and the absence of impact observed in other studies involving lettuce seedlings[59]. In our study, the simultaneous application of selenium and iodine improved chlorophyll biosynthesis. In this regard, iodine concentration did not indicate any effect on chlorophyll content of the leaves ($P > 0.05$); meanwhile, selenium concentration showed a positive effect ($P \leq 0.05$). The observed reduction in chlorophyll a content due to the combined treatment of selenium and iodine in *B. juncea* in Golubkina et al.[6] studies corresponds with data from buckwheat sprouts treated with both elements.

Conclusion

Through foliar spraying with iodine and selenium, there was an improvement in the plant yield from 40.80 to 50.80 (t/ha). Meanwhile. total soluble solids, dry matter (%), selenium, and iodine content of the leaves increased, significantly. The nutritional quality of spinach leaves, in terms of ascorbic acid, TPC, carotenoids, and antioxidant activity, enhanced remarkably. This enhancement was accompanied by a decrease in malondialdehyde (MDA) content from 30.3 to 14.4 µmol/g, which resulted from an increase in the leaves' antioxidant capacity. The

biochemical properties of spinach leaves, particularly in terms of catalase, ascorbate peroxidase, glutathione peroxidase, and superoxide dismutase activities, were enhanced which increased the leaves' capacity to detoxify hydrogen peroxide and provided greater protection against cellular oxidative damage. The composition of essential elements in the plant, including Ca, Mg, K, S, and P showed significant improvement, too. The results also indicated that iodine uptake was lower than that of selenium. From a consumer perspective, these biofortified spinach leaves can serve as an additional source of iodine and selenium.

Data availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

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Author contributions

Amirali Eslamiparvar: Investigation, Formal analysis. Mehdi Hosseinifarahi, Supervision, methodology, statically Analysis; Sedigheh Amiri: Writing - original draft, Writing - review & editing; Mohsen Radi: Conceptualization, Methodology, all authors reviewed the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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