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Effect of Selenium Form and Salicylic Acid on the Accumulation of Selenium Speciation Forms in Hydroponically Grown Lettuce

Iwona Kowalska 1,*, Sylwester Smolen 1, Małgorzata Czernicka 1, Maryia Halka 1, Kinga Kęska 1 and Joanna Pitala 2

1 Department of Plant Biology and Biotechnology, Faculty of Biotechnology and Horticulture, University of Agriculture in Kraków, 29 Listopada 54, 31-425 Kraków, Poland; sylwester.smolen@urk.edu.pl (S.S.); malgorzata.czernicka@urk.edu.pl (M.C.); maryskagalka@gmail.com (M.H.); kinga.keska@urk.edu.pl (K.K.)

2 Laboratory of Mass Spectrometry, Faculty of Biotechnology and Horticulture, University of Agriculture in Kraków, 29 Listopada 54, 31-425 Kraków, Poland; joanna.pitala@urk.edu.pl

* Correspondence: iwona.kowalska@urk.edu.pl; Tel.: +48-126625236

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Abstract: Selenium (Se) uptake by plants depends on its form and salicylic acid (SA) can increase the efficiency of plant biofortification in Se. This study investigated the effects of selenite (Na$_2$SeO$_3$) and selenomethionine (SeMet) applied individually or together with SA on a total content of Se, Se speciation forms, yield and content of sugars and ascorbic acid of lettuce, as well as activity of selenocysteine methyltransferase (smt) and methionine methyltransferase (mmt) genes of the Se metabolic pathway. Lettuce was grown in the nutrient film technique (NFT) system. Se and SA were used at doses of 0.5 and 10.0 mg dm$^{-3}$ of the nutrient solution, respectively. The treatments were: 1. control, 2. Na$_2$SeO$_3$, 3. Na$_2$SeO$_3$ + SA, 4. SeMet, 5. SeMet + SA, 6. SA. Se was accumulated more in the roots than the leaves. SeMet was more effective in biofortification than Na$_2$SeO$_3$. SA enhanced Se uptake, especially organic Se. Plants supplied with SeMet alone or SeMet + SA accumulated in their leaves mainly SeMet and methylselenocysteine (MeSeCys), while those treated with Na$_2$SeO$_3$ or Na$_2$SeO$_3$ + SA accumulated MeSeCys and selenite (SeO$_3^{2-}$). The roots showed no expression of smt, while the expression of two mmt genes was independent of either Se or SA. The leaves of plants supplied with Na$_2$SeO$_3$ demonstrated the strongest expression of mmt and smt.

Keywords: selenite; organic selenium; biofortification; smt; mmt

1. Introduction

Selenium (Se) is a necessary component of mammalian metabolism, but it is not a plant nutrient [1]. In humans and animals, Se is involved in the metabolism of the thyroid hormone and antioxidant protection system. Se deficiency enhances the risk of cardiovascular and neoplastic diseases [2]. According to estimates, it may be lacking in the diet of no less than one billion people worldwide [3]. Consumption of Se contained in plants is the most effective method of enriching the human diet in this element [4,5]. Therefore, in some countries where soils are poor in Se, it is added to the fertilizers used for agricultural production [6].

The Se content of plants depends mainly on the amount and absorbability of soil Se and on the plant species [1]. The efficiency of Se uptake by plants depends on the form of Se. Plants absorb Se as selenite, selenate, and organic forms [7]. Selenite (SeO$_3^{2-}$) is taken up to a much lesser extent than selenate (SeO$_4^{2-}$), which is due to its lower availability as it is bound by oxides and hydroxides of aluminum and iron and soil organic matter. Transport of SeO$_3^{2-}$ through the cell membranes in the
roots occurs via phosphate ion transporters [8,9]. Upon uptake, selenite is converted into organic forms such as selenocysteine (SeCys), selenomethionine (SeMet) or selenomethionine Se-oxide (SeOMet) and many other unidentified and water-insoluble forms of Se [8], which are hardly translocated into the shoots and are mostly retained in the roots [8,10,11]. Transport of SeO$_4^{2-}$ through the cell membranes in the roots takes place via sulfate ion transporters [7,12]. Uptaken SeO$_4^{2-}$ ions do not undergo any chemical modifications and are quickly transported via xylem to the shoots and leaves. Then, they are accumulated in the same form in vacuoles or converted into SeCys and SeMet [13]. Plants are also capable of absorbing organic Se and the process is much faster than for its inorganic forms [14]. A role of amino acid transporters in the uptake of organic Se was suggested [15,16]. The transporters that catalyze the uptake and movement of cysteine and methionine within plants might also transport SeCys and SeMet.

Plants differ in their Se accumulation capacity. In so-called non-accumulator plants, SeMet and SeCys non-specifically replace methionine and cysteine in proteins, which may disturb the protein structure and inhibit catalytic activity of some enzymes [17].

On the other hand, plants called accumulators or hyper-accumulators have the capacity to detoxify Se by biosynthesizing non-protein amino acids such as methylselenocysteine (MeSeCys) and γ-glutamyl-methyl-Se-cysteine (γ-GluMeSeCys). The presence of MeSeCys strongly correlates with the capacity to hyperaccumulate Se, and it was suggested that it is the biosynthesis of MeSeCys that prevents Se incorporation into proteins [13,17]. MeSeCys generation requires methylation of SeCys by S-methylmethionine, and this reaction is catalyzed by selenocysteine methyltransferase (SMT) [13]. The gene (smt) encoding SMT is constitutively expressed in roots and leaves of some Astragalus species, belonging to hyper-accumulators [18,19], and does not appear to be induced by Se. On the other hand, it was demonstrated [13] that smt was localized predominantly within the chloroplasts in Astragalus species and its activity closely correlated with plant ability to hyperaccumulate selenium.

In hyper-accumulators, MeSeCys is intensively transformed into volatile Se forms, mainly dimethylselenide (DMDSe) [7,20]. This may be regarded as a detoxification mechanism, as methylated volatile Se species are less toxic than their inorganic precursors [21], and volatilization offers a mechanism for removing excess intracellular Se. In hyper-accumulators, the formation of DMDSe is more abundant [20], whereas in non-accumulators the synthesis of dimethylselenide (DMSe), using SeMet as a starting point, is more important [12,20]. SeMet is methylated to methylselenomethionine (MeSeMet) and the key enzyme catalyzing this process is methionine methyltransferase (MMT) [7]. In Arabidopsis, MMT is encoded by a single gene mmt (GenBank Acc. NM_124359.4), whereas in Lactuca sativa L., two copies of mmt genes were identified by searching the Lettuce Genome Resource (https://lgr.genomecenter.ucdavis.edu). Formation of volatile Se compounds occurs mainly in the roots [22]. The highest rate of Se volatilization in the roots of rice, broccoli, Indian mustard, and sugar beet was found [22] when the plants were supplied with SeMet and SeO$_3^{-2}$ as compared with SeO$_4^{2-}$.

Since the uptake and translocation of Se differ among Se forms, the concentration of different forms of Se in various parts of plants may also vary [12]. In general, plant leaves accumulate Se at the highest level when Se is supplied as SeO$_4^{2-}$, followed by SeMet and then SeO$_3^{-2}$, while the highest concentrations of Se in the roots were shown when SeMet was supplied, followed by SeO$_3^{-2}$, and then SeO$_4^{2-}$ [22]. The distribution of different Se forms may also differ among plants and their physiological stages. For example, in a hyperaccumulator Indian mustard (Brassica juncea), the main Se species in the leaves was selenate when the plant was fed with selenate, whereas in plants fed with selenite, SeMet and SeOMet dominated [23].

While excessive doses of Se may be harmful to plants due to oxidative and nitrosative stresses and non-specific replacement of S with Se in proteins [17], providing plants with small amounts of Se may be beneficial [24] and improve yield [25] and plant antioxidant capacity [26,27]. The antioxidant effect correlates with enhanced activity of glutathione peroxidase (GSH-Px) [28]. The root application of Se remarkably increased the photosynthetic rate, biomass accumulation and tolerance to cadmium.
stress in rice [29]. The increasing medium concentration of Se (SeO$_4^{2-}$) from 0 to 120 µM enhanced the total nitrogen accumulation (TNA) in lettuce [30].

Salicylic acid (SA) is a signaling substance involved in plant response to biotic and abiotic stress factors [31] by, e.g., mitigating oxidative stress. Se induces the expression of genes involved in SA synthesis in plants, and the response is much stronger in hyperaccumulators than in non-accumulators [32]. The expression of these genes, as well as the genes of jasmonic acid (JA) and ethylene biosynthesis, is probably one of the mechanisms used by hyperaccumulators to tolerate and fight excessive Se and oxidative stress [32]. Some of these responses may be triggered by shared upstream signaling pathways, including SA. On the other hand, in non-accumulators, the expression of genes involved in SA biosynthesis and SA-responsive genes is much weaker. Therefore, we hypothesized that since supplying this group of plants with increased doses of Se required for biofortification may lead to oxidative stress [31], simultaneous administration of Se and exogenous SA could be justified to strengthen the plant antioxidant system. Simultaneous supply of Se and SA to oat [33] improved plant resistance to an herbicide. Similarly, the extent of iodine biofortification of lettuce plants was increased in the presence of SA and Se [34]. The presence of SA did not affect biofortification in terms of total Se but enhanced the content of SeMet and SeCys in lettuce leaves and roots. The effects of simultaneous administration of Se in the form of selenite or organic Se together with SA on plant growth and Se content in plant organs have not been studied so far. We assumed that supplying plants with Se increases the content of MeSeCys and methylselenomethionine (MeSeMet) in the leaves, and SA reduces possible plant stress caused by Se accumulation. SeMet was chosen as an organic form. Selenite was chosen as the inorganic form, considering that this form is more easily transferred into the organic form than selenate, which stays mostly unchanged after transportation to the leaves [13,22]. Lettuce was chosen as the research object as this non-accumulator species is relatively difficult to biofortify in Se [35]. However, lettuce is widely cultivated and consumed in many countries around the world and its enrichment in Se would be highly desirable. Plants were grown hydroponically using the nutrient film technique (NFT) system, which allows the conduction of model studies on the effect of nutrient supply to plants while avoiding the confounding effects of other factors such as soil sorption.

2. Materials and Methods

2.1. Plant Material and Treatments

A two year experiment was initiated in the spring, in a high tunnel at the Faculty of Biotechnology and Horticulture, University of Agriculture in Kraków. Lettuce (Lactuca sativa L. var. capitata, cv. “Valeska”; Enza Zaden, Enkhuizen, The Netherlands) was grown in six independent units of a nutrient film technique (NFT) hydroponic system. Selected units of the NFT system were supplied with selenium in the form of Na$_2$SeO$_3$ or selenomethionine with or without salicylic acid. Na$_2$SeO$_3$ (Chempur, Karlsruhe, Germany) and selenomethionine (SeMet; Carbosynth Ltd., Compton, UK) were used at a dose of 0.5 mg Se dm$^{-3}$ (i.e., 6.3 µM Se) of the nutrient solution, and SA (Avantor Performance Materials, Gliwice, Poland) was introduced at a dose of 10.0 mg dm$^{-3}$ (i.e., 72.4 µM SA). The treatments were as follows: 1. control, 2. Na$_2$SeO$_3$, 3. Na$_2$SeO$_3$ + SA, 4. SeMet, 5. SeMet + SA, 6. SA. The control treatment involved a complete nutrient solution without Se or SA. All plants received the nutrient solution containing the same concentration of macro- and micronutrients, i.e., N 150, P 50, K 200, Mg 40, Ca 170, Fe 2.0, Mn 0.5, B 0.3, Cu 0.10, Mo 0.05 mg dm$^{-3}$. pH and EC of the medium was maintained at 5.5–6.0, EC—1.8 mS cm$^{-1}$.

Each year, the seeds of lettuce were sown into rockwool multiblocks on March 1. At the four-leaf stage, the seedlings (21 days after sowing) were placed in holes (25 cm spacing) made in Styrofoam slabs filling NFT beds. No additional substrate was used. From the day of planting in the NFT system, the plants were growing in the medium enriched with Se and SA as per the experimental design. The experiment was conducted according to a randomized block design with four replications—six plants per replicate in each treatment (24 plants per treatment). Each cultivation set included a 200 dm$^3$
container for the medium. The medium was prepared using tap water, the chemical composition of which was taken into account when calculating the amounts of individual minerals. The medium was replaced with a new one once in the growing season, i.e., after 25 days of cultivation. The fresh medium had the same content of micro- and macronutrients and Se and SA as in the beginning of the experiment. The frequency of watering was adjusted to the growth stage of lettuce and weather conditions.

Air temperature was maintained at 15–18 °C and 10–12 °C day/night. From the seed emergence until the end of March, natural light was supplemented between 6.00 a.m. and 9.00 a.m. with the use of 600 W high-pressure sodium lamps.

Lettuce plants were harvested once they were ready for harvest (45 days after planting the seedlings in the NFT system). Plants were collected separately from each replication, weighed, and the mean weight of the head was calculated for each replication. Roots were collected separately from each repetition of each variant, and their fresh weight was not determined.

2.2. Plant Analysis

2.2.1. Plant Analysis in Fresh Samples

All lettuce heads from each replication were collected for chemical analysis of the plant material. One quarter of each lettuce head was chopped in a blender and mixed in order to obtain a representative sample of all leaves (old and young) from all heads in each treatment. The material was then used to determine dry weight content by drying it at 105 °C until constant weight.

**Determination of sucrose, glucose, and fructose content.** The content of these sugars was determined after preservation of the plant material by cooking it in 96% ethanol (using a reflux condenser). The sugars were determined by capillary electrophoresis with DAD detection (Diode-Array Detection; Beckman Coulter PA 800 plus Pharmaceutical Analysis System). Capillaries of Ø 25 µm, o.d. (outer diameter) 365 µm and total length of 60 cm (50 cm to the detector) were used. A negative power supply of 30 kV normal polarity was applied. The sugar content was measured at 205 nm. The running buffer solution contained 15 mL of 36 mmol Na₂HPO₄, 130 mmol NaOH, and 1.93 mmol β-cyclodextrin (pH 12.7) [34].

**Determination of ascorbic acid content.** The second quarter of lettuce head was minced with a plastic knife. Then, 20 g of the plant material was homogenized in 80 mL of 2% oxalic acid (Avantor Performance Materials). Next, the samples were centrifuged for 15 min at 4500 rpm, at 5 °C. The supernatants were filtered through 0.25 µm cellulose acetate membrane filters and analyzed using PA 800 Plus capillary electrophoresis system (Beckman Coulter, Brea, CA, USA) with DAD detection. Capillaries of i.d. (internal diameter) 50 µm, o.d. 365 µm and total length of 50 cm (40 cm to the detector) were used. A negative power supply of −25 kV was applied. The running buffer solution contained 30 mmol NaH₂PO₄, 15 mmol Na₂B₄O₇ and 0.2 mmol CTAB (cetyltrimethylammonium bromide; pH 8.80) [36].

**Quantitative real-time RT-PCR analysis of smt and mmt genes in leaves and roots.** The third youngest leaf and root samples containing apices were collected directly before harvest from single plants. There were four biological samples collected simultaneously from each experimental treatment. The samples were frozen in liquid nitrogen immediately after collection and stored at −80 °C until use. Total RNA extraction was carried out with Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer’s instructions. Quality and integrity of total RNA samples were assessed by electrophoresis in 1% agarose gel in denaturing conditions. Quantity of RNA was estimated spectrophotometriically using NanoDrop 2000c (Thermo Scientific™, Wilmington, DE, USA) at 260 and 280 nm, and 260/280 nm ratio within the range of 2.00–2.15 was retained. To avoid DNA contamination and RNA degradation, RNA samples were treated with RNase-free Dnase I (1U µL⁻¹, Ambion Inc., Austin, TX, USA) and RiboLock RNase Inhibitor (40 U µL⁻¹, Thermo Fisher Scientific, Wilmington, DE, USA). One microgram of total RNA from all samples was transcribed into cDNA using iScript synthesis kit (BioRad laboratories, Hemel Hempstead, UK) as stated in the manufacturer’s instructions.
**Arabidopsis thaliana** nucleotide sequences encoding homocysteine methyltransferase 2 (GenBank Acc. no. NM_202753.2) and methionine S-methyltransferase (GenBank Acc. no. NM_124359.4) were used in the Basic Local Alignment Search Tool (BLAST) search against Lettuce Genome Resource (LGR) (https://lgr.genomecenter.ucdavis.edu). As a result, smt gene was found on chromosome 5 of *L. sativa*, i.e., Lsat_1_v5_gn_5_186100.1, and two mmt genes were identified on chromosome 9, i.e., Lsat_1_v5_gn_9_83520.1 Lsat_1_v5_gn_9_125160.1 (Table 1). For mmt genes, highly specific primers were designed (Table 1, and submitted to BLAST to verify their specificity, whereas the formation of primer-dimers, structural hairpins and melting temperature (Tm) were assessed with OligoAnalyzer 3.1 software (www.idtdna.com). The primer sequences for selenocysteine methyltransferase gene (smt) were adopted from our previous study (34). Transcript levels were normalized using two reference genes, i.e., *act* and protein phosphatase 2A regulatory subunit A3 (*pp2a3PP2AA3*), for which primers were described by [34] and [37], respectively (Table 1). The utility of the designed primers was validated using PCR and confirmed by electrophoresis in 1% agarose gel. Primer specificity was verified by analyzing a single peak in the melting curves. Real-time PCR reaction mixture consisted of 12.5 µL of Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo Fisher Scientific, Wilmington, DE, USA), 0.7 µM of each primer (forward and reverse), 2 µL of 5 times diluted cDNA template, and up to 25 µL of nuclease-free DEPC-treated water (diethylpyrocarbonate; Thermo Fisher Scientific, Wilmington, DE, USA). PCR was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in 96-well plates with the following cycling parameters: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 52 °C for 1 min, followed by a standard melting curve (95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s with a reading at every 0.3 °C). Each run included a negative control and a cDNA reaction without reverse transcriptase to rule out DNA contamination. Each qPCR reaction was repeated twice: a single qPCR reaction was conducted in three technical replicates among four biological ones (4 biological repetitions × 3 technical instrumental replicates × 2 qPCR reaction repetitions). Standard curves of the investigated genes were determined for 10-fold dilutions of pooled cDNA. Efficiency of each gene was calculated from the slope of the standard curve using the formula \[ E = 10^{-\frac{1}{\text{slope}}} \] and converted into percentage values according to the formula \[ \%E = (E - 1) \times 100\% \]. The \( \Delta\Delta CT \) method [38] was used to normalize and calculate ratios of expression levels (relative fold changes) in accordance with housekeeping genes, i.e., *act* and *pp2a3*, the expression of which did not change in response to changes in the composition of the nutritional solution.

**Table 1.** Sequences of genes and primers used in the study.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Reference/Gene Model in Lettuce Genome Resource *</th>
<th>Primer Sequences F: 5′–3′</th>
<th>R: 5′–3′</th>
<th>Amplicon Length (bp)</th>
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</thead>
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<td>ACT</td>
<td>actin</td>
<td>Smolen et al. (2016) Lsat_1_v5_gn_8_116260.1</td>
<td>AGGTGTACATGTTGCGATGGGA</td>
<td>TGTCTTCAGGGCCGACACG</td>
<td>180</td>
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<tr>
<td>PP2AA3</td>
<td>protein phosphatase 2A regulatory subunit A3</td>
<td>Sgamma et al. (2016) Lsat_1_v5_gn_8_38881.1</td>
<td>CATGCAATGGTTACAAGACAAGGTAT</td>
<td>CAAACTCCTCCGCAAGTCTCTC</td>
<td>80</td>
</tr>
<tr>
<td>SMT</td>
<td>selenocysteine methyltransferase</td>
<td>Smolen et al. (2016) Lsat_1_v5_gn_5_186100.1</td>
<td>ACACAGGAGCTGGAGAATGGAA</td>
<td>CTGTGATGGTGGTGTTCG</td>
<td>108</td>
</tr>
<tr>
<td>MMT</td>
<td>selenomethionine Se-methyltransferase</td>
<td>- Lsat_1_v5_gn_9_83520.1</td>
<td>CGCTACACTGCAAAGAAA</td>
<td>GTCATGTTTCGAACGCTCT</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Lsat_1_v5_gn_9_125160.1</td>
<td>CAGCTGAAAGACACTGG</td>
<td>GGCTTCCAAGAACGCAAAG</td>
<td>141</td>
</tr>
</tbody>
</table>


2.2.2. Plant Analysis after Sample Drying

The remaining parts of the lettuce heads were dried in a forced-air dryer at 65 °C for 24 h. The same procedure was followed for the roots that were first washed twice in distilled water. Air-dried material (leaves and roots) was pulverized in a variable speed rotor mill Pulverisette 14, FRITSCH using a 0.5 mm sieve.
Sample preparation by enzymatic extraction. Total Se and its speciation forms were determined in air-dried and pulverized leaves and roots. First, 0.1 g of plant material was placed in 7 mL polypropylene tubes. Then, 2.5 mL of protease (8 g dm\(^{-3}\)) and lipase (4 g dm\(^{-3}\)) solutions were added, and the samples were incubated at 37 °C for 16 h. After incubation, the samples were left to cool down and centrifuged at 4500 rpm for 15 min at 5 °C. The supernatant was pipetted into 1.5 mL Eppendorf tubes and centrifuged again at 10,000 rpm for 5 min. For analysis, the samples were diluted 10 times with demineralized water [36].

Total Se in enzymatic extracts was determined using inductively coupled plasma mass spectrometry (iCAP TQ ICP-MS) and 40 ppb (Part per billion) tellurium solution as an internal standard. Se detection mode of S-TQ-O\(_2\) allowed us to determine the presence of \(^{80}\text{Se}\text{^{16}O}\).

Determination of Se speciation forms (in enzymatic extracts), i.e., \(\text{SeO}_3^{2-}\), \(\text{SeO}_4^{2-}\), selenocysteine (Se\(_2\text{Cys}\)), SeMet, MeSeCys, SeOMet was carried out using high performance liquid chromatography combined with inductively coupled plasma mass spectrometry (HPLC-ICP-MS/MS). Separation of Se compounds was performed using a HPLC Thermo Scientific Ultimate 3000, on a Dionex IonPac AS11 anion exchange column with a Dionex IonPac AG11 precolumn thermostated at 30 °C. The mobile phases included 50 mM NaOH, demineralized water and 0.5% TMAH (tetramethylammonium hydroxide). The separation procedure used a gradient flow with constant concentration of NaOH (1.2%) and TMAH concentration increasing from 0 to 98.8%. The flow rate was 1.5 mL min\(^{-1}\) (Table 2) [39]. The eluate flow from the chromatograph was directed to a mass spectrometer ICP-MS (iCAP TQ ICP-MS). Standards of Se forms were prepared by solving Na\(_2\text{SeO}_3\) (Chempur, Karlsruhe, Germany), Na\(_2\text{SeO}_4\) (Sigma Aldrich, St. Louis, MO, USA), Se\(_2\text{Cys}\) (Acros Organics, Carlsbad, CA, USA), SeMet (Carbosynth Ltd., Compton, UK), and MeSeCys (Sigma Aldrich, St. Louis, MO, USA) in demineralized water. The oxidized form of selenomethionine (SeOMet) was prepared by reacting selenomethionine with hydrogen peroxide.

Determination of salicylic acid

SA content was determined in 0.2 g samples of air-dried plant material mixed with 5 mL of demineralized water. Following vortexing for 1 min., the samples were placed in a water bath at water temperature of 60 °C. After 30 min, they were removed, cooled down to room temperature and centrifuged for 15 min. at 4500 rpm at 5 °C. Centrifuged samples were filtered through a cellulose...
Agriculture 2020, 10, x FOR PEER REVIEW 7 of 21 acetate filter. The filtrate was analyzed by capillary electrophoresis in a PA 800 Plus system (Beckman Coulter, Brea, CA, USA). Capillaries of 75 µm i.d. and 365 µm o.d. and a total length of 30 cm (20 cm to the detector window) were used. Separation was conducted at −25 kV and detection of SA at 205 nm. The running buffer solution contained 10 mM Tris (Sigma-Aldrich, St. Louis, MO, USA) at pH 2.78 set by formic acid (Avantor Performance Materials, Gliwice, Poland) [40].

Statistical analysis

Statistical analysis was performed with Statistica 12.0 PL. There was no effect of the year of the study, which is why the results presented are a mean of two years. Before analysis, all data were screened for normality using the Shapiro–Wilk test. The data with normal distribution (yield, sucrose, glucose, fructose, total sugars, ascorbic acid and smt, mmt, SeO₃²⁻, SeO₄²⁻, Se₂Cys in the leaves and roots) were subjected to one-way analysis of variance. Tukey’s test was then carried out to determine the significance of differences between the means. The data without normal distribution (SeMet, MeSeCys, SeOMet, Se-total in the leaves and roots, and SA in the roots) were subjected to the Kruskal–Wallis test with multiple comparisons using Conover–Iman’s test to evaluate significant differences between the means. Results were considered significant at 0.05 probability level (p < 0.05).

3. Results

3.1. Yield of Lettuce

Lettuce yield was expressed as the average mass (g) of a single head (Figure 1). We found no significant differences in the yield, except for plants supplied with SeMet and SeMet + SA. Application of SA with SeMet (SeMet + SA) significantly reduced yield as compared with SeMet alone. The differences in the average head weight between these variants was 42 g.

![Figure 1. Yield—mass of a single lettuce head. Means followed by different letters indicate significant differences (p < 0.05). The bars indicate standard error (n = 4).](image)

3.2. Content of Sugars and Ascorbic Acid

Enriching the medium with Se or SA significantly reduced the content of sucrose, glucose, fructose, and total sugars in lettuce leaves as compared with the control (Table 3). The presence of SA in Se-enriched media intensified the reduction in sucrose, glucose, fructose, and total sugar levels in relation to the plants treated with Se alone. The differences were significant only for glucose and total sugars in SeMet + SA vs. SeMet variants.

Neither Se or SA affected the levels of ascorbic acid in lettuce leaves (Table 3), although it tended to be higher in plants exposed to SeMet with or without SA and SA alone.
Table 3. The content of ascorbic acid, sucrose, glucose, fructose, and total sugars in lettuce leaves.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sucrose (mg 100 g⁻¹ f.w.)</th>
<th>Glucose (mg 100 g⁻¹ f.w.)</th>
<th>Fructose (mg 100 g⁻¹ f.w.)</th>
<th>Total Sugars (mg 100 g⁻¹ f.w.)</th>
<th>Ascorbic Acid (mg 100 g⁻¹ f.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>87 ± 2.18 b</td>
<td>448 ± 8.69 c</td>
<td>620 ± 23.48 c</td>
<td>1155 ± 28.41 c</td>
<td>13.62 ± 1.20 *</td>
</tr>
<tr>
<td>Na₂SeO₃</td>
<td>57 ± 1.53 a</td>
<td>333 ± 25.76 b</td>
<td>437 ± 29.00 ab</td>
<td>828 ± 55.37 b</td>
<td>13.30 ± 0.62</td>
</tr>
<tr>
<td>Na₂SeO₃ + SA</td>
<td>44 ± 2.63 a</td>
<td>263 ± 32.32 ab</td>
<td>373 ± 32.51 ab</td>
<td>680 ± 66.94 ab</td>
<td>13.80 ± 1.02</td>
</tr>
<tr>
<td>SeMet</td>
<td>62 ± 7.67 a</td>
<td>336 ± 14.77 b</td>
<td>443 ± 13.10 b</td>
<td>841 ± 29.25 b</td>
<td>16.18 ± 1.23</td>
</tr>
<tr>
<td>SeMet + SA</td>
<td>53 ± 7.10 a</td>
<td>208 ± 12.39 ab</td>
<td>345 ± 11.87 ab</td>
<td>606 ± 25.84 a</td>
<td>15.77 ± 0.48</td>
</tr>
<tr>
<td>SA</td>
<td>47 ± 3.53 a</td>
<td>186 ± 5.75 a</td>
<td>339 ± 16.87 a</td>
<td>571 ± 25.90 a</td>
<td>15.06 ± 0.80</td>
</tr>
</tbody>
</table>

* Differences between means for ascorbic acid content were not significant. Means followed by different letters indicate significant differences (p < 0.05). Control—without selenium and salicylic acid.

3.3. Content of Total Se and Se Speciation Forms

Irrespective of the form of supplied Se, roots accumulated greater amounts of total Se than the leaves (Tables 4 and 5a,b). The leaf to root ratio of total Se content ranged from 0.17–0.20 for plants supplied with selenite (Na₂SeO₃) to 1.16 for control plants (Table 4). For plants supplied with organic Se alone, the ratio was 0.93. Application of SA decreased the ratio, especially for plants supplied with SeMet and it was about 2.2 times lower than for organic Se alone.

Table 4. The leaf to root ratio of total Se content.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leaves/Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.16</td>
</tr>
<tr>
<td>Na₂SeO₃</td>
<td>0.20</td>
</tr>
<tr>
<td>Na₂SeO₃ + SA</td>
<td>0.17</td>
</tr>
<tr>
<td>SeMet</td>
<td>0.93</td>
</tr>
<tr>
<td>SeMet + SA</td>
<td>0.42</td>
</tr>
<tr>
<td>SA</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Control—without application of Se and SA.

Leaves. The greatest amounts of Se in the form of SeO₃⁻² were detected in the leaves of plants growing in the medium supplemented with Na₂SeO₃+SA (Table 5a). The content of SeO₃⁻² was significantly higher than in control and the plants treated with SA alone. A similar relationship was found for leaf content of SeO₄⁻², even though the levels of SeO₄⁻² were very low in all experimental variants. Similarly, the content of selenocysteine (Se₂Cys) was also low and remained at the control level.

Enriching the medium with Se alone or in combination with SA significantly affected SeMet content (Table 5b). Leaves of the plants supplied with organic Se accumulated nearly 10 times more SeMet than those supplied with mineral Se. Plants exposed to SeMet and SA significantly enhanced their leaf SeMet content. SeMet levels in control and SA-only variants were below the level of quantification with HPLC-ICP-MS/MS.

The content of MeSeCys in the variants with Se was higher than in control and SA only (Table 5b). The highest content of MeSeCys was demonstrated in the variants with organic Se, while plant exposure to SeMet + SA significantly lowered the levels of MeSeCys as compared with the treatment with organic Se alone (SeMet).

SeOMet was detected only in the leaves of plants treated with SeMet and SeMet+SA (Table 5b). Moreover, the combination of SeMet and SA considerably increased the content of SeOMet as compared with organic Se alone. Leaf content of total Se differed in plants exposed to different Se forms and SA (Table 5b) and was the highest in variants supplied with SeMet and SeMet + SA. In the leaves of these plants, total Se content was about eight times higher than in those treated with inorganic Se with or without SA. Lettuce growing in the medium enriched with Na₂SeO₃ or Na₂SeO₃ + SA was also
more abundant in total Se than control and SA only variants. Total leaf Se levels were unaffected by SA application either in organic or inorganic Se variants.

Identified speciation forms of Se accounted for 12 to 43% of total Se in the leaves (Table 6).

The lowest share of these forms was detected in the leaves of plants grown in the presence of organic Se, i.e., SeMet and SeMet + SA, where they constituted only 12.6–15.1%. The main speciation forms involved SeO$_3^{2-}$ and MeSeCys in plants exposed to inorganic Se, and SeMet and MeSeCys in plants supplied with organic Se. Medium supplementation with SA increased the share of these forms in the variants with inorganic Se and had the opposite effect in the case of organic Se supplementation.

**Roots.** SeO$_3^{2-}$ was the most common form of inorganic Se in the roots (Table 5a). It was detected in the roots of all variants, and the content of SeO$_3^{2-}$ was the lowest in control and in the presence of SA. Plants exposed to inorganic Se accumulated significantly greater amounts of SeO$_3^{2-}$ than those supplied with organic Se compounds. The levels of SeO$_3^{2-}$ further increased under joint application of SA and Se. Although to a lower degree than in the case of selenite, the content of SeO$_3^{2-}$ in the roots of plants supplied with organic Se was much higher than in control plants.

All plants featured low content of SeO$_4^{3-}$, even though in Na$_2$SeO$_3$ and Na$_2$SeO$_3$ + SA variants it was significantly higher than in the other plants.

**Table 5.** The content of Se speciation forms and total Se in lettuce leaves and roots.

<table>
<thead>
<tr>
<th>Part of Plant</th>
<th>Treatment</th>
<th>SeO$_3^{2-}$</th>
<th>SeO$_4^{2-}$</th>
<th>Se$_2$Cys</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>Control</td>
<td>0.184 ± 0.062$^a$</td>
<td>0.040 ± 0.014$^a$</td>
<td>0.147 ± 0.019$^{ab}$</td>
</tr>
<tr>
<td></td>
<td>Na$_2$SeO$_3$</td>
<td>0.572 ± 0.0116$^{ab}$</td>
<td>0.066 ± 0.017$^{ab}$</td>
<td>0.068 ± 0.016$^a$</td>
</tr>
<tr>
<td></td>
<td>Na$_2$SeO$_3$ + SA</td>
<td>1.376 ± 0.409$^b$</td>
<td>0.193 ± 0.039$^b$</td>
<td>0.077 ± 0.021$^{ab}$</td>
</tr>
<tr>
<td></td>
<td>SeMet</td>
<td>0.633 ± 0.263$^{ab}$</td>
<td>0.160 ± 0.025$^{ab}$</td>
<td>0.127 ± 0.029$^{ab}$</td>
</tr>
<tr>
<td></td>
<td>SeMet + SA</td>
<td>0.737 ± 0.119$^{ab}$</td>
<td>0.092 ± 0.060$^{ab}$</td>
<td>0.204 ± 0.027$^b$</td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>0.190 ± 0.083$^a$</td>
<td>0.029 ± 0.012$^a$</td>
<td>0.074 ± 0.051$^{ab}$</td>
</tr>
<tr>
<td>Roots</td>
<td>Control</td>
<td>0.496 ± 0.027$^a$</td>
<td>0.117 ± 0.023$^a$</td>
<td>0.058 ± 0.000$^a$</td>
</tr>
<tr>
<td></td>
<td>Na$_2$SeO$_3$</td>
<td>6.015 ± 0.216$^d$</td>
<td>0.630 ± 0.040$^b$</td>
<td>0.146 ± 0.000$^b$</td>
</tr>
<tr>
<td></td>
<td>Na$_2$SeO$_3$ + SA</td>
<td>7.331 ± 0.163$^c$</td>
<td>0.483 ± 0.100$^b$</td>
<td>0.490 ± 0.029$^c$</td>
</tr>
<tr>
<td></td>
<td>SeMet</td>
<td>3.689 ± 0.286$^b$</td>
<td>0.059 ± 0.026$^a$</td>
<td>0.446 ± 0.022$^c$</td>
</tr>
<tr>
<td></td>
<td>SeMet + SA</td>
<td>4.771 ± 0.266$^c$</td>
<td>0.030 ± 0.011$^a$</td>
<td>0.968 ± 0.040$^d$</td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>0.289 ± 0.082$^a$</td>
<td>0.039 ± 0.015$^a$</td>
<td>0.170 ± 0.016$^b$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Part of Plant</th>
<th>Treatment</th>
<th>SeMet</th>
<th>MeSeCys</th>
<th>SeOMet</th>
<th>Se-Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>Control</td>
<td>0.000 ± 0.000$^a$</td>
<td>0.112 ± 0.008$^a$</td>
<td>0.000 ± 0.000$^a$</td>
<td>1.375 ± 0.395$^a$</td>
</tr>
<tr>
<td></td>
<td>Na$_2$SeO$_3$</td>
<td>0.167 ± 0.033$^b$</td>
<td>0.810 ± 0.125$^b$</td>
<td>0.000 ± 0.000$^a$</td>
<td>5.513 ± 0.710$^b$</td>
</tr>
<tr>
<td></td>
<td>Na$_2$SeO$_3$ + SA</td>
<td>0.209 ± 0.022$^b$</td>
<td>0.839 ± 0.162$^b$</td>
<td>0.000 ± 0.000$^a$</td>
<td>7.180 ± 0.873$^b$</td>
</tr>
<tr>
<td></td>
<td>SeMet</td>
<td>1.608 ± 0.025$^c$</td>
<td>5.344 ± 1.283$^d$</td>
<td>0.140 ± 0.069$^b$</td>
<td>52.900 ± 5.883$^c$</td>
</tr>
<tr>
<td></td>
<td>SeMet + SA</td>
<td>2.914 ± 0.155$^d$</td>
<td>1.614 ± 0.519$^c$</td>
<td>0.673 ± 0.287$^c$</td>
<td>49.615 ± 9.274$^c$</td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>0.000 ± 0.000$^a$</td>
<td>0.111 ± 0.007$^a$</td>
<td>0.000 ± 0.000$^a$</td>
<td>0.940 ± 0.135$^a$</td>
</tr>
<tr>
<td>Roots</td>
<td>Control</td>
<td>0.000 ± 0.000$^a$</td>
<td>0.000 ± 0.000$^a$</td>
<td>0.000 ± 0.000$^a$</td>
<td>1.188 ± 0.166$^a$</td>
</tr>
<tr>
<td></td>
<td>Na$_2$SeO$_3$</td>
<td>3.723 ± 0.662$^b$</td>
<td>0.295 ± 0.041$^b$</td>
<td>0.014 ± 0.008$^a$</td>
<td>27.315 ± 1.001$^c$</td>
</tr>
<tr>
<td></td>
<td>Na$_2$SeO$_3$ + SA</td>
<td>3.759 ± 0.638$^b$</td>
<td>1.002 ± 0.219$^b$</td>
<td>0.072 ± 0.017$^b$</td>
<td>41.990 ± 1.018$^d$</td>
</tr>
<tr>
<td></td>
<td>SeMet</td>
<td>2.857 ± 0.054$^b$</td>
<td>1.783 ± 0.128$^c$</td>
<td>1.514 ± 0.186$^b$</td>
<td>56.725 ± 2.073$^c$</td>
</tr>
<tr>
<td></td>
<td>SeMet + SA</td>
<td>7.521 ± 1.783$^c$</td>
<td>2.929 ± 0.214$^d$</td>
<td>0.708 ± 0.708$^a$</td>
<td>118.770 ± 8.211$^f$</td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>0.000 ± 0.000$^a$</td>
<td>0.000 ± 0.000$^a$</td>
<td>0.032 ± 0.012$^a$</td>
<td>2.585 ± 0.409$^b$</td>
</tr>
</tbody>
</table>

Means followed by different letters indicate significant differences (p < 0.05). Control—without application of Se.
Supplementation of the medium with Se enhanced root accumulation of SeMet (Table 5b). When SeMet was accompanied by SA, SeMet content in the roots was 2–2.5 times higher than in the remaining Se-treated plants. Such an effect of SA was not observed following the application of selenite. Adding SA to Se-enriched media also enhanced MeSeCys synthesis in the roots, and this compound was significantly more abundant in the SeMet + SA variant.

Even though roots differed significantly in their content of Se2Cys and SeOMet, the concentration of these Se forms was low in all plants (Table 5a,b). Adding SA to Se-enriched media significantly increased root content of Se2Cys.

The plants exposed to SeMet accumulated more total Se in the roots than those treated with Na2SeO3 (Table 5b). The presence of SA enhanced total Se concentration in all Se-treated variants.

The smallest share of Se speciation forms in relation to total Se was identified in the roots of plants treated with organic Se, i.e., SeMet and SeMet + SA (Table 6). In these variants, the identified speciation forms accounted for only 14.2–18.2% of total Se. The main speciation forms in the plants exposed to inorganic Se involved SeO3−2 and SeMet, while in those supplied with organic Se SeO3−2, SeMet and MeSeCys prevailed. The presence of SA reduced the share of these forms.

Unidentified Se speciation forms in the roots accounted for 43.5–85.8%, and they were the most abundant in the roots of plants exposed to organic Se. The chromatogram presented in Figure 2 shows the signals from unidentified Se compounds, one of them being particularly strong. The strength of the signal recorded by the HPLC-ICP-MS/MS analytical system indicated a significant share of this compound among Se speciation forms accumulated in the roots.

**Figure 2.** Chromatogram of lettuce extract derived from plants grown in selenomethionine (SeMet)-enriched medium and a chromatogram of the same lettuce extract with MeSeCys, SeMet, Se2Cys, SeO3−2 and SeO4−2 standards.

<table>
<thead>
<tr>
<th>Part of Plant</th>
<th>Se Form in the Medium</th>
<th>SeO3−2</th>
<th>SeO4−2</th>
<th>Se2Cys</th>
<th>SeMet</th>
<th>MeSeCys</th>
<th>SeOMet</th>
<th>Sum of Identif. Forms, %</th>
<th>% of Unidentif. Forms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leaves</strong></td>
<td>Control</td>
<td>13.4</td>
<td>2.9</td>
<td>10.7</td>
<td>0.0</td>
<td>8.2</td>
<td>0.0</td>
<td>35.1</td>
<td>64.9</td>
</tr>
<tr>
<td></td>
<td>Na2SeO3</td>
<td>10.4</td>
<td>1.2</td>
<td>1.2</td>
<td>0.0</td>
<td>2.7</td>
<td>0.0</td>
<td>30.5</td>
<td>69.5</td>
</tr>
<tr>
<td></td>
<td>Na2SeO3 + SA</td>
<td>19.2</td>
<td>2.7</td>
<td>1.8</td>
<td>2.9</td>
<td>11.7</td>
<td>0.0</td>
<td>37.5</td>
<td>62.5</td>
</tr>
<tr>
<td></td>
<td>SeMet</td>
<td>1.2</td>
<td>0.3</td>
<td>0.2</td>
<td>2.9</td>
<td>10.1</td>
<td>0.3</td>
<td>15.1</td>
<td>84.9</td>
</tr>
<tr>
<td></td>
<td>SeMet + SA</td>
<td>1.5</td>
<td>0.2</td>
<td>0.4</td>
<td>5.9</td>
<td>3.3</td>
<td>1.4</td>
<td>12.6</td>
<td>87.4</td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>20.2</td>
<td>3.1</td>
<td>7.9</td>
<td>0.0</td>
<td>11.8</td>
<td>0.0</td>
<td>43.0</td>
<td>57.0</td>
</tr>
<tr>
<td><strong>Roots</strong></td>
<td>Control</td>
<td>41.8</td>
<td>9.9</td>
<td>4.9</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>56.5</td>
<td>43.5</td>
</tr>
<tr>
<td></td>
<td>Na2SeO3</td>
<td>22.0</td>
<td>2.3</td>
<td>0.5</td>
<td>13.6</td>
<td>1.1</td>
<td>0.1</td>
<td>39.6</td>
<td>60.4</td>
</tr>
<tr>
<td></td>
<td>Na2SeO3 + SA</td>
<td>17.5</td>
<td>1.2</td>
<td>1.2</td>
<td>8.9</td>
<td>2.4</td>
<td>0.2</td>
<td>51.3</td>
<td>48.7</td>
</tr>
<tr>
<td></td>
<td>SeMet</td>
<td>6.5</td>
<td>0.1</td>
<td>0.8</td>
<td>5.0</td>
<td>3.1</td>
<td>2.7</td>
<td>18.2</td>
<td>81.8</td>
</tr>
<tr>
<td></td>
<td>SeMet + SA</td>
<td>4.0</td>
<td>0.0</td>
<td>0.8</td>
<td>6.3</td>
<td>2.5</td>
<td>0.6</td>
<td>14.2</td>
<td>85.8</td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>11.2</td>
<td>1.5</td>
<td>6.6</td>
<td>0.0</td>
<td>0.0</td>
<td>1.2</td>
<td>20.5</td>
<td>79.5</td>
</tr>
</tbody>
</table>

Control—without application of Se and SA.
3.4. Expression of smt and mmt Genes in Leaves and Roots

No expression of the selenocysteine methyltransferase (smt) gene was detected in lettuce roots (Figures 3 and 4), but it was confirmed in all variants in the leaves. The expression was the strongest in the plants exposed to inorganic Se without SA (Na$_2$SeO$_3$), and it was significantly greater than in the remaining variants. The SeMet + SA variant showed lowered smt expression in relation to the control, and significantly lower expression than in all other variants.

**Figure 3.** Log$_2$ fold change in selenocysteine methyltransferase (smt) and methionine methyltransferase (mmt) gene expression in lettuce roots and leaves. Relative gene expression levels were calculated as a ratio of treatment to control samples from roots and leaves independently. Data are presented as means of four biological samples. Different letters indicate significant differences between treatments. ns—nonsignificant differences among treatments. nd—gene expression not detected.
3.5. Content of Salicylic Acid

SA content was higher in the roots than in the leaves for all variants (Figure 5), and it increased significantly in both organs when supplied with the medium either alone or together with organic and inorganic forms of Se. The highest SA content in the roots was detected in the SeMet + SA variant, and it was 2.2 times higher than in the variant with SA alone.

![Figure 4](attachment:image.png)

Figure 4. Confirmation of the specificity of primers and amplicon sizes for smt and mmt genes based on gel electrophoresis of RT-PCR products. M: 1 kB ladder. See Table 1 for product sizes.

Contrary to the leaves, no effects of the experimental factors on the expression of two genes encoding methionine methyltransferase (mmt) were found in the roots. Both mmt and smt genes were most abundantly expressed in the leaves of the plants supplied with selenite alone (Na$_2$SeO$_3$). In the remaining variants, mmt genes were downregulated in comparison with control plants. In the plants supplemented with Na$_2$SeO$_3$ + SA and SeMet alone or with SA, the expression level of mmt in the leaves was lower than when the plants were treated with SA alone.

![Figure 5](attachment:image.png)

Figure 5. Effect of selenium form and salicylic acid application on the content of salicylic acid in the leaves and roots of lettuce. Means followed by different letters indicate significant differences ($p < 0.05$). The bars indicate standard error ($n = 4$).
4. Discussion

Lettuce belongs to non-accumulators, i.e., plants that have not developed mechanisms of tolerating high doses of Se in their root environment. Excessive Se concentrations not only negatively affect their yield but may also be toxic [17]. However, plants of this group, including lettuce, may tolerate and accumulate certain amounts of Se < 100 µg g⁻¹ d.w (Dry weight). [35,41]. Moreover, low doses of Se may even enhance yield and plant tolerance to oxidative stress [30,42]. Due to the role of lettuce in the human diet, its biofortification with Se could be a way of increasing Se consumption [25,30]. The most important question is how much and what form of Se should be supplied to lettuce so that it does not suppress yield and quality but allows for accumulation of the amount of Se that positively affects the human diet. According to [25], to meet the dietary demand for Se, lettuce should be grown in a medium containing up to 10 µM Se as selenite or up to 6 µM Se as selenate. To match the recommended daily allowance (RDA) for an adult, Se doses should be considerably higher, i.e., 40 µM and 10 µM Se in the medium in the form of selenate and selenite, respectively [43]. Our experiment aimed at biofortification with Se supplied in its organic form, and we compared effectiveness of this process with selenite. We assumed that by application of organic Se, the biofortification may be more effective than by using selenite (SeO₃⁻²). Firstly, the organic form of Se is taken up by plants at an over 20 times higher rate than selenate or selenite [16]. Secondly, it is mostly transported to the edible part of lettuce. We additionally assumed that the SA addition to the medium would improve plant tolerance to stress evoked by possibly excessive dose of Se. The biofortification effect was investigated by analyzing the content of total Se and Se speciation forms in the roots and leaves, and the plant response to Se forms, and the presence of SA was assessed based on the yield and the content of sugars and ascorbic acid.

4.1. Yield and the Content of Sugars and Ascorbic Acid

Enriching the medium with organic or inorganic Se compounds at 6.3 µM (0.5 mg Se dm⁻³) did not affect lettuce yield. Many studies have shown positive effects of low Se doses on plant growth and developmental disturbances at higher doses [17,25]. Beneficial dose of Se depends mainly on the plant species, chemical form of Se and its application method, and cultivation conditions [25,30,44]. The stimulating effect of low doses of Se was reported for 5 µM selenite and 20 µM selenate [43], and for 2–10 µM selenite and 6 µM selenate [25]. On the other hand, relatively high Se doses were also found to be harmless or nearly harmless to lettuce [44]. In their study, triple soil fertigation with sodium selenite (Na₂SeO₃) at 50, 100 and 200 mg kg⁻¹ soil significantly enhanced yield and growth parameters of lettuce. In general, Se toxicity threshold is much lower in hydroponic cultures than in soil cultivation [30,43]. The thresholds at 15 µM for SeO₃⁻² and 20 µM for SeO₄⁻² [25] and at 10 µM for SeO₃⁻² and 40 µM for SeO₄⁻²[43] were established. Beneficial effects of Se on plant biomass may be due to Se’s influence on the content of assimilation pigments [9], stomatal conductance, intercellular CO₂ concentration, photosynthetic rate, chlorophyll content, and leaf area [9,25,29,45,46]. The leaf area in lettuce supplemented with 2–10 µM Se as selenite was considerably greater (by about 12–27%) than in control plants [25]. Low doses of Se in rice positively influenced photosynthesis, which resulted in increased grain yield [9]. Similar results were reported in other plants species treated with Se, such as winter jujube [47] or tomato [48]. The positive effects of low Se concentrations on plant biomass growth may be also due to improved antioxidant capacity of plants and their better stress tolerance [27,29,49].

Our experiment showed negative effects of Se on lettuce yield only when Se was applied together with SA, and the effect was observed only for the SeMet + SA variant. Yield reduction in this variant, not recorded for plants treated with SA alone or SA in combination with inorganic Se, could be due to over two times greater accumulation of total Se in the roots in comparison with application of SeMet alone. It can be assumed that the total Se accumulated in the roots in this variant was toxic to the plants, which manifested in a decrease in their yield. Other studies have demonstrated yield reduction as a result of absorbing excessive amounts of Se, which in non-accumulators leads to inclusion of selene-amino acids into peptides and disruption of protein synthesis [7,50]. The dual effect of Se on plant growth (beneficial or toxic) was also observed in lettuce by others [25,35,43].
Irrespective of its form, Se presence in the medium lowered leaf sugar content. This trend was more apparent in the presence of SA, in particular with application of SeMet. One of possible reasons for the sugar drop might be using sugars as energy sources to uptake and transport Se, and both of these processes were intensified in plants exposed to SA. The mechanism of SeMet absorption is metabolically controlled and requires energy [12].

Our experiment showed no effect of Se alone or Se in combination with SA on the content of ascorbic acid. We only observed a trend for a higher content of ascorbate in the presence of organic Se and SA. Many studies have suggested that Se induces ascorbate synthesis [43,47,51,52]. The beneficial effects of both inorganic Se forms on the content of ascorbic acid in lettuce and identified selenate (SeO$_4^{2-}$) as a more effective form were described [43]. The same study has also shown an increase in other antioxidant compounds, such as flavonoids or total phenolics. Their content and that of ascorbic acid grew together with Se concentration in the medium and was the highest at 120 µM SeO$_3^{2-}$ or SeO$_4^{2-}$. However, this dose resulted in a significant loss of biomass due to the toxic effects of excessive availability and uptake of Se. Supplying the lettuce with 15 µM selenite increased the accumulation of H$_2$O$_2$ and MDA (malondialdehyde), which indicated the occurrence of oxidative stress and could partially explain the toxic effects of higher Se doses [25]. Increasing the capability of scavenging free radicals is one of the plants’ strategies for limiting Se toxicity [17].

4.2. Speciation Forms of Se

Irrespective of Se form, our experiment showed considerably greater levels of total Se in the roots than in above-ground parts. The ratio of total Se in the leaves and roots of plants exposed to SeMet reached 0.42 and 0.93, and in the variant with Na$_2$SeO$_3$ it was lower and reached 0.17 and 0.20, respectively. Similar ranges, i.e., 0.6 and 1.0 in the presence of SeMet and below 0.5 in the presence of selenite, were found [22]. Selenite and organic Se such as SeMet are transported from roots to shoots at a much slower rate than selenate [11,12]. If we used selenate, the proportions of Se in the roots and aerial parts could be different, as selenate uptaken by root cells moves rapidly through the root symplast to the stele and is translocated to the shoot. On the other hand, selenite is converted to organoselenium compounds, e.g., SeCys, which often remain within the root [8,11,20,29,41]. SeCys can be metabolized to SeMet or methylated to Se-methylselenocysteine (MeSeCys) in the presence of selenocysteine methyltransferase (SMT). In our experiment, a considerable amount of absorbed SeO$_3^{2-}$ was probably converted into SeCys and then SeMet, as this form was abundantly detected in the roots. Even though the formation of organic Se forms is relatively rapid [8], the plants still contained significant amounts of selenite. Significantly higher content of SeO$_3^{2-}$ in the roots of plants supplied with selenite and SA than selenite alone may indicate a stimulating effect of SA on its absorption, further confirmed by a considerably high level of total Se in the roots of plants from this variant.

In the plants supplied with organic Se, greater content of Se in the roots than in the leaves resulted from accumulation of SeMet or its derivatives, including MeSeMet, in the roots and relatively slow translocation of organic forms into aerial parts of the plants. The presence of MeSeCys in the roots of plants supplied with organic Se might be due to the metabolism of SeMet, which is mainly converted into MeSeMet, or MeSeCys [20], to be further transformed into a volatile form, i.e., DMDSe. The formation of volatile Se compounds (volatilization) is one of the mechanisms plants use to exclude excessive Se [7,53]. Roots are the main site of methylation preceding volatilization [22]. Se volatilization by plant roots was 20–73 times faster in the plants supplied with SeMet than SeO$_3^{2-}$ [22]. It can be assumed that MeSeCys detected in the roots resulted from the process in which plants remove excessive Se.

Application of SA increased total Se content in the roots of plants supplied either with selenite or organic Se, since Se content in the roots was 1.5–2 times higher than in the plants supplied with selenite or organic Se alone. These values may indicate the effects of SA on enhanced uptake of organic and inorganic Se by plants. The effect was two times stronger for the organic form. As stated earlier, such a high intake of Se could have reduced lettuce yield in the presence of organic form. SA presence...
in the medium supplemented with organic Se also enhanced the content of SeMet, which may indicate increased uptake of this form by plants. SA also improved the content of MeSeCys in the roots of this variant, which suggests that SA may accelerate the process of Se conversion to MeSeCys on its way to volatilization.

SA is classified as a plant phytohormone or phytohormone-like compound, and is involved in the regulation of plant growth, development, and other physiological processes [31,54,55]. However, prolonged application of low doses of it or short-time treatment with high doses may be toxic to plants [56]. In our previous study, a combined application of Se (SeO\text{3}\text{2}^-), I (IO\text{3}\text{1}^-) and different doses of SA, i.e., 0.1, 1.0, and 10 mg dm\text{3}^- did not affect total Se levels in the roots [34], which contradicts the results of the present experiment. Enhanced absorption of Se in this experiment at the same dose of SA (10 mg dm\text{3}^-) may have resulted from biofortification with only one element (Se), using its organic form. It may be speculated that in our previous study [34] iodine affected the action of SA on the absorption of Se, and these effects may be related to the methylation of Se, I, and SA.

Unfortunately, the identified speciation forms constituted only 14–18% of total Se in plant roots supplied with organic Se alone or Se + SA. For this reason, it is difficult to closely monitor Se metabolism in the roots, and to explain why they contained Se as well as SeO\text{3}\text{2}^-.

Growing plants in the medium enriched with Se caused expected increase in total Se content in lettuce leaves. This effect was much more pronounced in the plants treated with organic Se, as plants growing in the presence of SeMet accumulated 7–10 times more Se. As in the case of roots, the fact that identified speciation forms accounted for 12–15% of total Se made it difficult to interpret the share of individual speciation forms in lettuce leaves. It can be assumed that some of those unidentified organic forms are methylated forms desired in the human diet. On the other hand, the presence of such forms such as MeSeMet or \(\gamma\)-GluMeSeMet (\(\gamma\)-glutamyl-MeSeMet) [7] may indicate an excess of Se that the plants need to remove. Formation of these forms from SeMet and their subsequent transformation to DMSe is the process in which non-accumulators eliminate excessive Se. SeMet and MeSeCys were the dominant identified speciation forms. While MeSeCys is a derivative of SeCys, another possible pathway of SeMet conversion into MeSeCys, as a result of continuous interconversion of SeMet and SeCys via methionine cycle and transsulfuration, was indicated [57].

In the variants supplied with selenite, SeO\text{3}\text{2}^- was the main speciation form in the leaves, but its absolute content was low, which was consistent with the results of other authors [35,58,59]. The reason for this was a considerable conversion of this inorganic form into the organic form in the roots, which is typical of selenite [7].

The addition of SA did not result in a visible increase in total Se in the leaves of plants supplied either with organic Se or selenite. Taking into account the significantly greater accumulation of Se in the roots of plants treated with SA, it can be assumed that SA stimulated Se uptake by the roots. However, the increased amounts of Se were not translocated to the leaves and remained in the roots.

It may also be assumed that SA inhibited the rate of MeSeCys synthesis in the plants supplied with organic Se, as its content in relation to total Se in the roots was lower than in the plants exposed to SeMet alone. MeSeCys is formed mainly in the SeCys pathway catalyzed by SMT [7].

SA is a signaling compound that activates the defense system [60]. Hyper-accumulators show a few times greater expression of genes responsible for the synthesis of JA, SA and ethylene than non-accumulators [32], which may be a part of the multiple mechanisms aimed at hyperaccumulation and hypertolerance of Se. Increased levels of SA in the hyper-accumulator \(A.\ thaliana\) following Se treatment were found [61]. In our experiment, exogenous SA could play the same role as endogenous SA in hyper-accumulators, i.e., activate the defense system to improve plant tolerance to Se. Feeding the plants with organic Se alone (SeMet) increased SA content by almost 15 times in relation to control and plants supplied with selenite, which may indicate plant response to increased uptake of Se. Plants absorbed more Se from the organic form than the inorganic form and this might have affected the synthesis of endogenous SA. It can be therefore assumed that the synthesis of endogenous SA mentioned above for hyper-accumulators may be also initiated in non-accumulators under increased supply of Se.
Our experiment showed that enhanced synthesis of endogenous SA occurred predominantly in the roots exposed to a greater excess of Se than the leaves.

Supplying plants with Se and SA further boosted SA content in the roots. In the plants treated with the inorganic form of Se, the increase in SA content in the roots was probably the result of SA uptake by plants. In the plants supplied with SeMet + SA, the presence of SA in the roots (2.2 times higher than in the plants treated with SA alone) could be due to absorption of exogenous SA and synthesis of endogenous SA. It can be assumed that due to greater absorption of Se the plants supplied with SeMet + SA synthesized more endogenous SA than the plants supplied with SeMet alone. The outcomes of this study demonstrate that the defense mechanism against excessive Se, involving synthesis of endogenous SA, is present not only in hyper-accumulators but also in non-accumulators such as lettuce. On the other hand, the anti-Se plant defense is based on the synthesis of ethylene and JA in relation to SA, the accumulation of which suppresses the acquisition of Se resistance in plants rather than enhancing it [62].

4.3. Expression of smt and mmt Genes

The study investigated the mRNA expression of three genes encoding the enzymes of Se transformation pathways, i.e., selenocysteine methyltransferase (smt) and methionine methyltransferase (mmt). smt is involved in a methylation process of SeCys to MeSeCys, whereas mmt gene participates in SeMet methylation to MeSeMet [3]. Gene expression was studied in both roots and leaves but smt expression was only detected in the leaves. This gene is predominantly expressed in the plastids of leaf cells [13].

Expression of the smt gene is one of the features of hyper-accumulator plants that enables them to transform SeCys into a non-protein amino acid. MeSeCys further converts into a volatile form DMDSe. Genes encoding functional SMT enzymes are not thought to exist in plants with little Se tolerance [3]. However, some non-accumulator species show low levels of SMT activity and contain marginal amounts of MeSeCys [13]. Our study revealed significantly higher expression of the smt gene in the leaves of plants supplied with selenite alone. It can be assumed that since the majority of Se absorbed with selenite was converted in the roots into organic forms of Se [8], including SeCys, and a part of Se pool was translocated to the leaves, it could be converted into MeSeCys by SMT. No increase in smt gene expression in plants treated with Na$_2$SeO$_3$ + SA is difficult to explain. Medium supplementation with SA alone had no significant effect on the expression of this gene. In our previous study [34], we reported an increased expression of smt at 10 mg dm$^{-3}$ SA applied together with Se and I.

Since smt is involved in SeCys metabolism, the lack of its gene expression in SeMet variants seems justified.

Although, as mentioned above, some selenite is transformed into SeCys, the basic organic forms of Se generated in the roots of plants supplied with selenite are SeMet and SeOMet [8]. SeMet was also abundantly detected in the roots of plants exposed to selenite in our study. However, its presence did not affect the expression of mmt genes encoding the enzymes involved in SeMet metabolism in the roots. Expression of both mmt genes in the leaves was, similar to smt, the greatest in the variants exposed to selenite alone. Although the content of MeSeMet in the leaves was not determined, it can be assumed that a significant part of SeMet translocated to the leaves in this variant (Na$_2$SeO$_3$) was transformed into MeSeMet in a process mediated by MMT. In this context, downregulation of mmt genes in SeMet variants is difficult to explain.

4.4. Biofortification in Se

Application of the inorganic and organic form of Se increased the content of this element in the leaves but the biofortification effect, which is determined based on total Se concentration, was considerably stronger when the plants were supplied with organic Se. Dominant Se speciation forms in the leaves of lettuce plants supplied with organic Se included organic SeMet and MeSeCys, which are highly desirable from a nutritional point of view. However, the share of identified speciation
forms in total Se following application of organic Se was low and did not exceed 15%. According to a review [63], unidentified forms of Se may account for up to 85% of total Se, and the main speciation form in this group is γ-glutamyl-MeSeCys, which was not determined in our study. The chromatogram showed the signals from unidentified Se compounds, with one of them being particularly strong (Figure 2). Considering the retention time of this signal and its closeness to the retention time of SeMet and MeSeCys, it can be assumed that it was an organic Se compound, e.g., the mentioned γ-glutamyl-MeSeCys or γ-glutamyl-MeSeMet. These compounds were not determined in our study due to a lack of commercially available standards. Apart from MeSeCys, selenite-treated plants accumulated Se as SeO₃. From the nutritional perspective, biofortification with selenite, even when associated with accumulation of some Se as organic forms (MeSeCys), was low mostly due to low content of total Se in the leaves. Contrary to that, biofortification of lettuce with organic Se enhanced total Se content by 7 to 10 times. Combined application of Se and SA did not induce changes in total Se content in the leaves or the share of speciation forms that could have a significant nutritional impact.

Taking into account the Se content in lettuce treated with organic Se, which in our study reached about 50 mg Se kg⁻¹ dry weight, mean content of dry weight in lettuce amounting to 5%, and daily consumption of lettuce at 23.6 g, i.e., mean daily consumption per an adult in the EU [64], this portion of the vegetable provides about 65 µg Se day⁻¹ and meets the daily recommended intake of Se, which is 55–70 µg Se day⁻¹ for adult humans [65].

5. Conclusions

The biofortification effect measured by total Se content was considerably greater when the plants were supplied with organic Se. A 0.5 mg dm⁻³ dose of organic Se in the medium allows for achieving Se content in plant tissue that covers daily dietary intake in adults consuming about 25 g of lettuce per day.

Dominant Se speciation forms in the leaves of lettuce plants supplied with organic Se included organic SeMet and MeSeCys, which are highly desirable from nutritional point of view. Plants treated with selenite accumulated mainly MeSeCys and SeO₃⁻².

SA enhanced Se uptake, especially of organic Se forms. Enhanced amounts of Se accumulated in the presence of SA were not translocated to the leaves but remained in the roots. SA improved the content of MeSeCys in the roots, which suggests its ability to accelerate the process of Se conversion to MeSeCys on its way to volatilization via methylation. Plant absorption of Se, and particularly organic Se, enhanced synthesis of endogenous SA, which probably served as a signal for activating defense mechanisms known in hyper-accumulating plants. Enhanced synthesis of endogenous SA took place mainly in the roots, which also accumulated more Se than leaves. Intensified expression of smt and two mmt genes was only observed in the leaves of plants supplied with selenite alone.

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