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Research article

Iodine biofortification through expression of *HMT*, *SAMT* and *S3H* genes in *Solanum lycopersicum* L.

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ABSTRACT

The uptake process and physiological reaction of plants to aromatic iodine compounds have not yet been documented. The aim of this research was to compare uptake by tomato plants of KI and KIO_3, as well as of organic iodine compounds – 5-ISA (5-iodosalicylic acid), 3,5-diISA (3,5-diiodosalicylic acid), 2-IBeA (2-iodobenzoic acid), 4-IBeA (4-iodobenzoic acid) and 2,3,5-triIBeA (2,3,5-triiodobenzoic acid). Only 2,3,5-triIBeA had a negative influence on plant development. All organic iodine compounds were taken up by roots and transported to leaves and fruits. Among all the compounds applied, the most efficiently transferred iodine was 2-IBeA – to fruits, and 4-IBeA – to leaves. The order of iodine accumulation in fruit cell compartments was as follows: organelles > cell walls > soluble portions of cells; for leaf and root cells, it was: organelles > cell walls or soluble portions, depending on the compound applied. The compounds studied influence iodine metabolism through expression of the *HMT* gene which encodes halide ion methyltransferase in leaves and roots. Also, their influence on modification of the activity of the *SAMT* and *S3H* genes that encode salicylic acid carboxyl methyltransferase and salicylic acid 3-hydroxylase was established. It was discovered that exogenously applied 5-ISA, 3,5-diISA, 2-IBeA and 4-IBeA are genuinely (endogenously) synthesised in tomato plants; to date, this has not been described for the tomato, nor for any other species of higher plant.

1. Introduction

Iodine (I) is an indispensable component of hormones produced by the thyroid gland, which play a vital role in many metabolic and developmental processes, as well as the growth process of humans and animals. This element is of particular importance during the prenatal period, contributing to the development of fetus' nervous system, as well as for newborns (Velasco et al., 2018).

Iodine content in soils is very low, thus its content in cultivated plants is insufficient in comparison to the nutritional needs of humans and animals (Eastman and Zimmerman, 2018). The major iodine sources in human diet are milk, dairy products (Van der Reijden et al., 2017), seaweeds and fish (Ershow et al., 2018). The iodine source in the human diet may also be a supplement or multivitamin, but there are not exact requirements for iodine content in these (Leung et al., 2012). However, many groups of people like vegans/vegetarians and people

Abbreviations: 2-IBeA, 2-iodobenzoic acid; 2,3,5-triIBeA, 2,3,5-triiodobenzoic acid; 3,5-diISA, 3,5-diiodobenzoic acid; 4-IBeA, 4-iodobenzoic acid; 5-ISA, 5-iodosalicylic acid; BeA, benzoic acid; SA, salicylic acid; HMT, adenosyl-l-methionine (SAM)-dependent halide methyltransferase; S3H, salicylic acid 3-hydroxylase; SAMT, *S*-adenosyl-L-methionine: salicylic acid carboxyl methylotransferase

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with lactose intolerance or low-salt diets may be at risk for low iodine intake (Booms et al., 2016). The commonly used method of introducing iodine into the human diet is iodinated table salt (WHO, 2008). The use of iodised table salt has significantly reduced the level of iodine deficiency disorders (IDD), but during storage, transport and packaging, iodine losses from table salt can be up to 20%, and another 20% can be lost during cooking and food preparation (WHO, 2004; Winger et al., 2008). In addition, salt is one of the most important determinants of high blood pressure, increased cardiovascular risk and other disease (Cappuccio, 2013). The WHO recommends limiting the daily dose of salt to 5 g/day (WHO, 2016), which will also involve less iodine uptake. Unfortunately, despite common introduction into the human diet of iodinated salt as a source of iodine, in many countries there is still an issue of iodine deficit in the diet (Iodine Global Network, 2017). An alternative source of iodine in the human diet is vegetables and other cultivated plants enriched with this element in the process of biofortification. Biofortification is widely used in many ways: enriching maize, rice, pearl millet and other cereals with microelements or provitamin A (al-Babili et al., 2001; Bouis and Welch, 2010; Sagare et al., 2018); and wheat with zinc (Sazawal et al., 2018) and iron (Riaz et al., 2017). Studies conducted by Tonacchera et al. (2013) indicated that biofortified vegetables may serve as the perfect iodine carrier and introduce iodine into people's system.

For many years, it had been considered that iodine is indispensable in the growth and development of embryophytes (land plants). Over the last decade, in many scientific facilities around the world, numerous research works have been carried out on the influences of, mainly, mineral iodine compounds on embryophytes. The results of those research works proved that iodine may influence growth, development and the course of some metabolic processes in plants. Thus, iodine has for a couple of years now been considered as one of the beneficial elements (Medrano-Macías et al., 2016).

Due to the high iodine concentration in seas and oceans, sea algae accumulate high amounts of iodine. This group of organisms has been well studied for the iodine uptake process and metabolism (Leblanc et al., 2006). In embryophytes, iodine content is several hundred or a thousand times smaller than in sea algae. This stems from drastically worse availability of iodine for plants in soil environments than in oceans. The much lower iodine accumulation level in embryophytes than in sea algae hinders the conduct of research into iodine uptake mechanisms and metabolism in cultivated plants and their species growing wild. To date, only fragmentary processes connected with iodine metabolism in embryophytes have been documented (Medrano-Macías et al., 2016). For example, it was established that iodine oxidation in the methylation process (similar to that in sea algae) takes place in them. Oxidation of iodine in the methylated form of CH_3I reduces its level of accumulation in plants. The iodine oxidation process is controlled by S-adenosyl-L-methionine (SAM)-dependent halide methyltransferase (HMT) or SAM-dependent halide/thiol methyltransferase (HTMT) enzyme using iodide as a substrate (Gonzali et al., 2017). The iodine oxidation process is observed, for example, in the following embryophytes: *Arabidopsis thaliana* (Rhew et al., 2003), rice (Redeker et al., 2000) and daikon radish (Itoh et al., 2009). Until now, no studies have been conducted on the activity of genes responsible for iodine methylation in tomato roots and leaves, conditioned by the iodine form that is applied to plants. As far as mineral iodine compounds (KI and KIO_3) are considered, there are several hundred research works, but the case of applying organic compounds with iodine bound with an aromatic ring to plants is a novel issue. These types of compound are ubiquitous in soils (iodine bound with soil organic matter – SOM), but their influence on plants has not yet been recognised. Iodosalicylates and iodobenzoates are compounds with iodine bound to an aromatic ring. In earlier research works of our team members, tests covered the influence of 5-iodosalicylic acid on lettuce (Smoleń et al., 2017) and of iodosalicylates and iodobenzoates on young tomato plants in the seedling phase (Halka et al., 2018). There are, however, no research works covering tomato in the generative growth and fructification phases.

Iodosalicylates and iodobenzoates are derivatives of salicylic acid (SA) and benzoic acid (BeA) which is a SA precursor in plants. We suspect that the application of these compounds in plants may influence iodine uptake and accumulation, as well as SA metabolism in plants. SA in plant systems is considered to have an action similar to that of plant hormones. SA plays a role in controlling processes connected with induction of immunity to abiotic and biotic stress factors, among others: salinity, high- and low-temperature stress, drought, heavy metals and UV radiation (Hayat et al., 2010). In the context of the problems discussed in the presented paper, it is vital that concurrent application of mineral forms of iodine, KI and KIO_3, together with SA, triggers improved iodine accumulation in tomato fruits in comparison to application of KI and KIO_3 alone, without SA (Smoleń et al., 2015). The authors, however, did not evidence which molecular, physiological and biochemical processes cause such an effect. This research work, inter alia, makes it possible to explain this problem, too.

Application of large exogenous doses of SA may cause excessive uptake of this compound by plants. After uptake, SA may be volatilised from plants in methylated form, i.e. methyl salicylate (MeSA). The MeSA production process in plants is controlled by the enzyme S-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase, which is encoded by the *SAMT* gene (Ross et al., 1999; Tieman et al., 2010). Additionally, SA in the plant's system may be subject to other transformations, e.g. glycosylation and conjugation to amino acids (Dempsey et al., 1999). In *A. thaliana*, SA may be converted to 2,3-dihydroxybenzoic acid (2,3-DHBA) or 2,5-dihydroxybenzoic acid (2,5-DHBA) in a hydroxylation reaction controlled by salicylic acid hydroxylase (Zhang et al., 2013, 2017).

The aim of this research was to compare the efficiency of iodine uptake (mineral vs iodosalicylates and iodobenzoates), as well as that of SA by vegetative and generative parts of tomato plants. Another aim of this research was evaluation of the activity of the *HMT*, *SAMT* and *S3H* genes, plus whether and in which directions the metabolism of endogenous and exogenous iodosalicylates and iodobenzoates in tomato plants is affected.

2. Materials and methods

2.1. Plant material and treatments

Tomato (*Solanum lycopersicum* L.) plants, of the 'Kmicic' variety, were grown in a foil tunnel, over the spring and summer period, and seedlings were produced in a greenhouse. The experiment was conducted in a greenhouse of the Faculty of Biotechnology and Horticulture, University of Agriculture in Krakow (50° 05′ 04.1" N, 19° 57′ 02.1" E). The experiment was repeated twice. Tomato seeds were sown in boxes (400 × 320 × 90 mm) filled with peat substrate. The sown seeds were covered with sand. After sprouting, the plants were bedded in multi-pallets (multi-pots), with dimensions of 305 × 515 mm. Over the period between sprouting of seeds and the end of seedling production, the plants were watered only with tap water. Plants were lit with a 600 W high-pressure sodium lamp. The photoperiod was 12 h for the day and 12 h for the night. The temperature over the period of seedling production varied in the range of 16–25 °C. Tomato plant seedlings (in the phase of 4–5 leaves proper) were planted in pots (with a volume of 7.5 dm^3 and diameter of 26 cm), filled with perlite (Perlit Polska Sp. z o. o., Poland) and placed in a foil tunnel. For 2 weeks, following the planting in pots, plants were watered with nutrient solution containing nutritional macrocomponents in the amount of (in $mg \cdot dm^{-3}$): N 180, P 40, K 245, Mg 50 and Ca 180 (Wysocka-Owczarek, 2001) and microelements in the form of 30 mg dm^{-3} Micromix Bentley fertiliser (Tradecorp, Spain). The nutrient solution's pH acidifying value was set with 38% nitric acid. Throughout the entire period of cultivation, the pH value remained within the range of

5.6–5.8, and of EC in the range of 1.81–2.35. Lower pH and EC values were employed immediately following planting, and higher ones after ca. 3 weeks, when the plants started to grow intensively. At the beginning of fluorescence, in the first cluster, the nutrient solutions with iodine compounds, and SA and BeA, were introduced in the following array: 1) control group (only basic nutrient solution, without I, SA and BeA content); 2) KI – potassium iodide; 3) KIO_3 – potassium iodate; 4) SA – salicylic acid; 5) BeA – benzoic acid; 6) 5-ISA – 5-iodosalicylic acid; 7) 3,5-diISA – 3,5-diiodosalicylic acid; 8) 2-IBeA – 2-iodobenzoic acid; 9) 4-IBeA – 4-iodobenzoic acid; and 10) 2,3,5-triIBeA – 2,3,5-triiodobenzoic acid. All iodine compounds and SA and BeA were added at a concentration of 25 μM, calculated as 25 μM of iodine and as 25 μM of SA or BeA, respectively. During the experiment, treatments with application of SA and BeA only were isolated, as there were tests conducted on the influence on plants of derivatives of those compounds, i.e. iodosalicylates and iodobenzoates. These compounds were applied to plants as a positive control – comparison treatment against iodosalicylates and iodobenzoates. Organic compounds were first dissolved in a few drops of 1 M NaOH, and then dissolved in basic nutrient solution (for nutrient solutions for other treatments, a similar amount of 1 M NaOH was applied). The plants were watered manually in the morning, with nutrient solutions containing the researched compounds. Nutrient solutions were introduced directly to the substrate (perlite). In afternoons, the plants were provided with a standard nutrient solution via the dripping system in order to supplement water deficit in the substrate. The studied compounds were applied to plants for a period of 50 days, during which a total of 50 dm^3 of nutrient solutions was introduced for every single plant (1 dm^3 per day, during single application for a single plant), with iodine, SA and BeA compounds dissolved in them. Iodine content in the tap water was 0.0012 μM, and the total iodine content in the control nutrient solution was 0.0205 μM (I from tap water and fertilisers). Each of the studied treatments covered four runs, six plants per each run (24 plants per treatment).

2.2. Plant analysis

One day after the last application of the studied compounds, leaf and root samples were taken for molecular analysis, and the following day, a one-time collection of all fruits from all the plants was conducted. In order to evaluate the crop yield, both ripe and unripe tomato fruits were collected in order to determine the earliness of crop yield (percentage share of ripe fruit yield in relation to total yield of fruits). Immediately after the collection of fruit, the biomass of the upper parts of the plant – shoot system without fruits (only stems and leaves) was also measured.

2.3. Analysis of fresh plant material

2.3.1. HMT, SAMT and S3H gene expression assay

In order to conduct the *HMT*, *SAMT* and *S3H* gene expression assay, fresh samples of tomato plant material (leaves and roots) were taken from a plant. Samples of well-developed, healthy leaves from the middle part of a shoot were taken in three biological replications. Root samples (portions of 5–10 cm, containing apexes) were taken directly from the root system also in the biological probes. The samples were immediately frozen in liquid nitrogen and stored at a temperature of −80 °C, until isolation of RNA.

Total RNA from leaf and root samples was extracted with a Direct-zol™ RNA MiniPrep Plus RNA isolation kit (Zymo). Then, RNase-free DNAseI treatment (Thermo Scientific) was conducted, and the quantity and quality of RNA probes were verified with the use of a spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific). Additionally, to confirm RNA purity and quality, electrophoresis in 1% agarose gel was conducted. The cDNA synthesis was conducted using an iScript cDNA synthesis kit (BioRad), following the manufacturer's instruction; 1 μg RNA was added for each sample. In the subsequent stage, cDNA samples were adequately diluted and used for the qRT-PCR reaction that was conducted using a QuantStudio™ 3 System with Maxima™ SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific). The total volume of the reaction mixture was 15 μL and included: 5 μL H_2O, 0.6 μL forward (5 μM) and 0.6 μL reverse (5 μM) primers (Table S1), 7.5 μL SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific) and 1.3 μL template cDNA. Amplification conditions were as follows: 3 min of initial denaturation at 95 °C and then 40 cycles at 95 °C for 10 s and 55 °C for 45 s (Halka et al., 2018). The qPCR reaction was conducted in three biological and three technological replicates. The qRT-PCR reaction products were confirmed by electrophoresis in 1% agarose gel. Relative quantification of gene expression was calculated according to the qBase method (Hellemans et al., 2007) with Ct value normalisation to endogenous reference genes, i.e. actin (act) and elongation factor 1 (ef1), and to untreated control samples.

2.4. Analysis of dry plant material

During crop collection, ripe fruits as well as leaf and root samples were collected for chemical analysis. The plant material was washed in tap water and then in distilled water. Pinnate leaves were separated from petioles. Pinnate leaves, petioles and roots were dried in a laboratory dryer with forced air circulation at a temperature of 50 °C, and then ground in a Fritsch Pulverisette 14 grinder using a 0.5 mm sieve. Due to the high water content and trouble with drying (among other reasons: caramelisation of sugars and long drying time due to high water content), the ripe tomato fruits were lyophilised with the use of a Gamma 1–16 LSC lyophilising cabinet (Martin Christ, Germany). The prepared dry plant material was used to analyse the iodine content in separate parts of the plant, and the SA, BeA, 5-ISA, 3,5-diISA, 2-IBeA, 4-IBeA and 2,3,5-triIBeA content. Additionally, in root and leaf samples, iodine content in cell walls, organelles and the soluble fraction of cells was determined.

2.4.1. Analysis of iodine content

In order to determine the iodine content in leaf, petiole and root samples, 0.5 g of dry plant material was weighed out, and for tomato fruit samples, 0.1 g was weighed out; then, 10 mL of distilled water and 1 mL of 25% TMAH (tetramethylammonium hydroxide) was poured onto the sample. The samples were incubated at 70 °C for 3 h, chilled afterwards and topped up with distilled water to 30 mL. In the next stage, the samples were centrifuged for 15 min at 4500 rpm, at 5 °C (PN-EN 15111–2008; Smoleń et al., 2016). The iodine content in leaves, petioles and roots was determined with ICP-OES (Prodigy spectrometer, Leeman Labs, USA), and in tomato fruits with the ICP-MS method (TQ ICP-MS spectrometer/ICP-MS triple quadrupole; ThermoFisher Scientific, Bremen, Germany). The iodine content in tomato fruits was much lower than in vegetative parts of the plant, thus the more sensitive ICP-MS technique was employed. To evaluate the accuracy of the analysis by ICP-MS, the I content in certified spinach leaf reference material (CRM: NCS ZC73013) was additionally determined. The results obtained were as follows: 0.33 ± 0.08 mg $I \cdot kg^{-1}$ d. w. (n = 6) for the certified value of 0.36 ± 0.12 mg $I \cdot kg^{-1}$ d. w. Additionally, the iodine recovery from fortified samples after incubation with TMAH was determined (by ICP-OES spectrometer), and was approximately 95%.

In order to determine the iodine content in individual cell fractions of fruits, leaves and roots, 2 g of dry plant material was weighed out (fruits, leaves and roots) and put into 30 mL phials; then, 10 mL of extraction buffer, consisting of 250 mM sucrose, 50 mM Tris-HCl (pH 7.5) and Cleland reagent (dithiothreitol), was added. The prepared samples were centrifuged for 1 min at 1210 rpm, at 4 °C, and following this, the supernatant liquid was drawn off. The sediment obtained, being a fraction of cell walls and their remains, was dried at a temperature of 50 °C and used for further analysis. The supernatant liquid following the first centrifugation was moved into 15 mL phials and centrifuged once more for 30 min at 10,000 rpm, at 2 °C. The

supernatant liquid following the second centrifugation, being a soluble fraction, was moved into new phials, and the sediment, i.e. organelle fraction, was left in 15 mL phials and dried at a temperature of 50 °C (Weigel and Jager, 1980; Weng et al., 2008). To calculate the percentage of iodine in the soluble cell fraction, the dry matter content was analysed by drying samples at 105 °C. The iodine content in all the fractions was measured by the ICP-OES method using a Prodigy spectrometer (Leeman Labs, USA).

2.4.2. Analysis of SA, BeA, iodosalicylate and iodobenzoate content

For the purpose of analysis, 50 mg of plant material was prepared (fruits, leaves and roots) in 7 mL PP test tubes which were topped up with 5 mL of 96% ethanol. For each sample, 25 µL of internal standard – 10 µg mL^{-1} of deuterated salicylic acid (SA-d4) was added. Samples prepared in such a way were left for incubation at room temperature for 1 h. Then, the extracts were filtered through a syringe filter (FilterBio NY Syringe Filter, nylon, pore size 0.22 µm). The concentration of SA, BeA, 5-ISA, 3,5-diISA, 2-IBeA, 4-IBeA and 2,3,5-triIBeA was determined by means of a high-performance liquid chromatograph (Ultimate 3000, Thermo Scientific) coupled with a mass spectrometer (QTrap 4500, Sciex). Chromatographic separation was carried out on a Luna 3 µm Phenyl-Hexyl 100 Å column (150 mm × 3 mm, i. d. 3 µm; Phenomenex). The mobile phase was: A – water with 0.3% formic acid (60% at the beginning), and B – acetonitrile with 0.3% formic acid (40%). After 2 min, the proportions of the mobile phase were increased linearly up to 98% phase B at 8 min and held for 4 min. The starting proportions were restored over a 3 min period after the 15 min analysis. The injection volume was 10 µL. The mobile phase was directed to an MS ion source between the 1st and 14th minute of separation. For detection, electrospray ionisation (ESI) in negative ion mode was used. Tandem mass spectrometry (MS/MS) was used for quantitative studies; 136.8/93.1, 120.9/76.9, 262.9/126.7, 388.8/126.7, 246.9/126.6, 246.9/144.7, 498.7/454.4 and 141/96.8 transitions were monitored for SA, BeA, 5-ISA, 3,5-diISA, 2-IBeA, 4-IBeA, 2,3,5-triIBeA and SA-d4, respectively. The LC-MS/MS system was controlled using Analyst 1.7 with HotFix 3 software, which was also used for data processing.

2.5. Statistical analysis

All data were statistically verified using the ANOVA module of Statistica 12.0 PL software at a significance level of $p < 0.05$. In the case of significant effects, homogenous mean groups were distinguished on the basis of the Tukey test.

3. Results

3.1. Yield of tomato plants

Except for 2,3,5-triIBeA, none of the compounds studied, in comparison with the control group, had a significant effect on the yield of fruits or mass of the shoot system (Table 1 and Fig. 1). In relation to the control group, 2,3,5-triIBeA caused a considerable decrease in the total fruit yield (by 30.8%) and marketable yield (by 41.2%), as well as in the biomass of overground parts of the plant (shoot system without fruits, by 23.1%). However, plants treated with 2,3,5-triIBeA had a higher percentage for the earliness of crop yield in relation to control plants.

3.2. Iodine content in parts of tomato plants and in cell fractions

The iodine compounds studied – both mineral and organic – conditioned an increase in iodine content in particular parts of tomato plants: fruits, petioles, leaves and roots (Fig. 2A–D). Tomato fruits had the lowest iodine content (Fig. 2A, Table S2). After application of mineral forms of iodine (KI and KIO_3), this element accumulated in comparable levels in the root system and in the overground parts of a plant, i.e. leaves and petioles. On the other hand, after application of organic forms (iodosalicylates and iodobenzoates), a higher level of iodine accumulation was observed in roots than in leaves and petioles (Fig. 2B–D). The highest iodine content in tomato fruits was obtained after treatment with 2-IBeA – it was 2026.82% higher in comparison to control plants (Fig. 2A). The iodine content in tomato fruits constituted 1.65–37.5% of the recommended daily allowance (RDA) of iodine, counted for 100 g of fresh tomato fruits, correspondingly for control plants and 2-IBeA (Table S2). Iodine content in leaves averaged between 1.37 mg I·kg^{-1} dry weight for control plants and 270.63 mg I·kg^{-1} dry weight for plants treated with 4-IBeA (Fig. 2B). Iodine content in roots showed the following trend for individual treatments: control, SA, Be < KIO_3 < KI < 3,5-diISA < 4-IBeA < 2,3,5-triIBeA < 5-ISA < 2-IBeA (Fig. 2D) and it averaged between ca. 2 and 727.62 mg I·kg^{-1} dry weight.

The iodine content in individual cell fractions in fruits, leaves and roots indicated that iodine accumulates first and foremost in the cell wall or organelle fraction, depending on the form of compounds applied (Fig. 3).

In fruits, a lower iodine content was observed in the soluble portion of cells (Fig. 3A), but iodine location in the cell wall fraction and organelles depended on the compound tested. After iodosalicylate and iodobenzoate treatment, iodine was mainly accumulated in the cell wall fraction, but for mineral form (KI and KIO_3) and SA and BeA, iodine was mainly located in the organelle fraction. Trace iodine content in control plants was more or less equal in cell wall and organelle fractions.

In leaf samples, the most iodine in the organelle fraction accumulated after application of 2,3,5-triIBeA, and for control plants and those treated with SA and BeA (Fig. 3B). In leaves, the iodine content in the organelle fraction for these treatments amounted to almost 81–94%. The lowest iodine content in the soluble portion of cells was indicated after 2,3,5-triIBeA, SA and BeA treatment, and amounted to 1.58% for

Table 1
Yield of tomato fruit and biomass of shoot system and roots after harvest.

Treatment	Total yield of fruit (kg·m^{-2}) ± SE	Commercial yield of fruit per one plant (kg·$plant^{-1}$) ± SE	Total yield of fruit (kg·$plant^{-1}$)	Commercial yield of fruit per one plant (kg·$plant^{-1}$) ± SE	Early yielding (%) ± SE	Biomass of shoot system[a] (kg·$plant^{-1}$) ± SE
Control	10.4 ± 0.58^{b}	8.5 ± 0.74^{b}	3.9 ± 0.22^{b}	3.2 ± 0.28^{b}	44.9 ± 3.51^{a}	1.3 ± 0.04^{ab}
KI	9.4 ± 0.36^{ab}	8.1 ± 0.60^{ab}	3.5 ± 0.14^{ab}	3.0 ± 0.20^{ab}	48.4 ± 4.21^{ab}	1.2 ± 0.07^{ab}
KIO_3	10.4 ± 0.36^{b}	8.9 ± 0.45^{b}	3.9 ± 0.13^{b}	3.3 ± 0.17^{b}	47.4 ± 2.90^{ab}	1.5 ± 0.10^{b}
SA	11.2 ± 0.52^{b}	9.6 ± 0.69^{b}	4.2 ± 0.20^{b}	3.6 ± 0.26^{b}	48.4 ± 4.29^{ab}	1.6 ± 0.14^{b}
BeA	10.4 ± 0.92^{b}	8.3 ± 1.16^{ab}	3.9 ± 0.35^{b}	3.1 ± 0.40^{ab}	49.9 ± 1.82^{ab}	1.5 ± 0.04^{b}
5-ISA	10.0 ± 0.48^{b}	8.1 ± 0.31^{ab}	3.7 ± 0.18^{b}	3.0 ± 0.10^{ab}	49.1 ± 1.53^{ab}	1.4 ± 0.09^{b}
3,5-diISA	9.7 ± 0.37^{b}	7.8 ± 0.66^{ab}	3.7 ± 0.14^{b}	2.9 ± 0.20^{ab}	49.7 ± 4.14^{ab}	1.4 ± 0.05^{b}
2-IBeA	10.5 ± 0.52^{b}	9.0 ± 0.64^{b}	3.9 ± 0.20^{b}	3.4 ± 0.24^{b}	50.5 ± 3.14^{ab}	1.4 ± 0.06^{b}
4-IBeA	10.4 ± 0.61^{b}	9.0 ± 0.88^{b}	3.9 ± 0.23^{b}	3.4 ± 0.33^{b}	49.0 ± 2.52^{ab}	1.6 ± 0.06^{b}
2,3,5-triIBeA	7.2 ± 0.13^{a}	5.0 ± 0.46^{a}	2.7 ± 0.05^{a}	1.7 ± 0.17^{a}	61.9 ± 2.00^{b}	1.0 ± 0.05^{a}

Means followed by the same letters are not significantly different for $p < 0.05$, (n = 8).
[a] Biomass of shoot system without fruits (only stems and leaves).

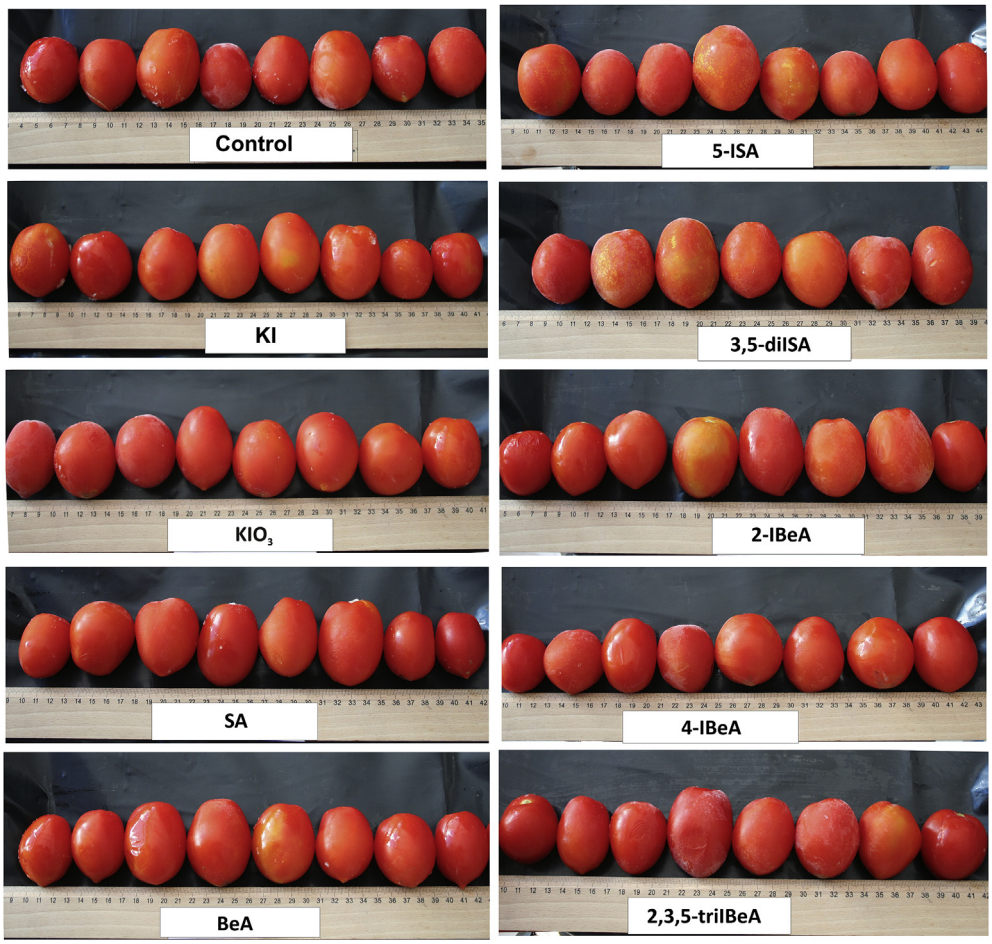

Fig. 1. The fruits of tomato plants after SA, BeA and iodine compounds application.

2,3,5-triIBeA, up to 5.45% for SA. KI treatment provided the highest iodine content in the soluble portion of cells and amounted to 43.96% for the other fractions. Iodine content in the soluble portion of cells for KIO_3, iodosalicylates and iodobenzoates, except 2,3,5-triIBeA, was at the same level (from 16.70% for 2-IBeA up to 31.23% for KIO_3).

In roots, iodine mainly accumulated in organelles and content was from 40.79% for 4-IBeA up to 88.98% for SA (Fig. 3C). Organic forms of iodine caused a higher iodine content in the soluble portion of cells compared to mineral forms as KI and KIO_3 and also for SA, BeA and control plants. In organelles, iodine was accumulated much better after KI application than after KIO_3 and organoiodine compound treatments.

3.3. Content of SA, BeA and organoiodine compounds

All of the iodosalicylates and iodobenzoates studied were observed to occur naturally in tomato plants, which is confirmed by their content in roots, leaves and fruits of control plants – except for 2-IBeA and 4-IBeA in fruits, which might be conditioned by too little sensitivity of the testing equipment for determining them in fruits (Table 2). After application of individual iodosalicylates and iodobenzoates, the highest content of 5-ISA, 3,5-diISA, 2-IBeA, 4-IBeA and 2,3,5-triIBeA was found in roots, leaves and fruits, correspondingly for individual compounds – a considerable increase in content in comparison to the control group and other combinations tested. An exception was finding the highest 5-ISA content in leaves after application of BeA, and not 5-ISA – it was, respectively, 3.11 and 2.06 μg of 5-ISA·100 g^{-1} dry weight for application of BeA and 5-ISA. The content of the SA, BeA and organoiodine compound metabolites analysed, as a reaction to the applied compounds, was different for tomato roots, leaves and fruits (Table 3).

Application of exogenous 5-ISA and 3,5-diISA to tomato plants resulted in an increase in the content of these compounds in fruits, leaves and roots (Table 2). Trace amounts of these compounds were evidenced in roots, leaves and fruits in the remaining treatments studied. Except for the application of 5-ISA, the increase of 5-ISA content in leaves was also influenced by exogenous BeA and 2-IBeA (they caused increased synthesis). In the case of roots, only application of 5-ISA resulted in a considerable increase in the content of this iodosalicylate. In the remaining combinations studied, the 5-ISA content in roots was the same as in the control group.

A comparative analysis was also conducted for SA, BeA and organoiodine compound content between tomato roots, leaves and fruits, and willow (*Salix cortex*) bark, as it has a naturally high SA content. The analysis proved the presence of SA and BeA as well as all organoiodine compounds in willow bark (Table 2). It is important that the content of all those compounds in willow bark was much higher in tomato leaves and fruits – even in the case of plants which during cultivation had been treated with SA, BeA and organoiodine compounds.

3.4. Expression of HMT, SAMT and S3H genes

Application of the iodine compounds studied, as well as SA and BeA, had a considerable influence on expression of the *HMT*, *SAMT* and *S3H* genes in leaves and roots – except for the activity of *SAMT* in leaves (Fig. 4A–F).

The tested iodosalicylates and iodobenzoates, and SA alone, caused a considerable increase in expression of the *HMT* gene in tomato leaves (Fig. 4A). In the case of root samples, a considerable increase in expression of the *HMT* gene was only influenced by the application of

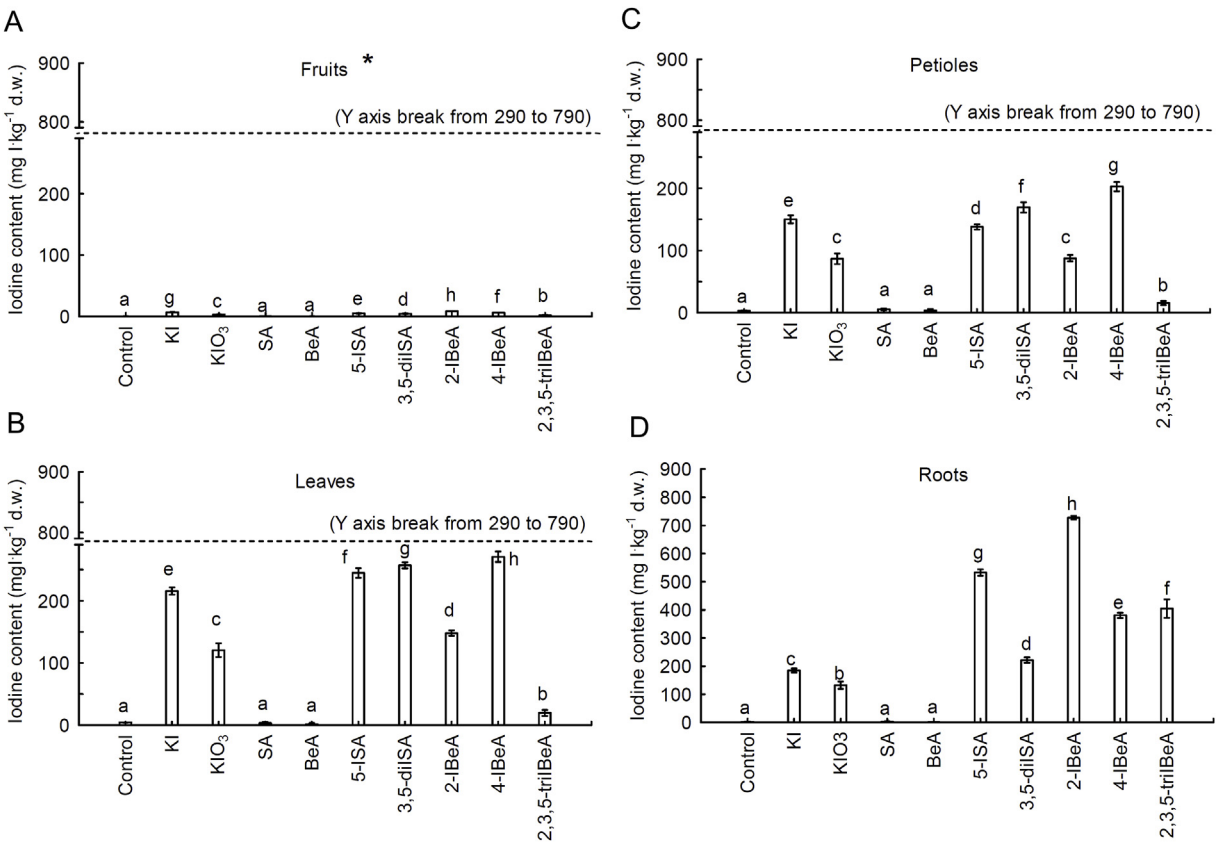

Fig. 2. Iodine content in fruits (A), leaves (B), petioles (C) and roots (D) of tomato plants. Means followed by the same letters are not significantly different for $p < 0.05$. Bars indicate standard error (n = 8). The lines indicate breaks in the Y axis from 290 to 790
*- due to the low content of iodine in fruits of tomato plants the results are also presented in Table S1 in supplementary materials.

exogenous 3,5-diISA, 2-IBeA and 2,3,5-triIBeA, as well as 5-ISA. The greatest increase in *HMT* gene activity in roots was evidenced for 3,5-diISA – it was 1.7-fold higher than in the control group (Fig. 4B). After application of BeA, a 2.5-fold decrease in expression of the *HMT* gene in leaves and 11-fold decrease in tomato roots was observed in relation to control plants (Fig. 4A and B).

There was no influence of the studied compounds observed on *SAMT* expression in tomato leaves (Fig. 4C); on the other hand, in roots, a tendency for the expression level to decrease was observed for all treatments except for SA (Fig. 4D). Application of BeA to plants led to the highest (155-fold) decrease in expression of the *SAMT* gene in tomato roots in relation to the control group. To the smallest extent, a statistically significant decrease in expression of *SAMT* took place after application of KIO_3 and 3,5-diISA.

Application of organic iodine compounds, i.e. 3,5-diISA, 2-IBeA, 4-IBeA and 2,3,5-triIBeA (and, to a lesser extent, 5-ISA), caused a considerable growth in expression of the *S3H* gene in tomato plant leaves (Fig. 4E). Following treatment of plants with 3,5-diISA, 2-IBeA and 4-IBeA, an increase in expression of this gene was noted in leaves, by ca. 26%, and the highest increase, by 51%, was evidenced for treatment of the plants with 2,3,5-triIBeA. As a result of application of BeA in plants, the expression of *S3H* was decreased in leaves and roots (Fig. 4F). Other compounds studied did not indicate any statistically significant expression of the *S3H* gene in tomato roots.

4. Discussion

4.1. Influence of iodine compounds, SA and BeA on growth and development of tomato plants

Kiferle et al. (2013) indicated that iodine applied at a dose below 10 mM of KI and KIO_3 does not trigger negative effects for the growth and development of tomato plants. Higher doses of these compounds may, however, lead to the development of chloroses, epinasties and plant wilting (Kiferle et al., 2013). Until now, there has been no research conducted on the application of iodine with the use of organic forms of iodosalicylates and iodobenzoates in tomato cultivation from the beginning of its growth at a permanent site until ripening of fruits. Our previous study, pertaining to application of these iodine compounds in tomato plants in the seedling phase, indicated a negative influence of 3,5-diISA and 4-IBeA, applied at a dose of 25 μM of I (excluding SA, BeA, 5-ISA and 2-IBeA) to young tomato plants (Halka et al., 2018). Earlier research on *A. thaliana* indicated that *p*-iodobenzoic acid (4-IBeA synonym), as well as its derivative, may inhibit the lignification process via inhibition of cinnamate 4-hydroxylase – a key enzyme of the phenylpropanoid pathway, which leads to the creation of lignin polymer blocks (Van der Wouwer et al., 2016). However, the results presented in this paper do not evidence any negative influence of 3,5-diISA and 4-IBeA at the same dose of I on tomato plants in the generative growth phase. This might stem from the method of their application (periodical application) in subsequent phases of plant growth. In this research, a negative influence on yield and fruit quality, as well as on the biomass of the overground vegetative part of plants, was caused only by 2,3,5-triIBeA (Fig. 1, Table 1). 2,3,5-TriIBeA is considered a known auxin inhibitor – otherwise referred to as TIBA (Saniewski et al., 2014). This compound inhibits the polar transportation of auxins, responsible for regulation of many development processes in plants: elongation growth, vascular tissue formation, embryogenesis, tropisms and many other processes, including root formation (Roberts and Friml, 2009). Additionally, in some plants, 2,3,5-triIBeA (TIBA) may induce abscission activity, i.e. natural separation of leaves, floral parts and fruits, as well as foliage branches (Weintraub et al.,

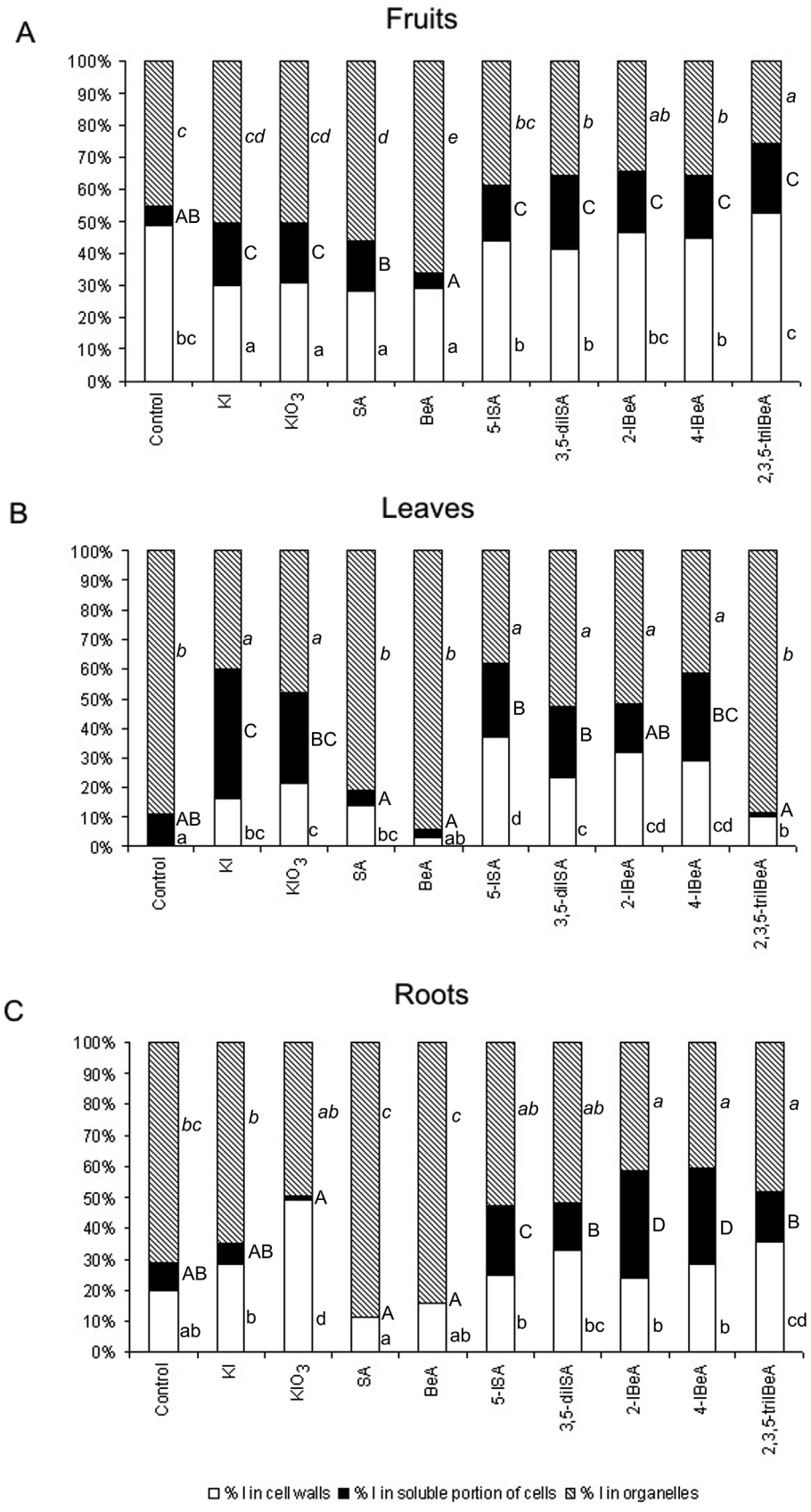

Fig. 3. Percent of iodine content in cell walls, organelles and the soluble fraction of tomato fruits(A), leaves (B) and root (C) cells. Means followed by the same letters are not significantly different for $P < 0.05$. Bars indicate standard error (n = 8, for each fraction). ANOVA calculations were performed independently for each fraction.

Table 2
Comparison the salicylic acid (SA), benzoic acid (BeA), iodosalicylates (5-ISA and 3,5-diISA) and iodobenzoates (2-IBeA, 4-IBeA and 2,3,5-triIBeA) content tomato fruits, leaves and roots of control plants with willow bark (*Salix* L.).

Treatment	Parts of plants	SA (μg.100 g^{-1}d.w.) ± SE	BeA (μg.100 g^{-1} d.w.) ± SE	5-ISA (μg.100 g^{-1} d.w.) ± SE	3,5-diISA (μg.100 g^{-1} d.w.) ± SE	2-IBeA (μg.100 g^{-1} d.w.) ± SE	4-IBeA (μg.100 g^{-1} d.w.) ± SE	2,3,5-triIBeA (μg.100 g^{-1} d.w.) ± SE
Control	Fruits	41.12 ± 3.63^{a}	337.21 ± 9.36^{ab}	0.13 ± 0.05^{a}	0.25 ± 0.28^{ab}	< LOQ	< LOQ	0.29 ± 0.03^{a}
KI		374.75 ± 12.00^{e}	2565.46 ± 194.36^{e}	0.23 ± 0.02^{a}	0.18 ± 0.02^{ab}	0.44 ± 0.14^{ab}	< LOQ	0.44 ± 0.09^{a}
KIO_3		249.39 ± 9.84^{d}	1749.24 ± 605.01^{de}	0.09 ± 0.02^{a}	0.12 ± 0.01^{a}	0.42 ± 0.09^{ab}	< LOQ	0.10 ± 0.02^{a}
SA		254.30 ± 17.58^{d}	1397.05 ± 47.24^{cd}	0.15 ± 0.01^{a}	0.12 ± 0.01^{a}	0.29 ± 0.05^{ab}	< LOQ	0.14 ± 0.05^{a}
BeA		471.72 ± 33.93^{f}	1679.19 ± 158.89^{d}	0.33 ± 0.03^{a}	0.11 ± 0.01^{a}	0.24 ± 0.03^{ab}	< LOQ	0.19 ± 0.05^{a}
5-ISA		148.02 ± 0.12^{c}	299.21 ± 16.43^{a}	5.48 ± 1.83^{b}	0.20 ± 0.13^{ab}	0.14 ± 0.02^{a}	< LOQ	0.25 ± 0.14^{a}
3,5-diISA		49.02 ± 4.79^{ab}	344.39 ± 11.70^{ab}	0.09 ± 0.01^{a}	0.67 ± 0.07^{b}	1.01 ± 0.10^{b}	< LOQ	0.11 ± 0.02^{a}
2-IBeA		123.44 ± 12.08^{b}	399.97 ± 36.85^{bc}	0.05 ± 0.01^{a}	0.07 ± 0.01^{a}	7.58 ± 0.36^{c}	< LOQ	0.20 ± 0.08^{a}
4-IBeA		136.45 ± 4.49^{c}	384.48 ± 53.08^{b}	0.06 ± 0.00^{a}	0.03 ± 0.00^{a}	0.17 ± 0.02^{a}	0.05 ± 0.01	0.18 ± 0.06^{a}
2,3,5-triIBeA		40.60 ± 10.85^{a}	1227.57 ± 429.80^{c}	0.25 ± 0.22^{a}	0.04 ± 0.01^{a}	< LOQ	< LOQ	3.02 ± 0.08^{b}
Control	Leaves	85.05 ± 3.70^{b}	1236.57 ± 146.37^{ab}	0.80 ± 0.24^{ab}	3.80 ± 0.86^{c}	0.24 ± 0.04^{a}	0.06 ± 0.03^{a}	0.11 ± 0.01^{a}
KI		55.35 ± 4.03^{a}	1605.97 ± 135.09^{b}	0.79 ± 0.03^{ab}	2.14 ± 0.71^{b}	0.27 ± 0.00^{a}	0.04 ± 0.01^{a}	0.07 ± 0.00^{a}
KIO_3		88.02 ± 2.33^{bc}	1527.16 ± 150.66^{b}	0.75 ± 0.02^{ab}	1.26 ± 0.29^{ab}	0.39 ± 0.04^{a}	0.03 ± 0.01^{a}	0.08 ± 0.01^{a}
SA		76.96 ± 3.74^{ab}	1182.06 ± 39.15^{ab}	1.15 ± 0.07^{b}	0.85 ± 0.13^{ab}	0.37 ± 0.03^{a}	0.03 ± 0.01^{a}	0.19 ± 0.01^{a}
BeA		61.08 ± 1.34^{ab}	1502.69 ± 241.31^{b}	3.11 ± 0.24^{e}	0.45 ± 0.15^{a}	0.25 ± 0.02^{a}	0.04 ± 0.01^{a}	0.08 ± 0.01^{a}
5-ISA		105.03 ± 2.14^{d}	1179.67 ± 89.17^{ab}	2.06 ± 0.08^{d}	1.18 ± 0.08^{ab}	0.41 ± 0.02^{a}	0.03 ± 0.00^{a}	0.22 ± 0.00^{a}
3,5-diISA		96.36 ± 6.51^{c}	1094.12 ± 53.51^{ab}	0.97 ± 0.04^{ab}	7.21 ± 0.52^{d}	0.42 ± 0.03^{a}	0.52 ± 0.10^{b}	0.17 ± 0.01^{a}
2-IBeA		70.93 ± 1.47^{ab}	705.71 ± 60.23^{a}	1.45 ± 0.05^{c}	0.59 ± 0.02^{ab}	3.77 ± 0.09^{b}	0.02 ± 0.01^{a}	0.18 ± 0.01^{a}
4-IBeA		96.99 ± 2.44^{c}	1434.05 ± 122.64^{b}	0.74 ± 0.03^{ab}	0.33 ± 0.12^{a}	0.37 ± 0.06^{a}	2.94 ± 0.11^{c}	0.12 ± 0.00^{a}
2,3,5-triIBeA		174.47 ± 6.78^{e}	662.59 ± 11.03^{a}	0.54 ± 0.03^{a}	0.51 ± 0.12^{a}	0.28 ± 0.02^{a}	0.06 ± 0.02^{a}	4.88 ± 0.12^{b}
Control	Roots	102.69 ± 3.72^{b}	342.45 ± 9.43^{a}	0.29 ± 0.04^{a}	1.16 ± 0.80^{b}	1.19 ± 0.06^{a}	0.33 ± 0.09^{a}	0.94 ± 0.02^{a}
KI		111.13 ± 5.85^{b}	359.26 ± 11.10^{a}	0.51 ± 0.03^{a}	0.36 ± 0.05^{ab}	0.42 ± 0.02^{a}	0.06 ± 0.01^{a}	0.10 ± 0.02^{a}
KIO_3		182.41 ± 7.07^{e}	471.59 ± 36.05^{ab}	0.26 ± 0.01^{a}	0.43 ± 0.13^{ab}	0.86 ± 0.04^{a}	1.91 ± 0.09^{b}	0.77 ± 0.03^{a}
SA		163.80 ± 7.69^{d}	661.85 ± 31.32^{c}	0.23 ± 0.01^{a}	0.01 ± 0.00^{a}	1.84 ± 0.39^{a}	4.33 ± 1.20^{c}	0.81 ± 0.08^{a}
BeA		151.76 ± 7.49^{c}	609.13 ± 47.00^{c}	0.55 ± 0.03^{a}	0.01 ± 0.00^{a}	0.22 ± 0.02^{a}	0.07 ± 0.02^{a}	0.12 ± 0.02^{a}
5-ISA		141.69 ± 4.63^{c}	562.09 ± 10.29^{bc}	27.86 ± 0.40^{b}	0.01 ± 0.00^{a}	0.37 ± 0.02^{a}	0.08 ± 0.02^{a}	1.61 ± 0.12^{a}
3,5-diISA		62.19 ± 1.64^{a}	436.25 ± 23.94^{ab}	0.45 ± 0.05^{a}	22.00 ± 0.31^{d}	0.87 ± 0.03^{a}	0.04 ± 0.01^{a}	0.11 ± 0.02^{a}
2-IBeA		63.99 ± 3.03^{a}	418.06 ± 53.70^{ab}	0.28 ± 0.02^{a}	1.94 ± 0.16^{c}	57.64 ± 2.26^{c}	2.40 ± 0.14^{b}	0.11 ± 0.02^{a}
4-IBeA		52.58 ± 1.14^{a}	363.06 ± 17.48^{a}	0.45 ± 0.02^{a}	0.53 ± 0.01^{ab}	4.25 ± 0.20^{b}	58.33 ± 0.79^{d}	1.89 ± 0.04^{a}
2,3,5-triIBeA		132.76 ± 4.33^{bc}	964.15 ± 46.05^{d}	0.28 ± 0.05^{a}	0.39 ± 0.01^{ab}	1.59 ± 0.08^{a}	0.77 ± 0.02^{ab}	247.87 ± 7.44^{b}
Comparison with willow bark (*Salix* L.) (n = 4)								
Willow (*Salix* sp. L)	Bark	1540.04 ± 14.55	1054.66 ± 26.05	32.64 ± 0.27	54.92 ± 3.58	21.24 ± 1.68	27.81 ± 0.57	30.84 ± 0.94

Means followed by the same letters are not significantly different for $p < 0.05$, (n = 8).
Limit of quantification (LOQ) for 2-IBeA analysis was 0.10 and for 4-IBeA was 0.013.
Ground willow bark was produced by Unit of Plant Herbs „Kawon-Hurt" Nowak Sp.J. Gostyń, Poland (number of sample 002.2018/35).

1952). Of very high importance is the stage at which plants are treated with 2,3,5-triIBeA (TIBA), as evidenced by the results of research conducted by Ramesar-Fortner and Yeung (2006) on microspore-derived embryos of *Brassica napus*. Treatment of embryos in the preglobular and globular phases led to the union of seed lobes, and in subsequent phases to radial symmetry. Exogenous TIBA did not have any effect on the development of embryos in relation to the control group (Ramesar-Fortner and Yeung, 2006).

However, for some compounds, derivatives of SA and BeA, e.g. 3-iodobenzoic acid (3-IBeA), a positive effect may be observed, for example: treatment of seedlings of *Cucumis sativus* L. with 0.05 mM 3-IBeA monoethanolamine salt stimulated elongation growth of roots, and increased the ability to develop side roots. However, on increasing the concentration of 3-IBeA monoethanolamine salt – 0.5 and 1 mM – the length of the shoots and roots decreased (Crisan, 2008). Thus, a conclusion may be drawn that the influence of the compounds studied and their doses on the growth and development of plants depends highly on species and plant development phase.

4.2. Effectiveness of iodine uptake, distribution in plants and biofortification of tomato fruits

The results of our research allowed us to define uptake via roots and distribution of mineral forms of iodine (KI and KIO_3) as well as iodosalicylates and iodobenzoates to overground vegetative and generative (fruits) parts of tomato (Figs. 5 and 6).

After the application of mineral compounds (KI and KIO_3), iodine was subject to even distribution over the entire root system, and in leaves and petioles. On the other hand, after application of organic forms of iodine, this element – to a greater extent than in comparison to application of KI and KIO_3 – was accumulated in the root system. Quantitatively, the iodine accumulation level in petioles and leaves followed the trend: 4-IBeA > 3,5-diISA > 5-ISA > KI > 2-IBeA > KIO_3 > 2,3,5-triIBeA > control group, SA and BeA. Iodine content in roots had the following pattern: 2-IBeA > 5-ISA > 2,3,5-triIBeA > 4-IBeA > 3,5-diISA > KI > KIO_3 > control group, SA and BeA (Fig. 6). Earlier research works carried out by Halka et al. (2018) on tomato plants in the seedling phase indicated that the accumulation level of iodine in leaves has the following pattern: KI > 4-IBeA > 5-ISA = 2-IBeA > 3,5-diISA > control group, SA and BeA, and in roots: 2-IBeA > 4-IBeA > 5-ISA > KI > 3,5-diISA > control group, SA and BeA (Halka et al., 2018). On this basis, we draw a conclusion that uptake and distribution of mineral and organic iodine in tomato roots and leaves depends on the plant's development phase.

The level of iodine accumulation in tomato fruits had the following pattern: 2-IBeA > KI > 4-IBeA > 5-ISA > 3,5-diISA > KIO_3 > 2,3,5-triIBeA > control group, SA and BeA (Fig. 6). Thus, we draw a conclusion that after application of 2-IBeA (in the generative phase of plant

Table 3
Summary of the effect of application SA, BeA, iodosalicylates and iodobenzoates on level of this compounds in different parts of tomato plants.

Organic compound applied to plant	Level of SA, BeA, iodosalicylates and iodobenzosates in tomato plant compared to control							
	SA	BeA	5-ISA	3,5-diISA	2-IBeA	4-IBeA	2,3,5-triIBeA
SA	↑ in fruits and roots	↑ in fruits and roots	↑ in leaves	↓ in fruits, leaves and roots	↑ in fruits	↑ in roots	no influence
BeA	↑ in fruits and roots	↑ in fruits, leaves and roots	↑ in leaves	↓ in leaves and roots	no influence	no influence	no influence
5-ISA	↑ in fruits, leaves and roots	↑ in roots	↑ in fruits, leaves and roots	↓ in leaves and roots	no influence	no influence	no influence
3,5-diISA	↑ in leaves, ↓ in roots	no influence	no influence	↑ in fruits, leaves and roots	↑ in fruits	↑ in leaves	no influence
2-IBeA	↑ in fruits, ↓ in roots	↑ in fruits	↑ in leaves	↓ in leaves ↑ in roots	↑ in fruits, leaves and roots	↑ in roots	no influence
4-IBeA	↑ in fruits and leaves, ↓ in roots	↑ in fruits and leaves	no influence	↓ in leaves	↑ in leaves and roots	↑ in fruits, leaves and roots	no influence
2,3,5-triIBeA	↑ in roots	↑ in fruits and roots	no influence	↓ in leaves	no influence	no influence	↑ in fruits, leaves and roots

development), iodine in this form is more effectively transported to fruits in relation to the other iodine compounds tested by us. There was no negative effect of 2-IBeA on tomato fruit, so it can be considered that this compound may be a good source of iodine in tomato biofortification. Enriching tomatoes in iodine can help reduce the problem of malnutrition in this plant. In many countries, the tomato is a very economically important vegetable. It is widely consumed all over the world – both raw and in processed form. In our study, intake of 100 g of tomato fruits enriched with iodine with the use of 2-IBeA might cover ca. 37.5% of iodine RDA (Iodine Global Network, 2015) (Table S2). A high iodine content was also observed in tomato fruits after application of KI – iodine RDA coverage reached 35.6% for a 100 g portion of fresh tomato fruit. For other treatments, the calculated iodine RDA values were lower than for 2-IBeA and KI: 27.7%, 22.2%, 18.8%, 12.1% and 9.0% for 4-IBeA, 5-ISA, 3.5-diISA, KIO_3 and 2,3,5-triIBeA, respectively. Nevertheless, these values were still high enough that the use of these iodine compounds could be useful for the development of biofortification protocols for tomato plants. We believe that the enrichment of iodine fertilisers with organic or mineral iodine in tomato cultivation can effectively reduce iodine malnutrition. However, our method for applying iodoorganic compounds was only tested in hydroponic cultivation. The use of fertilisation with organic or mineral iodine compounds during cultivation of tomatoes in soil (in field or greenhouse conditions) requires additional research on the effectiveness of this type of iodine biofortification method.

It is considered that iodine in the form of KI in soil-less cultivations is – in comparison to KIO_3 – more easily assimilated by plants, but it may have more toxic actions. When taken up through roots, iodine in the form of IO_3^- is first reduced to I^-, and only then assimilated by plants (Kato et al., 2013; Medrano-Macías et al., 2016). This may also be an explanation for the two-fold lower iodine content in tomato fruits after application of KIO_3 in comparison to KI. A higher iodine enrichment level of tomato fruits with the use of KI stems from a confirmed theory that in many species of plants, I^- ions are preferred in comparison to IO_3^- when it comes to uptake via roots (Caffagni et al., 2011; Kiferle et al., 2013; Zhu et al., 2003).

Iodine accumulated by plants is unstable and may be subject to volatilisation in the form of methylated CH_3I compound. The iodine methylation process is controlled by the enzymes HMT and HTMT (Itoh et al., 2009; Redeker et al., 2004). The process of iodine volatilisation can be associated with low stability in plants, as well as reducing the iodine dose for people consuming biofortified tomato fruits. In our research, we determined the expression level of the *HMT* gene which encodes HMT in tomato leaves and roots (Fig. 4). An increase in expression of this gene was observed in leaves and roots of plants treated with organic iodine compounds, in comparison to application of KI and KIO_3. An increase in expression of the *HMT* gene after application of organic forms of iodine may be evidence of more intensive iodine volatilisation in those treatments, which finds ground in our determination of the iodine accumulation level in vegetative parts of plants.

A high level of expression of the *HMT* gene in the case of application of SA may be explained by the fact that this compound may also be volatilised in methylated form, and this process is controlled by the enzyme S-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase (SAMT) (Ross et al., 1999). The enzymes SAMT and HMT fall into the same class, i.e. methyltransferases, and both are dependent on SAM (Itoh et al., 2009; Redeker et al., 2004; Ross et al., 1999).

After application of organic iodine forms, the lowest iodine content in roots was found in plants treated with 3,5-diISA, though in leaves and petioles its reached the same level as in the case of application of 5-ISA and 4-IBeA. At the same time, for the 3,5-diISA treatment, an almost two-fold increase in *HMT* gene expression was observed in plant roots in relation to control plants. In the face of the above, the lower iodine content in roots after application of 3,5-diISA may be explained by its transportation from roots to leaves, where it might be volatilised in the form of CH_3I. In leaves and petioles, as well as tomato plant

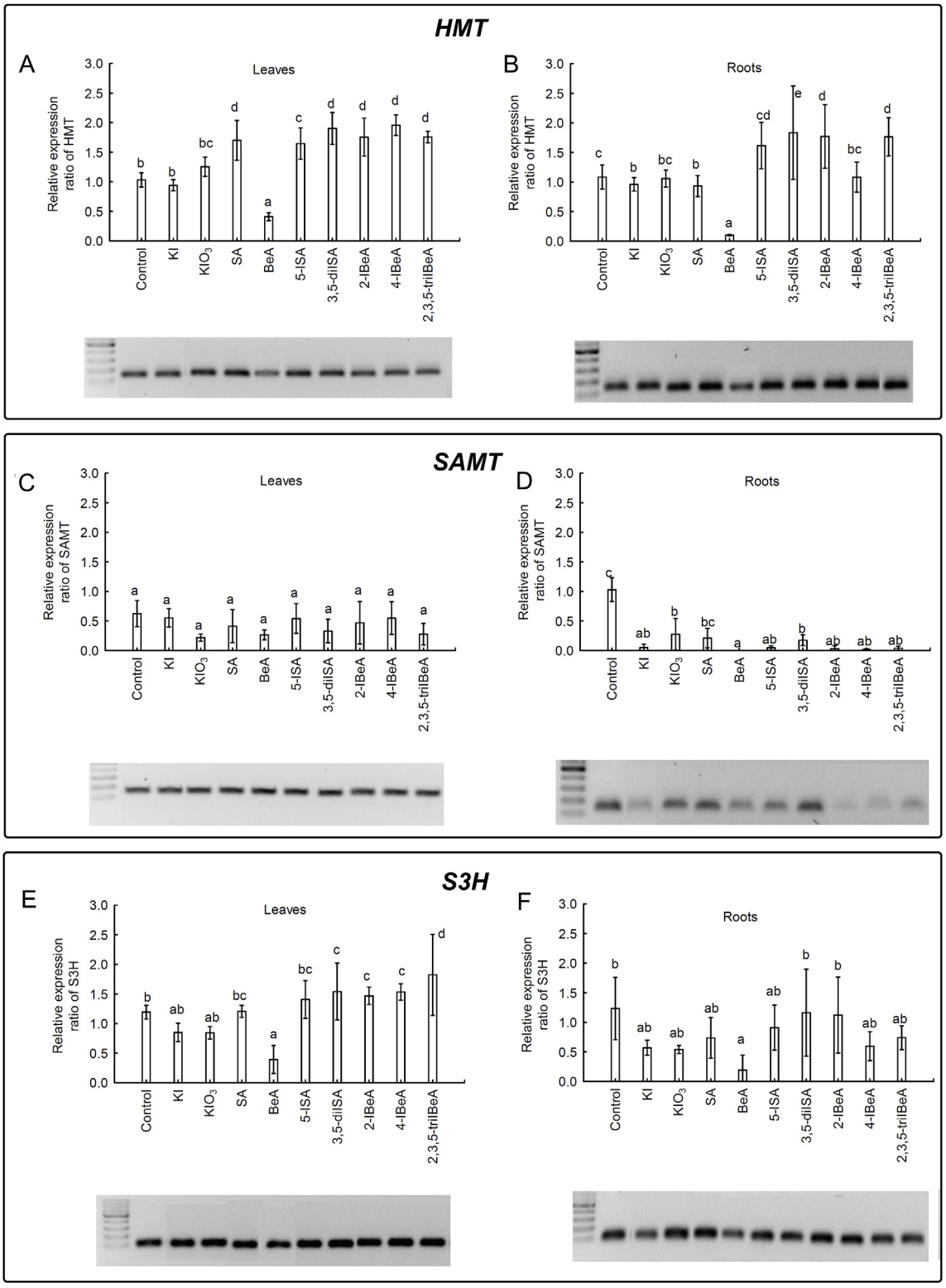

Fig. 4. Relative expression of HMT (A, D), SAMT (B, E) and S3H (C, F) genes in leaves and roots of tomato plants and photos of the qRT-PCR reaction products confirmed by electrophoresis in 1% agarose gel (pictures of bands on the gel below each figure). Means followed by the same letters are not significantly different for $p < 0.05$. Bars indicate standard error (n = 9).

fruits, the lowest iodine content was observed after application of 2,3,5-triIBeA, and in roots, the content of this element was much higher at 404.69 mg·100 g^{-1} dry weight. This may prove that due to the more complex structure in relation to the other forms of iodine applied, transportation of 2,3,5-triIBeA to overground parts of plants is highly restricted. Additionally, in the case of both tomato leaf and root samples, after application of 2,3,5-triIBeA, increased *HMT* gene expression was observed, which, after application of this compound, may be connected with the volatilisation of iodine to the atmosphere.

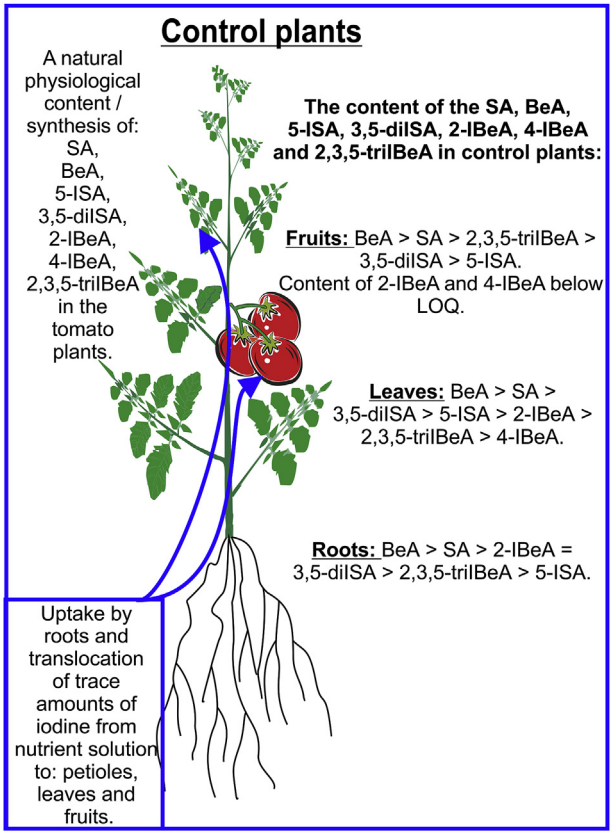

Fig. 5. Summary of the most important results with regard to control plants.

4.3. Iodine localisation in the cell

Iodine taken up by plants may be accumulated in various compartments of plant cells. Research conducted by Weng et al. (2008) on cabbage plants indicated that iodine applied in the form of NaI and $NaIO_3$ at various doses may be accumulated in cell wall, organelle and soluble cell portion fractions. It was observed that in the case of roots, stems and leaves of cabbage plants, iodine was accumulated the following way: soluble portion > insoluble cell walls > organelles; its content in the soluble portion amounted to 60%, in organelles – 19%, and in the cell wall fraction – ca. 20.4–21.8% (Weng et al., 2008). In our research – in the case of fruit samples – we observed the following tendency to accumulate iodine in individual fractions: organelles > cell walls > soluble portion for mineral iodine form, SA and BeA, but for organoiodine compounds it was: cell walls > organelles > soluble portion of cells (Fig. 3A). Iodine content in leaves and roots depended on the compound applied; for control, SA, BeA and 2,3,5-triIBeA in leaves and for all treatments except 2-IBeA and 4-IBeA in fruit samples, the tendency observed was: organelles > insoluble cell walls > soluble portion (Fig. 3B and C). After application of a mineral form (KI and KIO_3), iodine in leaves was accumulated in a different way: organelles > soluble portion > cell walls. Iodine content in organelles for fruit samples amounted to ca. 25–66%, for leaves 38–94% and in roots – to ca. 41–89%. Research works conducted by Halka et al. (2018) on tomato plants in the seedling phase indicated a tendency to accumulate iodine in individual cell fractions, both in the case of the mineral form of KI, as well as organic forms of iodosalicylates and iodobenzoates. In the context of the above data, we reason that localisation of iodine in individual cell fractions is conditioned by the plant species, organ and growth phase.

4.4. Accumulation of SA, BeA and its derivatives, and SA metabolism in tomato plants

In our studies, iodosalicylates and iodobenzoates were observed in roots, leaves and fruits (Table 3, Fig. 5). This proves that these compounds are naturally synthesised in plants (Table 2, Fig. 5). Exogenous iodosalicylates and iodobenzoates triggered a considerable increase in their content in tomato roots, leaves and fruits (Fig. 6). The highest content of organoiodine compounds was observed in plant roots, and the lowest– except for 5-ISA – in tomato fruits. This proves the possibility of iodosalicylate and iodobenzoate uptake via roots and their subsequent transportation to overground vegetative and generative parts of tomato plants. This caused the lowest level of accumulation of 5-ISA, 3,5-diISA, 2-IBeA and 4-IBeA in roots, leaves and fruits (respectively for application of individual compounds).

SA was discovered following its isolation from willow bark (Hayat et al., 2010). The bark of this plant is naturally rich in SA and BeA. Although referencing many works in the literature, we have not found any data on the occurrence of 5-ISA, 3,5-diISA, 2-IBeA and 4-IBeA in plants. Thus, as well as analysis of tomato plant samples, we conducted a comparative analysis of willow bark for the purpose of determining the 5-ISA, 3,5-diISA, 2-IBeA, 4-IBeA and 2,3,5-triIBeA content. We had only suspected that willow bark might contain iodosalicylates and iodobenzoates. Analysis of results confirmed our hypothesis. Willow bark is used in medicine for the preparation of infusions in order to treat fever and pain in mild rheumatic ailments (Laekeman and Neef, 2017). Getting back to the primary aim of our paper's experiment, it is important that fruits of plants treated with 5-ISA, 3,5-diISA, 2-IBeA, 4-IBeA and 2,3,5-triIBeA are adequately enriched with these compounds, though in the case of application of mineral iodine compounds, KI and KIO_3, the content of iodosalicylates and iodobenzoates in fruits reached the same levels as in the control group. Taking the above into account, our studies show that tomato fruits biofortified with iodine, with the use of iodosalicylates and iodobenzoates, may potentially have better health-promoting characteristics than those biofortified solely with KI and KIO_3. We draw this conclusion also from the fact that we obtained an increased content of iodosalicylates and iodobenzoates, that are present in willow bark. The content of these compounds in fruits was found at a lower level, which indicates the possibility of their intake by consumers for the purpose of promoting a better health condition, both in relation to supplementation of a system with iodine, and in the scope of similar, as featured by willow bark. Additionally, scientific research works confirm also that derivatives of SA and aniline, i.e. salicylanilide derivatives, occur naturally in plants and may have antiproliferative activity in relation to *Hep-G2* cell lines, typical for liver cancer. Research works conducted by Zhu et al. (2011) show that synthesised derivatives of 5-ISA indicate the highest antiproliferative activity over *Hep-G2* cell lines, as well as influencing inhibition of epidermal growth factor receptor (EGFR) which plays a key role in signal transduction of pathways responsible for division and diversification of cells, which in the case of a cancer results in a rapid growth of tumour cells. It must be emphasised that these results evidence that iodine is of high importance for antiproliferative activity, when replaced in the position of a 5-aromatic ring (Zhu et al., 2011).

Research conducted by Jensen et al. (2004) on Wistar rats, which were administered with 2-, 3- and 4-iodobenzoic acids at a dose of 50 mg kg^{-1} of body weight of one rat, indicates that the organic iodine compounds studied may be assimilated and metabolised in their systems, which is evidenced in their increase iodine content in urine and bile. Additionally, the research confirmed that iodine administered in this form is present in rats' systems in the form of iodobenzoates (Jensen et al., 2004). Thus, we draw a conclusion that plants, after application of iodosalicylates and iodobenzoates, may be a direct source of iodine, and they may have a positive influence on consumers' health condition.

When comparing the effects of solely exogenous SA and BeA, a higher SA content in tomato fruits was observed after application of

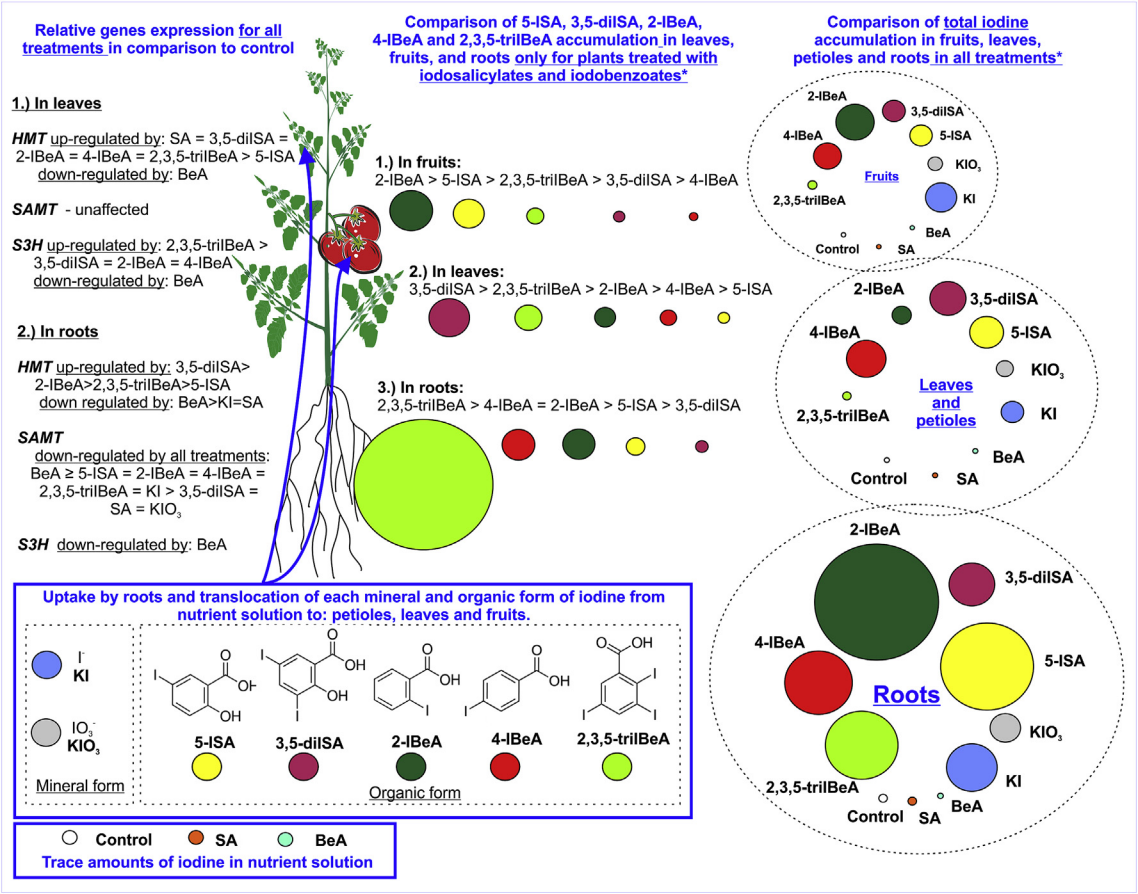

Fig. 6. Graphical summary of study – comparison of total iodine, iodosalicylates and iodobenzoates accumulation and metabolism, in different parts of tomato plants in each of the experiment treatments.

BeA, which is a precursor of SA. However, after the application of exogenous SA, the SA content in tomato fruits was almost two-fold lower than after application of BeA. In tomato roots, the highest SA content was observed after application of exogenous SA, and in leaves after the introduction of 2,3,5-triIBeA to the nutrient solution. The high content of SA after application of 2,3,5-triIBeA may be connected with the negative influence of this compound on plants (inhibition of auxins; Saniewski et al., 2014), which triggers the accumulation of endogenous SA as a response to stress caused by an increased concentration of 2,3,5-triIBeA. Increased SA content in a plant may be connected with the plant's response to various types of stress factor and result in volatilisation of this compound in the methylated form, i.e. MeSA. High emission of MeSA is correlated with high expression of the *SAMT* gene that encodes SAMT (Tieman et al., 2010). In our experiment, no statistically significant differences in *SAMT* gene expression were observed in the case of tomato leaves; however, with respect to the control group, a decrease in the level of expression of this gene in roots was observed for all treatments studied, except for SA (Fig. 4C and D). A decrease in *SAMT* gene expression following treatment of plants with mineral and organic compounds of iodine and BeA might have been caused by directing metabolic processes of SA not towards the methylation process, but towards its transformation into other SA derivatives in plants. A decrease in the expression level of the *SAMT* gene in roots may be also connected with the probably intensified transportation of SA to plant leaves and fruits, which is confirmed by a higher SA content than in the control group: in the case of application of KI, KIO_3, SA, BeA, 5-ISA, 2-IBeA and 4-IBeA in fruits, and in the case of application of 5-ISA, 3,5-diISA, 4-IBeA and 2,3,5-triIBeA in tomato leaves.

SA in plants may be also the subject of conversion into, among others, the compounds 2,3-DHBA and 2,5-DHBA (Zhang et al., 2013). Transformation of SA into these compounds in a plant is executed also as a protective measure against excessive accumulation of SA in a plant. This process is controlled by the enzyme salicylic acid 3-hydroxylase encoded by the *S3H* gene. This mechanism also plays a very important role in the process of ageing of *A. thaliana* leaves (Zhang et al., 2013). In the present research, a statistically significant increase in *S3H* gene expression was observed in tomato leaves for all organoiodine compounds (Fig. 4E). The highest increase in expression in leaves was observed after application of 2,3,5-triIBeA, which is also connected with the highest SA content in leaves after application of this compound in relation to other treatments (Table 2). The tendency for up-regulation of the *S3H* gene on treatment with iodosalicylates and iodobenzoates may be connected to the possibility of transforming these compounds into SA, and subsequently into 2,3-DHBA and 2,5-DHBA, or direct transformation of organoiodine compounds, SA derivatives, into these products. Also, down-regulation of the *S3H* gene was observed in leaf and root samples after application of BeA, which is a direct precursor of SA. Thus, it may be assumed that after application of BeA in tomato plants, the compound will enter other metabolic pathways, not subject to this experiment.

While 2,3,5-triIBeA is a known inhibitor of auxins, 5-ISA, 3,5-diISA, 2-IBeA and 4-IBeA tested by us do not serve this function, nor are they precursors of 2,3,5-triIBeA synthesis in plants. We draw this conclusion as 5-ISA, 3,5-diISA, 2-IBeA and 4-IBeA did not result in an increased 2,3,5-triIBeA content in roots, leaves and fruits. We also postulate that 2,3,5-triIBeA is not degraded in plants to 5-ISA, 3,5-diISA, 2-IBeA and 4-IBeA, as exogenous 2,3,5-triIBeA did not trigger an increase in their content in plants. Additionally, a visual symptom of 2,3,5-triIBeA activity was a strong twisting of plants' shoots (inhibition of auxins), and this effect was not observed for 5-ISA, 3,5-diISA, 2-IBeA, 4-IBeA, SA and

BeA.

5. Conclusion

Our study showed that iodosalicylates and iodobenzoates can be a good source of iodine for tomato biofortification. Most of these compounds, as well as mineral forms KI and KIO_3, did not have a negative effect on growth and development of tomato plants and led to an increase of the iodine content in leaves, petioles, roots and fruits of tomato plants. The best iodine accumulation of iodine in the edible part of tomato plants was observed after 2-IBeA application. Intake of 100 g of tomato fruits enriched with iodine using 2-IBeA might cover ca. 37.5% of iodine RDA (for KI and KIO_3 it would be 35.6% and 12.1%, respectively). In addition, many scientific studies show that some iodosalicylates and iodobenzoates can have health-promoting properties. The use of iodosalicylates and iodobenzoates for iodine biofortification of tomato plants can have a double positive effect due to their health-promoting properties as well as a being source of iodine in the human diet. After application of individual exogenous iodosalicylates and iodobenzoates, a diversified reaction of plants was established in view of their metabolic capabilities and/or reaction of plants consisting of the synthesis of other compounds than the applied iodosalicylate and iodobenzoate in tomato plant fruits, roots and leaves. A summary of these metabolic reactions/processes taking place in plants is presented in Table 3 and Fig. 6.

On the basis of the results obtained, we may precisely define how enzymatic/biochemical pathways are responsible for the metabolism of each of the studied organic iodine compounds in plants.

Author contribution

Smoleń S. conceived of the present idea and supervised the work. Halka M. and Smoleń S. are responsible for planning the experiment, plants cultivation and chemical analysis. Halka M., Czernicka M. and Klimek-Chodacka M. are responsible for planning and realization the molecular part of the experiment. Smoleń S., Pitala J., and Tutaj K. are responsible for the development of iodosalicylates and iodobenzoates determination method and its analysis. Halka M., and Smoleń S. are responsible for data analysis interpretation of the results. All authors are responsible for interpretation of the results, writing and editing manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.028.

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