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Grain Endogenous Selenium and Moderate Salt Stress Work as Synergic Elicitors in the Enrichment of Bioactive Compounds in Maize Sprouts

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Abstract: Salt stress and selenium are known to elicitate the production of plant secondary metabolites with antioxidant properties. On this basis, maize grains obtained from mother plants fertilized or not fertilized with selenium were sprouted at different levels of salinity (0, 25, and 50 mM NaCl) to evaluate the effects on the sprout yield, inorganic and organic Se species, minerals, and secondary metabolites, as revealed by a metabolomics analysis. Grain endogenous selenium (135 mg kg^{-1} vs. 0.19 mg kg^{-1} of the non-enriched grain) and salinity affected the sprout yield and composition, with salinity having the greatest effect on secondary metabolites. Most of the Se in sprouts was in an inorganic form, despite Se-enriched grains only containing organic Se. Some synergic effect was observed between Se and salinity. The best combination was obtained with Se-enriched grains sprouted at 25 mM NaCl, since this provided a good yield (not lower than in the untreated control), while sprout shoots were enriched in selenocystine and pro-nutritional semipolar compounds with antioxidant properties. Therefore, using grains from Se-fertilized crops and sprouting them under mild salt stress might represent a promising technique for improving the nutritional value of sprouts.

Keywords: elicitation; abiotic stress; metabolomics; phytochemical; antioxidant

1. Introduction

Recent research on sprouts has focused on the use of elicitors to stimulate the production of secondary metabolites, since many of these compounds, also referred to as phytochemicals or bioactive compounds, are known to have a role as antioxidants, with several positive effects on human health [1,2]. In this context, several studies have investigated the elicitation of phytochemicals obtained by sprouting seeds under different NaCl concentrations, e.g., for legumes [3,4], *Brassica* spp. [5,6], and cereals [7,8]. Meanwhile, selenium biofortification has also been found to boost the phytochemical concentration of sprouts from the same groups of species, i.e., legumes [9], *Brassica* spp. [10,11], and cereals [12]. Furthermore, many works have reported that selenium biofortification has a role in increasing plant tolerance against abiotic stresses, such as drought stress and salinity [13–15]. Therefore, for example, selenium fertilization could represent a promising tool to improve plant stress tolerance and reduce the

irrigation requirements of crops in drylands, such as Mediterranean areas [16]. Finally, Se fertilization implies the accumulation of organic forms of selenium in plant products, which may represent an advantage in human feeding, because selenium itself has antioxidant properties. In fact, the production of Se-enriched food, including sprouts, is currently exploited and may actually increase market chances [17–19]. Based on these considerations, we deemed that studying the combined effect of both selenium and NaCl on the nutraceutical profile of sprouts was of interest. Among plants, maize (*Zea mays* L.) was selected for this study. This species is the third most cultivated crop all over the world, with countless uses in food, feed, industry, and pharmaceutics. Several studies have reported that its sprouts, which are widely consumed worldwide, are rich in phenolic compounds (i.e., flavonoids, phenolic acids, etc.) and other antioxidants [20–22]. Se-biofortification of maize sprouts by the application of Se to the growing substrate has already been attempted by Diowksz et al. (2014) [23]. More recently, in a study on basil microgreens, Puccinelli et al. (2019) [24] introduced the idea of investigating the effect of endogenous Se, i.e., using seeds with a high constitutive Se concentration, obtained by fertilizing the mother plant with Se. Similarly, maize grains with an increased concentration of endogenous Se were obtained by D'Amato et al. (2019) [25], by fertilizing the field crop with sodium selenite, and these grains were made available for our experiment on sprouting. In this context, a metabolomics study appeared to be the most suitable approach for evaluating the global response at a chemical level as a consequence of several biological processes. To date, metabolomics studies of Se-enriched plants have only been carried out for strawberry, which revealed an increase in primary (amino acids, organic acids, and sugars) and secondary (flavonoids) compounds [26], while a systematic and global metabolomics study on staple crops such as maize is still missing.

Therefore, the present work was aimed at investigating the effect of endogenous Se on physiological responses and secondary metabolites of maize sprouts grown under moderate salinity.

2. Materials and Methods

2.1. Sprout Production

Maize (*Zea mays* L. cultivar Dekalb DKC4316, FAO 300) crop cultivation, Se fertilization, and grain production were performed as described by D'Amato et al. (2019) [25]. In this experiment, we used grains harvested in 2016 from crops subjected to non-limiting irrigation and subjected to two Se fertilization treatments: Se fertilization with 200 g Se ha^{-1} as sodium selenite (Se_{YES}) and no Se fertilization (Se_{NO}).

Grains from both Se_{YES} and Se_{NO} were sown on plastic trays containing filter paper wetted with distilled water or with 25 or 50 mM NaCl solution, labeled as $NaCl_0$, $NaCl_{25}$, and $NaCl_{50}$, respectively. An additional filter paper wetted with water was used to cover the seeds, in order to guarantee constant water availability for germination. Trays were incubated in a growth chamber at 20 °C in the dark. After germination, the filter paper covering seeds was removed and the trays were placed in a dark:light regime of 10:14 h and light intensity of 200 µmol m^{-2} s^{-1}, according to a completely randomized design with four replicates (trays), and were then regrouped two by two for analysis.

Distilled water was periodically added to trays to restore the initial tray weight, assuming that the weight change was mainly due to water evaporation (i.e., considering the biomass weight change as negligible), so approximately maintaining the initial NaCl concentration of each treatment [6–8]. Maize sprouts from each replicate were harvested at the euphylla stage [20], before the complete expansion of the euphylla. Maize sprouts of $NaCl_0$ and $NaCl_{25}$ were collected 7 days after sowing (DAS), while sprouts of $NACl_{50}$ were collected 8 DAS, because increasing the salinity slightly slowed seedling growth. Roots were separated from shoots. Fresh and dry matter (FM and DM, respectively) of sprouts, as well as their shoot and root lengths, were measured for ten individuals per replicate. The dry mass was determined after drying at 60 °C for 48 h in a ventilated stove. The lengths were measured using a micrometer with an accuracy of 0.02 mm. A sub set of fresh shoots and roots from each replicate was immediately used for Se speciation. The remaining root and shoot samples were

immediately frozen in liquid nitrogen; subsequently, they were freeze-dried, grinded, and stored at −20 °C in polypropylene tubes until analysis. Three separate subsamples were taken from each replicate, and each subsample was analyzed once. Therefore, the mean value of each treatment is the result of six separate measurements (i.e., 2 replicates per treatment × 3 subsamples per replicate × 1 measurement per subsample).

2.2. Se Speciation

Se speciation was achieved in fresh roots and shoots of maize sprouts following the method used by D'Amato et al. (2019) [25]. The results were expressed as micrograms per gram of dry mass (μg g–1 DM). The total inorganic and organic Se concentrations were calculated as the sum of single inorganic (i.e., selenite, SeO_3^{-2}, and selenate, SeO_4^{-2}) and organic (i.e., selenocystine, $SeCys_2$; Se-(methyl)selenocysteine, SeMeSeCys; selenomethionine, SeMet) compounds, respectively. The total Se concentration (TSeC) was calculated as the sum of total inorganic and total organic compounds.

2.3. Ion Concentrations

Ion concentrations was determined in shoots by ion chromatography with conductivity detection (Portlab Hplc System Stayer, Milan, Italy), using the method of Bocchini et al. (2018) [14]. The concentrations of anions (Cl^-, NO_3^-, PO_4^{3-}, and SO_4^{2-}) and cations (Na^+ and K^+) were both calculated as mg kg^{-1} for DM.

2.4. Metabolomics Analyses

The extraction and LC-HRMS of polar and non-polar metabolomes of maize sprouts under NaCl and Se treatments were analysed as previously described [27–30], with the exception of ionization settings that were as follows: polar and non-polar metabolites were ionized in heated electrospray ionization (HESI) and atmospheric-pressure chemical ionization (APCI) sources, respectively, with nitrogen used as sheath and auxiliary gas, set to 35 and 20 units (HESI) and 30 and 10 units (APCI). The vaporizer and capillary temperatures were set to 260 and 310 °C, and 270 and 250 °C for polar and non-polar metabolomes, respectively. The discharge current was set to 5 μA, and the S-lens RF level was set to 50. The acquisition was performed in the mass range of 110–1600 m/z, both in positive and negative ion mode, with the following parameters: resolution, 70,000; microscan, 1; AGC target, $1 \times e^6$; and maximum injection time, 50. An ad hoc metabolomics database of polar and non-polar compounds was compiled using in-house and literature data [31].

2.5. Statistical Analysis

All data were analysed by two-way ANOVA according to a randomized blocks design with two replicates. The average values ± standard deviation are depicted. Means were compared by using the Fisher's least significant difference (LSD) at $P < 0.05$.

Heatmap and symmetrical correlation matrices were generated the same way as in previous studies [32,33].

3. Results

3.1. Growth Indexes

Germination was over 90% in all treatments, with only a non-significant decrease for $NaCl_{50}$, independent of Se treatment (Table 1). Root length was not affected by Se treatments, while it was significantly decreased by the highest salinity level. Regarding the shoot length, an "Se x NaCl" interaction was observed, with the lowest values being recorded for $Se_{NO}NaCl_0$ and $Se_{YES}NaCl_{50}$. However, the fresh and dry mass of both shoots and roots was not affected by either selenium or salinity.

Table 1. Germination percentage, shoot and root lengths, individual total fresh mass, and dry matter concentration of maize sprouts obtained at 0, 25, or 50 mM NaCl (i.e., $NaCl_0$, $NaCl_{25}$, and $NaCl_{50}$, respectively) from grains produced without or with Se fertilization (i.e., Se_{NO} and Se_{YES}, respectively).

Treatment	Germination (%)	Lengths (mm)		Fresh Mass (mg)		Dry Matter (%)	
		Roots	Shoot	Roots	Shoot	Roots	Shoot
$Se_{NO}NaCl_0$	99 (1.2)	59 (14.9)	26 (9.3)	133 (33.6)	83 (32.2)	8.9 (2.25)	11.9 (4.61)
$Se_{NO}NaCl_{25}$	96 (3.5)	65 (10.8)	37 (7.2)	147 (24.4)	98 (19.1)	9.1 (1.51)	12.3 (2.39)
$Se_{NO}NaCl_{50}$	94 (2.0)	52 (17.3)	32 (3.5)	126 (41.9)	103 (11.3)	10.3 (3.43)	11.8 (1.29)
$Se_{YES}NaCl_0$	97 (2.3)	78 (22.8)	39 (13.2)	180 (52.6)	99 (33.5)	6.9 (2.02)	11.6 (3.93)
$Se_{YES}NaCl_{25}$	99 (2.3)	68 (19.5)	35 (5.8)	161 (46.2)	109 (18.4)	8.9 (2.55)	11.9 (1.97)
$Se_{YES}NaCl_{50}$	93 (3.1)	53 (16.5)	26 (3.2)	91 (28.3)	84 (10.3)	10.2 (3.18)	15.4 (1.90)
F Test							
Se fertilization (Se)	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
NaCl treatment (NaCl)	*n.s.*	**	*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Se X NaCl	*n.s.*	*n.s.*	*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
LSD of the interaction	*3.44*	*15.22*	*6.75*	*69.6*	*40.39*	*4.58*	*5.21*

Standard deviation in brackets. Different letters within each column indicate statistically significant differences at $p \leq 0.05$. F test: ** significant at $p < 0.01$, * significant at $p < 0.05$, n.s. not significant.

3.2. Selenium Concentration

The TSeC in Se_{YES} sprouts was over six times higher than that in Se_{NO} sprouts. Higher values of Se_{YES} were obtained for both shoots and roots and both inorganic and organic Se species (Table 2). Within Se treatments, the TSeC of both shoots and roots was not affected by salinity.

On average, over all treatments, the inorganic species represented the greatest part of TSeC (75% in roots and 81% in shoots), with selenite (SeO_3^{2-}) as the predominant species (90% of total inorganic species in both roots and shoots). Within organic species, SeMet was the most represented in roots (60% total organic species) and $SeCys_2$ and SeMet were the most represented in shoots (over 44% and 46% of total organic species, respectively).

Table 2. Inorganic and organic Se species and total Se concentration (TSeC) in shoots and roots of maize sprouts obtained at 0, 25, or 50 mM NaCl (i.e., $NaCl_0$, $NaCl_{25}$, and $NaCl_{50}$, respectively) from grains produced without or with Se fertilization (i.e., Se_{NO} and Se_{YES}, respectively).

Treatments	Se Species (μg kg^{-1} DM)							TSeC
	Inorganic Species			Organic Species				
	$SeO3^{2-}$	$SeO4^{2-}$	Total	SeCys2	SeMeSeCys	SeMet	Total	
Roots								
$Se_{NO}NaCl_0$	172 (20)	16 (1.2)	188 (19.3)	12 (2.2)	6 (2.8)	32 (3.8)	52 (7.8)	240 (14.2)
$Se_{NO}NaCl_{25}$	160 (6.9)	20 (1.6)	180 (5.3)	14 (2.0)	17 (7.9)	19 (4.1)	47 (11.4)	228 (16.7)
$Se_{NO}NaCl_{50}$	167 (27.5)	44 (9.8)	211 (35.8)	12 (0.7)	16 (4.2)	55 (8.4)	141 (69.5)	352 (73.1)
$Se_{YES}NaCl_0$	867 (87.2)	37 (7.6)	904 (92.1)	81 (22.0)	10 (5.3)	139 (43.6)	236 (92.5)	1140 (179)
$Se_{YES}NaCl_{25}$	943 (160.5)	82 (21.3)	1025 (139.2)	77 (19.1)	13 (3)	237 (60.0)	382 (63.6)	1407 (196.9)
$Se_{YES}NaCl_{50}$	876 (136.2)	29 (20.5)	904 (156.1)	131 (13.6)	14 (2.9)	226 (24.8)	317 (39.5)	1221 (190.3)
F Test								
Se fertilization	**	**	**	**	*n.s.*	**	**	**
NaCl treatment	*n.s.*	**	*n.s.*	**	*	*	*n.s.*	*n.s.*
Se x NaCl	*n.s.*	**	*n.s.*	**	*n.s.*	*n.s.*	*n.s.*	*n.s.*
LSD of interaction	167	9	169	23	8	76	100	197
Shoots								
$Se_{NO}NaCl_0$	221 (34.8)	16 (0.65)	237 (34.3)	13 (0.7)	9 (0.58)	31 (1.8)	52 (2.6)	289 (35.9)
$Se_{NO}NaCl_{25}$	279 (25.5)	53 (10.9)	332 (14.6)	86 (11.2)	14 (1.9)	95 (25.8)	195 (34.8)	527 (48.3)
$Se_{NO}NaCl_{50}$	168 (58.8)	64 (3.4)	232 (56.6)	34 (12.2)	8 (0.6)	33 (3.5)	75 (16.2)	306 (53.6)
$Se_{YES}NaCl_0$	1512 (153.4)	30 (2.7)	1541 (153.4)	94 (7.0)	15 (0.8)	104 (5.8)	212 (2.2)	1754 (153.2)
$Se_{YES}NaCl_{25}$	1512 (70.6)	31 (0.7)	1543 (69.9)	112 (1.1)	21 (2.2)	63 (3.7)	196 (5.6)	1738 (64.3)
$Se_{YES}NaCl_{50}$	1489 (173.5)	24 (1.5)	1512 (174.7)	96 (10.2)	15 (1.2)	93 (5.0)	204 (11.8)	1716 (162.9)
F Test								
Se fertilization	**	**	**	**	**	**	**	**
NaCl treatment	n.s.	*	*n.s.*	**	**	*n.s.*	*n.s.*	*n.s.*
Se x NaCl	n.s.	**	*n.s.*	**	*n.s.*	**	*n.s.*	*n.s.*
LSD of interaction	184	23	*149*	15	*2*	*20*	196	222

SeO_3^{2-}, selenite; SeO_4^{2-}, selenate; $SeCys_2$, selenocystine; SeMeSeCys, Se-(methyl)selenocysteine; SeMet, selenomethionine. Standard deviations in brackets. Different letters within each column indicate statistically significant differences at $p \leq 0.05$. F test: ** significant at $p < 0.01$, * significant at $p < 0.05$, n.s. not significant.

3.3. Mineral Concentrations

The mineral contents were generally significantly affected by salinity, while the effect of Se was only significant for K and nitrates (Table 3). Specifically, increasing the salinity increased Na and chlorides, while the highest nitrate, phosphate, and sulphate contents were recorded with $NaCl_{25}$ and then decreased with $NaCl_{50}$, regardless of the Se treatments. Endogenous Se increased the K content and reduced the Na/K ratio.

Table 3. Mineral concentrations in shoots of maize sprouts obtained at 0, 25, or 50 mM NaCl (i.e., $NaCl_0$, $NaCl_{25}$, and $NaCl_{50}$, respectively) from grains produced without or with Se fertilization (i.e., Se_{NO} and Se_{YES}, respectively).

Treatments	Mineral Concentrations (mg kg^{-1} DM)					
	K	Na	Chlorides	Nitrates	Phosphates	Sulphates
$Se_{NO}NaCl_0$	853.0 (32.9)	2011 (27)	37.9 (5.4)	1.86 (0.50)	91.2 (15.5)	14.5 (0.9)
$Se_{NO}NaCl_{25}$	991.7 (82.2)	9689 (1959)	64.0 (5.6)	63.9 (2.5)	210.7 (40.9)	44.2 (1.7)
$Se_{NO}NaCl_{50}$	1576.7 (153.6)	20988 (980)	114.6 (11.0)	2.9 (1.4)	117.7 (70.2)	21.9 (7.1)
$Se_{YES}NaCl_0$	1641.7 (520.9)	2104 (164)	30.4 (6.9)	1.5 (0.8)	122.9 (31.1)	19.0 (8.8)
$Se_{YES}NaCl_{25}$	1490.7 (271.6)	12176 (2972)	50.6 (28.3)	200.4 (2.4)	233.3 (4.0)	40.4 (1.4)
$Se_{YES}NaCl_{50}$	1667.0 (85.2)	18443 (1727)	95.5 (14.8)	1.4 (0.2)	36.0 (0.9)	13.6 (0.6)
F Test						
Se fertilization	**	*n.s.*	*n.s.*	**	*n.s.*	*n.s.*
NaCl treatment	*	**	**	**	**	**
Se x NaCl	*n.s.*	*n.s.*	*n.s.*	**	*	*n.s.*
LSD of interaction	449.4	2963	25.7	2.8	64.2	8.4

Standard deviations in brackets. Different letters within each column indicate statistically significant differences at $p \leq 0.05$. F test: ** significant at $p < 0.01$, * significant at $p < 0.05$, n.s. not significant.

3.4. Polar and Non-Polar Metabolomics

We measured, by LC-HRMS, the levels of 51 non-polar and 144 semipolar compounds, belonging to plant primary and secondary metabolism. They included either nutritional metabolites, such as several isoprenoid classes (carotenoids, chlorophylls, tococromanols, and quinones), amino acids, ascorbic acid, phenolics precursors, flavonoids and benzoxazinoids, or antinutritional metabolites like undesirable lipids (LPCs) and some alkaloids. The choice of these metabolic classes was made by taking into consideration previous studies indicating positive [34–36] and negative [37,38] effects on human health.

The entire non-polar and semipolar databases are reported in Tables S1 and S2, respectively. Principal component analysis (PCA) was first exploited to evaluate global alterations of both metabolic fractions: overall, in both non-polar and semipolar metabolomes, component 1 (explaining 99.31% and 97.86% of the total variance, respectively) allowed a clear separation of the two tissues (root and shoot), in comparison to the NaCl/Se treatments (Figure 1a,b). However, component 2 of semipolar compounds, explaining 1.68% of the variance, was able to discriminate $Se_{YES}NaCl_{50}$ shoots compared to the other samples (Figure 1b). At a metabolite level, component 1 of non-polar and semipolar fractions, explaining 98.60% (1.05) and 99.13% (0.65) of the total variance, respectively, highlighted a major contribution of pheophytin a and α-tocopherolquinone, followed by hydroxy-phytofluene, lutein, β-tocotrienol/γ-tocotrienol, chlorophyll a and b, and a series of chlorophyll-related metabolites (Figure 1c), as well as four amino acids (phenylalanine, proline, leucine, and tryptophan), two flavonoids (maysin and naringenin-hexoside), and five benzoxazinoids (DIMBOA, DIM2BOA, MBOA, MBOA-glucoside, and M2BOA) (Figure 1d) in the discrimination of tissues (root and shoot) and the treatments (Se and NaCl) under study.

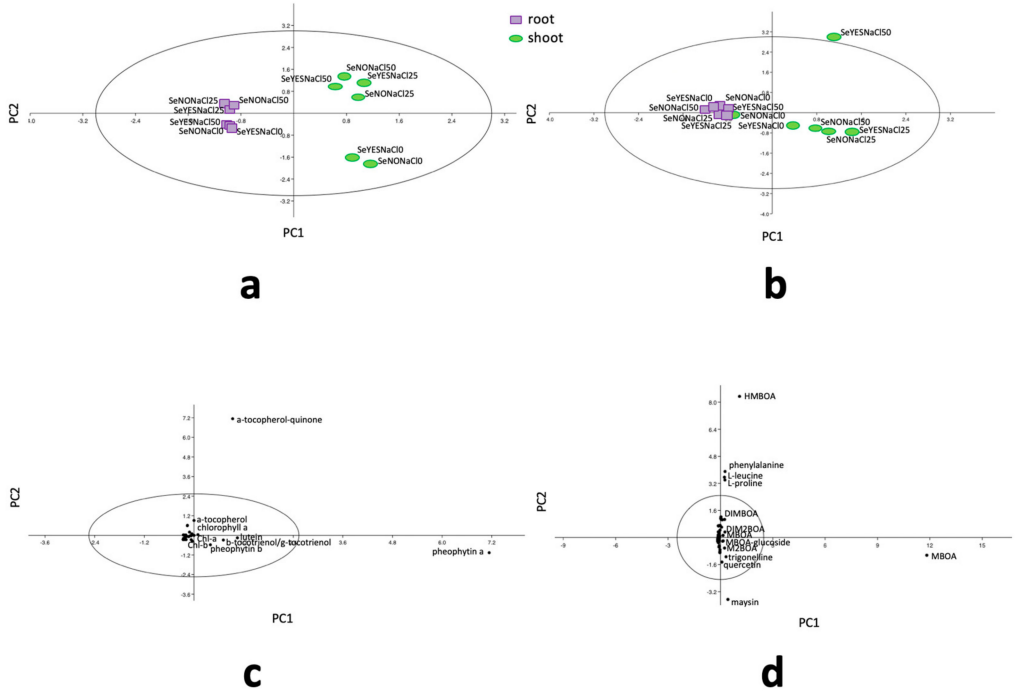

Figure 1. Principal component analysis of non-polar and semipolar metabolomes in terms of treatment (**a, b**) and metabolite (**c, d**) levels in maize sprouts obtained at 0, 25, or 50 mM NaCl (i.e., $NaCl_0$, $NaCl_{25}$, and $NaCl_{50}$, respectively) from grains produced without or with Se fertilization (i.e., Se_{NO} and Se_{YES}, respectively) in comparison to the control $Se_{NO}NaCl_0$.

We also took advantage of heatmap visualization coupled with hierarchical clustering (HCL) analysis, in order to investigate the relationships of non-polar and semipolar metabolomics fractions within the treatments under study (Figure 2, Figures S1 and S2). At a root and non-polar metabolome level, HCL highlighted a clear separation of the samples in two groups: $Se_{NO}NaCl_0$ and $Se_{YES}NaCl_0$ on the left side; $Se_{NO}NaCl_{25}$, $Se_{NO}NaCl_{50}$, and $Se_{YES}NaCl_{50}$ in the central region; and $Se_{YES}NaCl_{25}$ on the right extreme of the HCL, with no clustering for any of the other treatments (Figure 2a). Similarly, at a shoot non-polar metabolome level, the treatments clustered in the same way, with the exception of $Se_{YES}NaCl_{50}$, located at the right end of the HCL (Figure 2b).

On the contrary, semipolar metabolites clustered in the control and treatments in three groups, from the most similar to the most different: $Se_{NO}NaCl_0$ and $Se_{YES}NaCl_0$; $Se_{NO}NaCl_{25}$, $Se_{YES}NaCl_{25}$, and $Se_{YES}NaCl_{50}$; and $Se_{NO}NaCl_{50}$, located at the extreme right of the HCL (Figure 3a). This structure was partially maintained in the semipolar metabolome of the shoot samples, with the exception of $Se_{YES}NaCl_{50}$, which was the treatment showing the most divergent profile compared to the other samples, followed by $Se_{NO}NaCl_{50}$ (Figure 3b).

Statistical analysis (Student t-test) was performed to identify metabolites showing a differential accumulation following Se and/or NaCl treatments compared with the $Se_{NO}NaCl_0$ control (Tables S1 and S2); a summary of the total number of Up- and Dw-accumulated metabolites for each chemical class and treatment is reported in Table 4. The non-polar metabolome of root samples was characterized by a limited, but positive, effect in several antioxidant and vitamin A- and E-related compounds (the carotenoid and tocochromanol groups) in most of the treatments, while quinones and undesirable lipids (LPCs) exhibited an opposite trend (Table 4). A more dramatic effect was observed in treated shoots: early carotenoids (phytoene and phytofluene) and zeaxanthin decreased in the presence of Se or NaCl, while late carotenoids (violaxanthin, neoxanthin, and luteoxanthin) and chlorophyll a and b increased in most of the tested conditions, with the exception of $Se_{YES}NaCl_0$, or at low NaCl concentrations in the presence or absence of Se (such as α- and β-carotene, β-cryptoxanthin, lutein,

and plastoquinone). Finally, α-tocopherol increased in $Se_{YES}NaCl_0$, $Se_{NO}NaCl_{25}$, and $Se_{YES}NaCl_0$, and a group of lipids (mostly LPCs) was reduced in $Se_{YES}NaCl_{50}$ (Table 4 and Table S1).

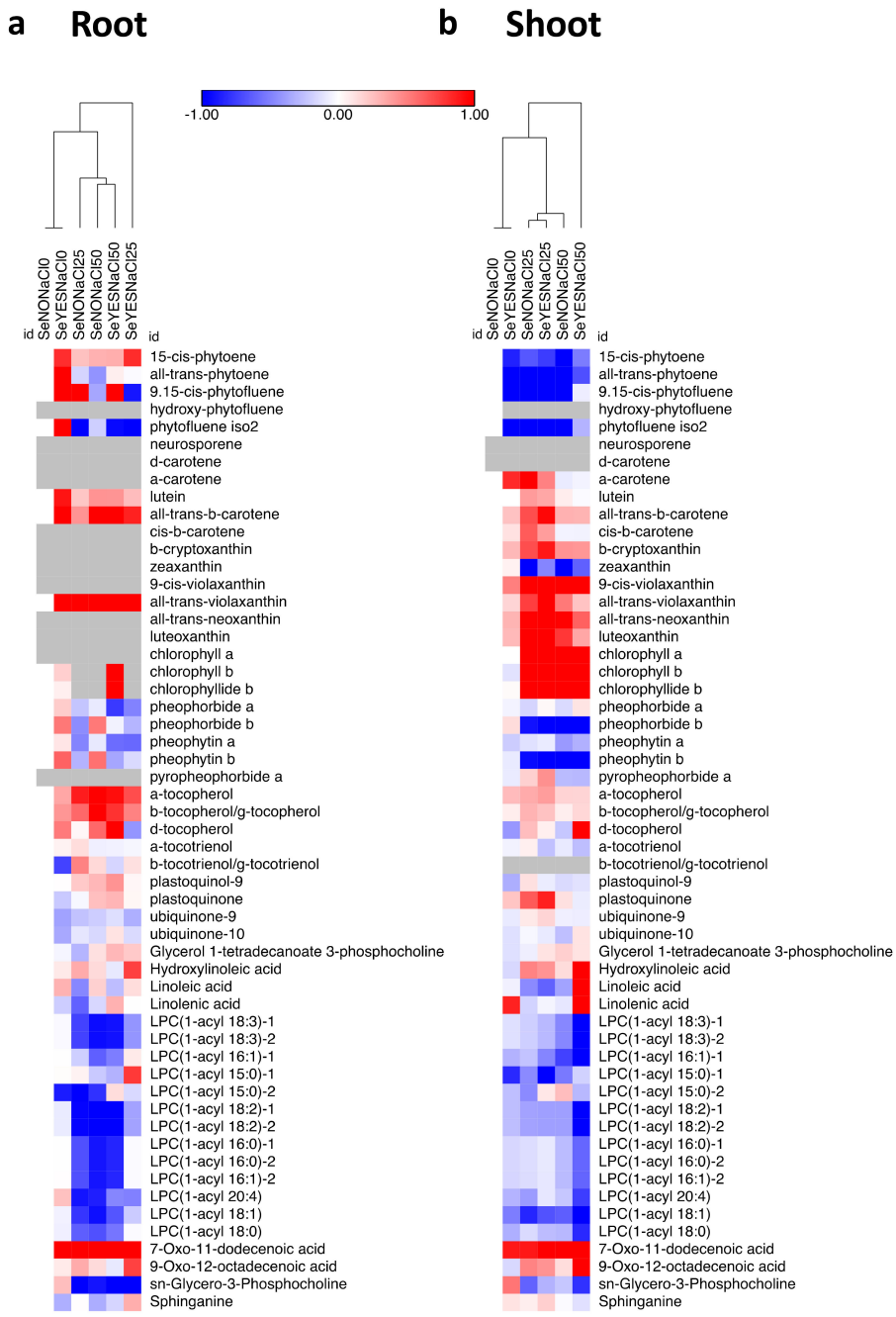

Figure 2. Heatmap visualization coupled with hierarchical clustering (HCL) analysis of non-polar metabolomes in the root (**a**) and shoot (**b**). Data are reported as log2 fold with respect to the $Se_{NO}NaCl_0$ condition. Only significant metabolites in at least one comparison (treatment vs. $Se_{NO}NaCl_0$), according to a Student t-test (with p-value < 0.05), are reported. Red and shades of red color indicate over-represented metabolites, whilst blue and shades of blue color indicate down-represented metabolites. Data characterized by no alteration in metabolite accumulation are shown in white. Grey boxes indicate metabolites that were not detected.

Figure 3. Heatmap visualization coupled with hierarchical clustering (HCL) analysis of semipolar metabolomes in the root (**a**) and shoot (**b**). Data are reported as log2 fold with respect to the $Se_{NO}NaCl_0$ condition. Only significant metabolites in at least one comparison (treatment vs. $Se_{NO}NaCl_0$), according to a Student t-test (with p-value < 0.05), are reported. Red and shades of red color indicate over-represented metabolites, whilst blue and shades of blue color indicate down-represented metabolites. Data characterized by no alteration in metabolite accumulation are shown in white. Grey boxes indicate metabolites that were not detected.

Table 4. Number of differentially accumulated metabolites in non-polar and semipolar metabolomes of maize sprouts obtained at 0, 25, or 50 mM NaCl (i.e., $NaCl_0$, $NaCl_{25}$, and $NaCl_{50}$, respectively) from grains produced without or with Se fertilization (i.e., Se_{NO} and Se_{YES}, respectively) in comparison to the control $Se_{NO}NaCl_0$.

Class of Metabolites	Roots											Shoots										
	$Se_{YES}NaCl_0$		$Se_{NO}NaCl_{25}$		$Se_{YES}NaCl_{25}$		$Se_{NO}NaCl_{50}$		$Se_{YES}NaCl_{50}$			$Se_{YES}NaCl_0$		$Se_{NO}NaCl_{25}$		$Se_{YES}NaCl_{25}$		$Se_{NO}NaCl_{50}$		$Se_{YES}NaCl_{50}$		
	UP-	DW-	UP-	DW-	UP-	DW-	UP-	DW-	UP-	DW-	TOT	UP-	DW-	UP-	DW-	UP-	DW-	UP-	DW-	UP-	DW-	TOT
Non polar																						
carotenoids	4	-	3	-	3	-	2	-	4	-	16	1	3	9	6	9	5	4	4	3	3	47
chlorophylls	2	-	-	2	-	2	2	2	1	1	12	-	-	3	2	4	2	3	3	3	3	23
tococromanols	2	1	2	-	2	-	3	-	2	-	12	1	2	2	1	1	2	-	1	1	2	13
quinones	-	2	-	-	-	-	-	-	-	1	3	-	-	1	-	1	-	-	-	-	-	2
lipids	1	1	3	12	3	1	3	11	1	7	43	3	2	3	3	4	3	1	4	2	8	33
Tot	9	4	8	14	8	3	10	13	8	9		5	7	18	12	19	12	8	12	9	16	
Semipolar																						
acids	1	1	2	2	1	1	2	2	1	2	15	-	1	-	1	2	-	-	-	-	-	4
alkaloids	-	3	-	4	-	1	-	1	-	1	10	-	-	-	-	1	-	1	-	1	-	3
amides	-	1	-	2	-	-	-	2	-	2	7	-	1	-	3	1	2	-	3	-	2	12
amino acids and derivatives	1	8	2	20	2	15	5	13	4	16	86	-	2	3	5	6	4	4	7	1	7	39
anthocyanins	-	-	-	-	-	-	-	1	-	-	1	1	-	-	-	1	-	1	-	1	-	4
benzoxazinoids	2	3	-	7	2	2	4	7	3	3	33	11	3	6	1	13	2	10	1	10	-	57
flavonoids	11	2	14	8	5	1	7	16	3	-	67	16	-	14	-	27	-	19	-	10	-	86
nucleosides and nucleotides	-	1	3	3	3	1	1	3	2	2	19	2	2	-	1	4	-	3	1	2	2	17
others	-	-	-	-	-	-	1	-	-	-	1	-	-	1	-	1	-	-	-	-	1	3
other phenylpropanoids	1	-	-	-	-	-	-	2	1	-	4	-	-	-	-	1	-	-	-	-	-	1
peptides	-	-	1	1	1	1	1	1	2	1	9	-	-	-	-	1	-	2	-	1	-	4
phenolics precursors	-	2	2	2	4	1	2	1	3	1	18	4	-	5	-	4	-	5	1	5	1	25
sugars and phosphates	1	-	-	2	-	-	-	2	-	2	7	-	-	-	-	1	-	-	-	-	-	1
vitamins	1	-	1	2	-	2	1	3	-	3	13	1	1	-	1	3	-	4		1	1	12
Tot	18	21	25	53	18	25	24	54	19	33		35	10	29	12	66	8	49	13	32	14	

The analysis of the semipolar metabolome revealed a dramatic reduction in several amino acids and their derivatives, either at a root and shoot level, with the exception of $Se_{YES}NaCl_{25}$, for which six compounds (N-Acetyl-L-glutamic acid, Caffeoyltryptamine, L-Carnitine, Coumaroylserotonin, 5-Hydroxyindoleacetic acid, and N-(3-Indolylacetyl)-L-alanine) and four compounds (asparagine, Feruloyltryptamine, L-Isoleucine, and Tryptamine) increased and decreased, respectively (Table 4 and Table S2). Interestingly, antinutritional metabolites largely changed in the treated samples: several alkaloids and amides were reduced in both roots and shoots, independent of the treatment. On the contrary, a non-univocal behaviour was observed for several pro-nutritional metabolites: for instance, the benzoxazinoid class, for which lower and higher levels were found in most of the Se-/NaCl-treated roots and shoots, respectively (Table 4 and Table S2). Similarly, different subclasses of phenylpropanoids (phenolic precursors and flavonoids) displayed the maximum number of positive changes: $Se_{YES}NaCl_0$ and $Se_{NO}NaCl_{25}$ were the treatments at a root level with the highest number of Up-differentially accumulated metabolites (11 and 16, respectively); conversely, $Se_{YES}NaCl_{25}$ and $Se_{NO}NaCl_{50}$ were the conditions characterized by the largest increase in 27 and 19 flavonoids, respectively (Table 4 and Table S2). In terms of the shoots, caffeic, coumaric, ferulic, hydroxyferulic, and sinapic acids showed an enhanced content compared to the control in most of the treatments (Table 4 and Table S2).

4. Discussion

The data in Table 1 indicate that neither endogenous Se nor salinity treatments substantially affected germination and sprout growth, except for a clear decrease in root length caused by salinity and a less clear "Se x NaCl" interaction for the shoot length. The effect of endogenous Se on growth parameters has never been studied in maize sprouts, so there is no literature to support the lack of effect we observed between Se_{YES} and Se_{NO}. Puccinelli et al. (2019) [24] did not find significant differences in biomass production in basil microgreens obtained from seeds with or without endogenous Se. The sensitivity of maize seedlings to salt stress has been widely studied and has been reported to vary among genotypes [39–41]. Specific comparisons with previous studies are difficult due to the different age of seedlings and salt concentration used. Our results on the germination performance were in line with those of Carpýcý et al. (2009) [39] and Sali et al. (2015) [42], who generally did not find significant differences between maize seedlings grown at 0 and 50 mM NaCl. The depressive effect of NaCl on both root and shoot growth parameters, which is well-known in the literature for many species, including maize [39–41], appeared slighter and sometimes contradictory at the low concentrations ($\leq$ 50 mM) we imposed. Some osmotic adjustment probably occurred with the moderate salinity we imposed, which led to the maintenance of high cellular turgor, cellular expansion, and growth. Osmotic adjustment was also observed by Yuan et al. (2010) [43] and Guo et al. (2014) [44] in *Brassica* sprouts grown at a low salt concentration. However, the differences we observed in both fresh and dry weights were not significant (Table 1).

The much higher TSeC measured in Se_{YES} compared to Se_{NO} treatments was expected, since we used the grains obtained by D'Amato et al. (2019) [25], having a TSeC of 1.35 mg kg^{-1} for Se_{YES} and 0.19 mg kg^{-1} for Se_{NO}. The observed TSeC in Se_{YES} treatments should not involve any risk of exceeding the recommended daily threshold for Se intake in human nutrition (50-55 µg Se) (EFSA, 2014) [45], because the daily consumption of sprouts is expected to be limited to a few dozens of grams of fresh matter. Considering a daily consumption of 50 g of fresh sprouts, even the treatments with the highest TSeC (i.e., Se_{YES} treatments) would involve a maximum intake of less than 10 µg of Se, i.e., less than 20% of the recommended daily allowance. It is worth noting that, as reported in D'Amato et al. (2019) [25], the grains we used contained only organic Se forms, whereas in the sprouts we obtained, inorganic species were the greatest part of TSeC. We might explain this finding by assuming a release of inorganic forms due to the hydrolysis of Se proteins (SeCys, SeMet, etc.) during the germination process. The observed increase of inorganic Se forms in sprouts appears to be novel evidence. In fact, most of

the Se biofortification strategies reported in the literature have dealt with exogenous applications of inorganic Se forms, which are bio-converted into organic Se forms by plants [17,46].

The effect of salinity on the Se content of sprouts was only significant in a few cases and it can hardly be explained due to a lack of similar experiments in the literature. We can only mention that the increase of organic Se species is thought to be a kind of detoxification mechanism that allows plants to adapt to stressful conditions [47]. The obvious increase of Na and chloride concentrations with salinity was also reported by Hassini et al. (2017 [48]. This increase should not alarm readers, given the small number of sprouts that are expected to be consumed daily, because it would account for a very small part of the daily Na intake in normal diets. The observed increase of nitrates, phosphates, and sulphates in $NaCl_{25}$ treatments, but not in $NaCl_{50}$ treatments, was quite surprising. This finding is not easy to explain. As reported by Roberts (2006) [49], the increase of anions under stress conditions may be due to the increased synthesis of proteins, especially those belonging to the anion channel in the plasma membrane of cells, and which are permeable to a range of physiological anions. All of these minerals are beneficial to human nutrition [50,51] and their increase may be regarded as positive. Only nitrates may imply negative effects, but their level, even in the $Se_{YES}NaCl_{25}$, was much lower than the threshold for leafy vegetables like lettuce [52], and, different from these vegetables, sprouts are intended to be eaten in very low amounts. We can exclude the idea that the high nitrate content of $Se_{YES}NaCl_{25}$ might be due to casual sprout contamination. In fact, both replicates showed similar values and it is worth remembering that each replicate was obtained from regrouping two separate trays. Moreover, $Se_{NO}NaCl_{25}$ also showed a high nitrate content, which would suggest an actual effect of NaCl at the low concentration of 25 mM.

The metabolic profiling of non-polar and semipolar metabolomics fractions was exploited to unravel changes in primary and secondary compounds, particularly those with pro-nutritional and anti-nutritional activities. Overall, the data evidenced a different behavior for roots and shoots for a series of metabolites of interest: for instance, shoots displayed a higher number of differentially accumulated metabolites in the carotenoid (including α- and β-carotene, the vitamin A precursors) and phenylpropanoid (either at phenolic precursors and flavonoids/anthocyanins). This divergent behavior is not surprising, since several reports have proved the existence of organ-specific mechanisms controlling metabolite synthesis and accumulation [53,54], as reviewed in Wang et al. (2019) [55]. The detailed investigation of metabolic alterations for each tissue/treatment allowed us to identify the best conditions for improving the nutritional contents of roots and shoots. At a root level, all of the treatments were able to increase the number of health-related non-polar compounds (particularly $Se_{NO}NaCl_{50}$ and $Se_{YES}NaCl_{50}$) (Table 5), while a more variegated effect was observed in the case of the semipolar metabolites, although $Se_{YES}NaCl_{0}$, $Se_{YES}NaCl_{25}$, and $Se_{YES}NaCl_{50}$ were characterized by the best ratios between up- and dw-accumulated compounds with pro-nutritional and anti-nutritional activities. The $NaCl_{25}$, regardless of the content of Se, was also the treatment allowing the highest number of positive non-polar metabolites, while pro-nutritional semipolar molecules were especially accumulated in $Se_{YES}NaCl_{25}$.

Table 5. The total number of differentially accumulated non-polar and semipolar metabolites with pro-nutritional and anti-nutritional properties in maize sprouts obtained at 0, 25, or 50 mM NaCl (i.e., $NaCl_0$, $NaCl_{25}$, and $NaCl_{50}$, respectively) from grains produced without or with Se fertilization (i.e., Se_{NO} and Se_{YES}, respectively) in comparison to the control $Se_{NO}NaCl0$. For each group and tissue/treatment, the ratio between up- and dw-accumulated compounds is reported.

Class of Metabolites	Roots										Shoots									
	$Se_{YES}NaCl_0$		$Se_{NO}NaCl_{25}$		$Se_{YES}NaCl_{25}$		$Se_{NO}NaCl_{50}$		$Se_{YES}NaCl_{50}$		$Se_{YES}NaCl_0$		$Se_{NO}NaCl_{25}$		$Se_{YES}NaCl_{25}$		$Se_{NO}NaCl_{50}$		$Se_{YES}NaCl_{50}$	
	UP-	**DW-**	**UP-**	**DW-**	**UP-**	**DW-**	**UP-**	**DW-**	**UP-**	**DW-**	**UP-**	**DW-**	**UP-**	**DW-**	**UP-**	**DW-**	**UP-**	**DW-**	**UP-**	**DW-**
non polar pro-nutritional	8	3	5	2	5	2	7	2	7	2	2	5	15	9	15	9	7	8	7	8
up-/dw- ratio	*2.67*		*2.50*		*2.50*		*3.50*		*3.50*		*0.40*		*1.67*		*1.67*		*0.88*		*0.88*	
non polar anti-nutritional	0	1	0	8	0	0	0	9	0	6	0	2	0	1	0	3	0	3	0	7
up-/dw- ratio	-		-		-		-		-		-		-		-		-		-	
semipolar pro-nutritional	18	17	25	47	18	24	23	51	19	30	35	9	28	9	63	6	48	10	31	11
up-/dw- ratio	*1.06*		*0.53*		*0.75*		*0.45*		*0.63*		*3.89*		*3.11*		*10.50*		*4.80*		*2.82*	
semipolar anti-nutritional	0	4	-	6	0	1	0	3	0	3	0	1	0	3	2	2	1	3	1	2
up-/dw- ratio	-		-		-		-		-		-		-		*1.00*		*0.33*		*0.50*	

Most of the alterations were positive, making the treated shoots an interesting source of biofortified antioxidants. This aspect affected pro-nutritional compounds produced through different pathways and cell compartments—isoprenoids synthesized by GGPP, phenylpropanoids from phenylalanine, and benzoxazinoids from indole—suggesting a general NaCl/Se capacity to promote deep changes in the metabolome of maize. Interestingly, most of these compounds have also been reported to take part in the plant tolerance to abiotic and biotic stress responses [56–59]; benzoxazinoids, in particular, are known to act as natural pesticides, with a strong activity against plant pests and pathogens [60]. Therefore, from this perspective, the observed metabolic alterations might also result in an improved agronomical performance of the treated seedlings. In addition, molecules with anti-nutritional characteristics, such as amides and LPCs, also decreased. Similarly, treated roots were enriched, although to a lesser extent compared to the shoots, in carotenoid and flavonoids, as well in the group of tocochromanols (mainly α- and β-/γ-tocopherols), thus increasing their vitamin E status. In addition, alkaloids, which are undesirable from a nutritional point of view, decreased in most of the treatments. Unfortunately, root samples were also characterized by a reduction in compounds of nutritional interest, such as amino acids and sugars, which are aspects to consider from the perspective of their commercial use. Although distinct biochemical phenotypes can occur according to the plant species, our data are in agreement with previous studies [26,61,62]. Therefore, the positive effect on antioxidant contents by selenium and salt can be explained by their capacity to induce a stress condition in the treated tissues, which react with the production of secondary metabolites to compensate for the stress status. Notably, an opposite biochemical phenotype has been observed in other crops, such as broccoli [63], indicating the presence of complex regulation between Se fertilization and phenolics accumulation. Interestingly, transcriptomics studies of *Brassicaceae* (*Arabidopsis* and *Stanleya pinnata*) have shown that Se can stimulate and perturb cell metabolism by up-regulating genes involved in S uptake and assimilation, as well as phenyalanine ammonia-lyase (PAL) (and other phenolic-related genes), and many antioxidant-related genes [64–66]. Although additional findings are needed, the molecular mechanism underlying these changes would imply the involvement of jasmonic acid- and ethylene-synthesis/response transcripts. We cannot exclude the occurrence of a similar phenomenon in maize, and this might represent a subject for future research.

Considering all of the data, it appears that a moderate concentration of NaCl (25mM), especially in grains with a high endogenous Se content, allowed the primary and secondary metabolism of maize sprouts to be redirected towards an improved accumulation of compounds with beneficial properties, particularly at a shoot level.

5. Conclusions

The results indicate that both grain endogenous selenium and salinity in the germination substrate affected the sprout yield and composition, with some interaction between treatments. Grain endogenous selenium (i.e., 1.35 mg kg^{-1} of grains, as obtained by fertilizing the maize field crop with 200 g ha^{-1} of Se as sodium selenite) mainly affected the sprout Se content, while salinity had the greatest effect on sprout secondary metabolites. A synergic effect was observed between treatments. Overall, using maize grains rich in endogenous selenium and imposing mild NaCl stress (i.e., 25 mM NaCl) during germination allowed a good sprout yield, increased the content of selenocystine, and improved the balance between pro-nutritional and anti-nutritional compounds, especially boosting the synthesis of pro-nutritional semipolar metabolites with antioxidant properties. Considering a consumption of 50 g of fresh sprouts in a day, the total Se ingestion with these kinds of Se-enriched sprouts would be less than 10 μg, corresponding to less than 20% of the recommended daily allowance. However, it has to be noted that most of the Se in sprouts was in an inorganic form, despite the grains only containing organic Se.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/5/735/s1/: Figure S1: Heatmap visualization of non-polar metabolomes. Data are reported as log2 fold with respect to $Se_{NO}NaCl_0$ treatment; Figure S2: Heatmap visualization of semipolar metabolomes. Data are reported as log2 fold

with respect to $Se_{NO}NaCl_0$ treatment; Table S1: LC-HRMS analysis of the non-polar metabolome in maize sprouts obtained at 0, 25, or 50 mM NaCl (i.e., $NaCl_0$, $NaCl_{25}$, and $NaCl_{50}$, respectively) from grains produced without or with Se fertilization (i.e., Se_{NO} and Se_{YES}, respectively). Data are represented as the mean (standard deviation) of two independent biological replicates, with three separate measurements per replicate. Asterisks indicate significant differences according to a t-test (pValue: *<0.05; **<0.01) carried out by comparing each treatment against the control treatment $Se_{NO}NaCl_0$. Red and green boxes refer to metabolites showing a higher and lower content compared to $Se_{NO}NaCl_0$, respectively; Table S2: LC-HRMS analysis of semipolar metabolome in maize sprouts obtained at 0, 25, or 50 mM NaCl (i.e., $NaCl_0$, $NaCl_{25}$, and $NaCl_{50}$, respectively) from grains produced without or with Se fertilization (i.e., Se_{NO} and Se_{YES}, respectively). Data are represented as the mean (standard deviation) of two independent biological replicates, with three separate measurements per replicate. Asterisks indicate significant differences according to a *t*-test (*p*-Value: * <0.05; ** <0.01) carried out by comparing each treatment against the control treatment $Se_{NO}NaCl_0$. Red and green boxes refer to metabolites showing a higher and lower content compared to $Se_{NO}NaCl_0$, respectively.

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