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# Effects of Se Application on Polyamines and Carbon–Nitrogen Metabolism of Pepper Plants Suffering from Cd Toxicity

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**Abstract:** Previous studies have shown that the application of selenium (Se) can efficiently mitigate the toxic effects of cadmium (Cd) on various crops. The objective of the present work is to decipher the mechanisms responsible for the efficiency of Se against the effects of Cd in pepper plants, with respect to the carbon and nitrogen metabolism. The following were analyzed: the concentrations of anions related with this metabolism, such as nitrates, nitrites, and ammonium, the activities of different enzymes such as nitrate reductase, nitrite reductase, and glutamate synthase, polyamines in their different forms, organic acid salts, amino acids, and sugars in the leaf and root tissues of the pepper plants grown in a hydroponics system. Four different treatments were applied: plants without Cd or Se applied (−Cd/−Se); plants grown with Cd added to the nutrient solution (NS) but without Se (+Cd/−Se); plants grown with Cd in the NS, and with the foliar application of Se (+CD/+SeF); and lastly, plants grown with Cd in the NS, and with Se applied to the root (+Cd/+SeR). The metabolites and enzymes related with carbon and nitrogen metabolism were analyzed 15 days after the application. The results showed the superiority of the +Cd/+SeR treatment with respect to the +Cd/+SeF treatment, as shown by an increase in the conjugated polyamines, the decrease in glutamate and phenylalanine, and the increase of malate and chlorogenic acid. The results indicated that SeR decreased the accumulation and toxicity of Se as polyamine homeostasis improved, defense mechanisms such as the phenylpropanoid increased, and the entry of Cd into the plants was blocked.

**Keywords:** cadmium toxicity; selenium; nitrogen metabolism; polyamines; metabolites



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## 1. Introduction

The concern for environmental contamination due to cadmium (Cd) has increased in the last few years, due to the toxicity of this metal to crops at low concentrations [1]. For plants defined as "not hyperaccumulators", the Cd toxicity threshold in the irrigation water has been set at 5–10 µM Cd and 5.0 mg $kg^{-1}$ in the soil. As for the leaf concentration, a toxicity threshold of 3–30 mg $kg^{-1}$ dry weight has been established [2]. The toxic effects of Cd in plants has been widely studied [3], and research is presently being conducted on how to reduce or mitigate the negative effects of this metal on plants grown in agricultural areas in which the irrigation water has a high content of Cd, due to the industrial sources of the water. Among these strategies, several studies have reported that Se application can decrease Cd uptake and/or alleviate the toxic effects of Cd on vegetables [4–6], such as in the cultivation of pepper [7]. In a previous study with these plants [8], it was observed that

Se applied to the root had beneficial effects due to the decrease in the foliar concentration of Cd, and the increase in the concentration of Se in every plant part, thus reducing the toxicity of Cd in the leaves, and maintaining the vegetative growth of the plants.

Plants have developed many strategies to avoid heavy metal toxicity in their tissues. These tolerance mechanisms are multi-factorial, and include the expression of specific groups of genes, the synthesis of proteins related to stress, and the accumulation of compatible solutes (such as sugars, amino acids, organic acid salts, betaines, and polyamines (PAs)), all of which are involved in the protection of cellular structures, the maintenance of ionic homeostasis, and osmotic adjustment [9–16]. Different studies have confirmed that the tolerance responses to Cd stress involve the synthesis of amino acids such as proline, glycine betaine, glutamic acid, gamma-aminobutyric acid (GABA), asparagine, and glutamine [17,18]. The role played by these amino acids on the tolerance to Cd excess has also been confirmed by the application of proline and glycine in tobacco [19] and wheat [20], GABA in corn [21], and glutamic acid in lentils [22].

In general, tolerance to heavy metals is determined by the reduction of the transport of the metal to the inside of the cell and/or a greater capacity to sequester these metals. The root is one of the main defense barriers, through the immobilization of Cd by the pectins found in the cell wall. The extracellular carbohydrates (mucilage and callose) of the root can also intervene in the immobilization of the metal [23,24]. The accumulation of the metal in the trichomes found on the surface of the leaf is also a mechanism of immobilization and cell defense [25,26]. Lastly, another mechanism consists of the reduction of the transport or increase in the extrusion of Cd by cation transporters found in the plasma membrane [27,28].

Once inside the cell, Cd and other heavy metals are sequestered by organic acid salts, amino acids, phytochelatins, and metallothioneins, and are afterwards compartmentalized in the vacuole to prevent toxicity. Phytochelatins are the main defense mechanisms against heavy metals. They are synthesized starting with glutathione, and their synthesis is induced by the presence of heavy metals [29,30]. Presently, the function of polyamines (Pas) as a response to biotic and abiotic stress in plants has become a new and interesting field in the regulation and involvement of these compounds. It has been suggested that their properties of free-radical capture, stabilization of membranes, protection of nucleic acids, and regulation of gene expression, are some of the mechanisms through which they can confer tolerance to these stresses [30,31]. Polyamines are aliphatic nitrogenous compounds with two or more amino groups found in every living cell. In plants, polyamines are mainly found in their free form and/or covalently conjugated with small size molecules (phenolic compounds), with macromolecules (nucleic acids, proteins, etc.), and with lignin through hydrogen and ionic bonds. Each form is associated with a multitude of regulation and cell signaling processes, which encompass a great range of biological functions [11,30].

Cd stress also affects nitrogen metabolism, and recent studies have shown that it increases the concentration of nitrates and decreases the quantity of ammonium in leaves, provoking alterations in N metabolism and their relationship with C metabolism [32]. Other studies have stated that Cd phytotoxicity increases the content of organic acid salts in plant tissues, which can be secreted by the root and bound to Cd, forming chelating-organometallic compounds that decrease the bioavailability of free toxic Cd [33]. This increase in organic acid salts can mitigate the phytotoxicity of Cd in plants and improve their tolerance, as they induce and increase the activity and respiration of the roots, which improves the absorption of nutrients and the antioxidant response [34]. Likewise, it has been observed that plants grown in soils with a high content of Cd increase their contents of sugars (glucose, sucrose, and fructose), to maintain the osmotic equilibrium in shoot and root cells [35].

The objective of the present work is to discover how Cd toxicity affects nitrogen and carbon metabolism of pepper plants, and to identify if, among the mechanisms involved in the tolerance of pepper plants to Cd when Se is applied, they are related with the synthesis and accumulation of polyamines and amino acids. This work is part of another publication

by Pérez-Millán et al. (2021) [8], which demonstrated that the application of Se reduces the concentration and toxicity of Cd in pepper plants.

## 2. Materials and Methods

### *2.1. Growing Conditions and Plant Material*

For this study, pepper (*Capsicum annuum* L.) seeds from the commercial variety 'Cristal' were germinated in sterile vermiculite trays inside a controlled-environment growth chamber. The information related to the germination and crop management have previously been described in Pérez-Millán et al. (2021) [8].

### *2.2. Cd and Se Treatments Applied to the Plants*

The pepper plants were grown in two different Cd conditions: (i) control treatment in which the nutrient solution did not contain cadmium (−Cd/−Se); and (ii) under an excess of cadmium (+Cd), in which the nutrient solution contained a final concentration of 3 mg $L^{-1}$ of cadmium, applied as $CdSO_4 \cdot 8H_2O$ (considered severe stress; [36]). This group of plants was divided into three sub-groups: plants grown without the application of Se (+Cd/−Se), plants treated with a foliar application of Se (+Cd/+SeF), and plants to which Se was applied to the root (+Cd/+SeR). The Se was applied as $Na_2SeO_4$ to a final concentration of 10 µM in both the foliar and root treatments. The details of the experimental design can be found in Pérez-Millán et al. (2021) [8]. The sampling began 15 days from the start of the Cd treatment for the corresponding analytical studies.

### *2.3. Analytical Parameters Measured during the Assay*

#### 2.3.1. Nitrogen Metabolism in Leaves and Root

For the quantification of $NO_3^-$, 25 mg of the dry and ground tissue was used to which 5 mL of ultrapure water were added. Afterwards, the samples were agitated for 30 min in the dark. Then, the contents were filtered through a 45 µm filter. Two-hundred microliters of the filtered sample were taken to measure the concentration with a $NO_3^-$ 11 LAQUAtwin nitrate meter (HORIBA, Irvine, CA, USA). The concentration of $NO_3^-$ was expressed as g of $NO_3^-$ 100 $g^{-1}$. The measurement of $NO_2^-$ was performed by following the protocol described by Hageman and Hucklesby (1971) [37], which quantified the formation of a purple-red color due to the diazotization-coupling reaction between sulfanilamide and N-(1-Naphthyl)ethylenediamine dihydrochloride (NED dihydrochloride) at pH between 2.0 and 2.5. In parallel, a calibration curve using different concentrations was made, which ranged from 0 to 17 mg $L^{-1}$ of potassium nitrite ($KNO_2$). On its part, the measurement of $NH_4^+$ was performed according to the process described by Kempers (1974) [38]. For this, 205 mg of dried and ground leaf material was used to which 5 mL of ultrapure water were added. Afterwards, the sample was agitated in the dark for 30 min. Then, the mixture was filtered through a 45 µm syringe filter. One hundred µL of the sample were mixed with 100 µL of reagents 1 and 2 coming from part of the KIT Ammonium Test (EMD Millipore Corporation, Germany). The samples were kept in the dark at 37 °C for 60 min. After this, the absorbance was read at 640 nm. At the same time, a calibration curve was made with ammonium chloride ($NH_4Cl$) at concentrations ranging from 0 to 32 mg $L^{-1}$. The $NH_4^+$ concentration in the leaves was expressed as µg of $NH_4^+$ $g^{-1}$ DW. In addition, leaves and roots were frozen in liquid nitrogen for the study of enzymatic activities of nitrate reductase (NR; [39]), nitrite reductase (NiR; [40]), glutamate synthase GOGAT-NADH, EC 1.4.1.14; [41]) and glutamate dehydrogenase (GDH, EC 1.4.1.2; [42]).

#### 2.3.2. Metabolic Analysis of Leaf and Root Tissue

The frozen leaf and root samples were utilized for a "non-targeted" metabolic study with the H-NMR technique in lyophilized leaf tissue, as described in Alfosea-Simón et al., (2020) [43]. The following compounds, which are involved in the primary and secondary metabolism of plants, were measured: (1) amino acids: 4-aminobutyrate (AB = GABA), alanine (Ala), asparagine (Asn), aspartate (Asp), glutamate (Glu), glutamine (Gln), isoleucine

(Iso), proline (Pro), tyrosine (Tyr), phenylalanine (Phe), and valine (Val); (2) organic acid salts: -citrate (Cit), formate (For), fumarate (Fum), malate (Mal), and chlorogenate (CGA); and (3) sugars: -fructose (Fru), glucose (Glu), and sucrose (Suc).

#### 2.3.3. Determination of Free, Soluble-Conjugated, and Insoluble-Bound Polyamines

Polyamines were measured with the procedure described in Flores and Galston (1982) [44] through thin layer chromatography (TLC). The leaf material (frozen leaves) was homogenized in 3.2 mL of 5% (*w*/*v*) cold perchloric acid (PCA) and incubated at 4 °C for 60 min, and then 1, 6-hexanediamine was mixed with the homogenate as the internal standard. Afterwards, the homogenate was centrifuged at 12,000× *g* at 4 °C for half an hour. The supernatant was used to measure free and soluble conjugated polyamine contents, whereas the pellet was used to estimate insoluble-bound polyamines. The polyamines were measured with a ultraperformance liquid chromatography coupled with tandem mass spectrometry (UPLC–MS/MS) by using UHPLC (Shimadzu, Nexera Series; Columbia, MD, USA) and mass spectrometer (Orbitrap Exploris GC 240 mass spectrometer, Waltham, MA, USA).

### *2.4. Experimental Design and Statistical Analysis*

The statistical analysis included a one-way ANOVA, performed with the SPSS v.24 statistical package (Chicago, IL, USA). The measurements shown are the means of six repetitions (n = 6). When the variables were significant ($p < 0.05$), the treatment means were separated using Tukey's multiple range test.

## 3. Results

### *3.1. Nitrogen Metabolism Study in Leaves and Root*

The concentrations of nitrate, nitrite, and ammonium in leaves and roots of the plants were significantly affected by the treatments applied. The presence of Cd in the nutrient solutions without the added Se (+Cd/−Se) produced an increase in the concentration of nitrate and ammonium in the root and nitrite in the leaves, while in the other cases, no differences were found between −Cd/−Se and +Cd/+Se.

The behavior described previously was different in some cases with the presence of Se. Thus, the application of Se, in both leaves and roots (+Cd/+SeR; +Cd/+SeF), increased the leaf concentration of nitrate and ammonium, while the concentration of nitrite only increased in the +Cd/+SeR treatment, as compared to the −Cd/−Se treatment (Figure 1). In the root, the concentration of nitrate was similar in the +Cd/+SeR and +Cd/+SeF treatments, and significant differences were not found with respect to −Cd/−Se. The concentration of nitrite increased with the +Cd/+SeF treatment with respect to the rest of the treatments, and the concentration of ammonium increased in the plants treated with Se, independently of the manner of application, with respect to −Cd/−Se).

The results from the enzymatic activity assays (Figure 2) showed that the Cd in the nutrient solution (+Cd/−Se) decreased the activity of NR and increased the activity of NiR in the leaves, while in the root, the NiR activity was affected, decreasing it with respect to −Cd/−Se. The application of Se changed this type of response, with an increase observed in NR in the leaves from the +Cd/+SeF treatment, and an increase of GOGAT activity in plant leaves treated with +Cd/+SeF and +Cd/+SeR. In the root, a significant increase was observed in NR and NiR activities with the +Cd/+SeR treatment, and that of GOGAT in both Se treatments, as compared to the control (−Cd/−Se).

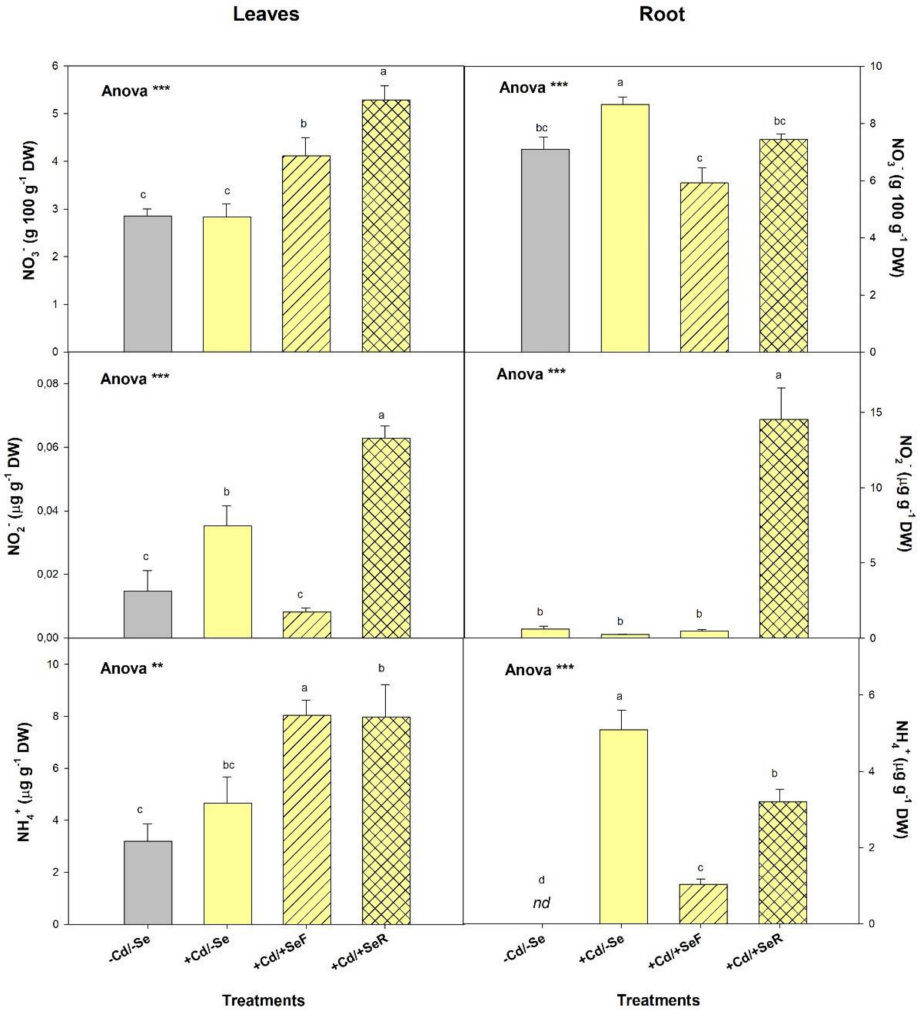


**Figure 1.** Concentration of nitrate ($NO_3^-$), nitrite ($NO_2^-$) and ammonium ($NH_4^+$) in leaves and roots from the pepper plant var. 'Cristal' grown in a hydroponic system under four different treatments which combined the application of Cd and Se (−Cd/−Se, +Cd/−Se; +Cd/+SeF and +Cd/+SeR). The results come from samples harvested two weeks after the start of the treatments. In the ANOVA: ** and *** indicate significant differences at $p < 0.01$ and 0.001, respectively. Lower case letters indicate significant differences between treatments at $p < 0.05$ established by Tukey's test. The bars indicate the SE of the mean ($n = 6$).

### *3.2. Free, Soluble-Conjugated, and Insoluble-Bound Polyamines in Leaves*

In the polyamine study, the following were found in the pepper leaves: spermine (Spm), spermidine (Spd), putrescine (Put), and cadaverine (Cad) in their three forms; free, soluble-conjugated, and insoluble-bound. In the Cd toxicity treatment without Se, +Cd/−Se, an increase of 56%, 18%, 26%, and 38% was observed in Spm, Spd, Put, and Cad, respectively, with respect to −Cd/−Se (Figure 3). The application of Se, either foliarly or through the root, also increased the concentrations of each of these polyamines, but this increase was greater than for the +Cd/−Se treatment, with increases of 309%, 126%, 130%, and 108% observed for the +Cd/+SeF treatment, and 204%, 136%, 85%, and 57% for the +Cd/+SeR treatment for Spm, Spd, Put, and Cad, respectively, relative to −Cd/−Se.

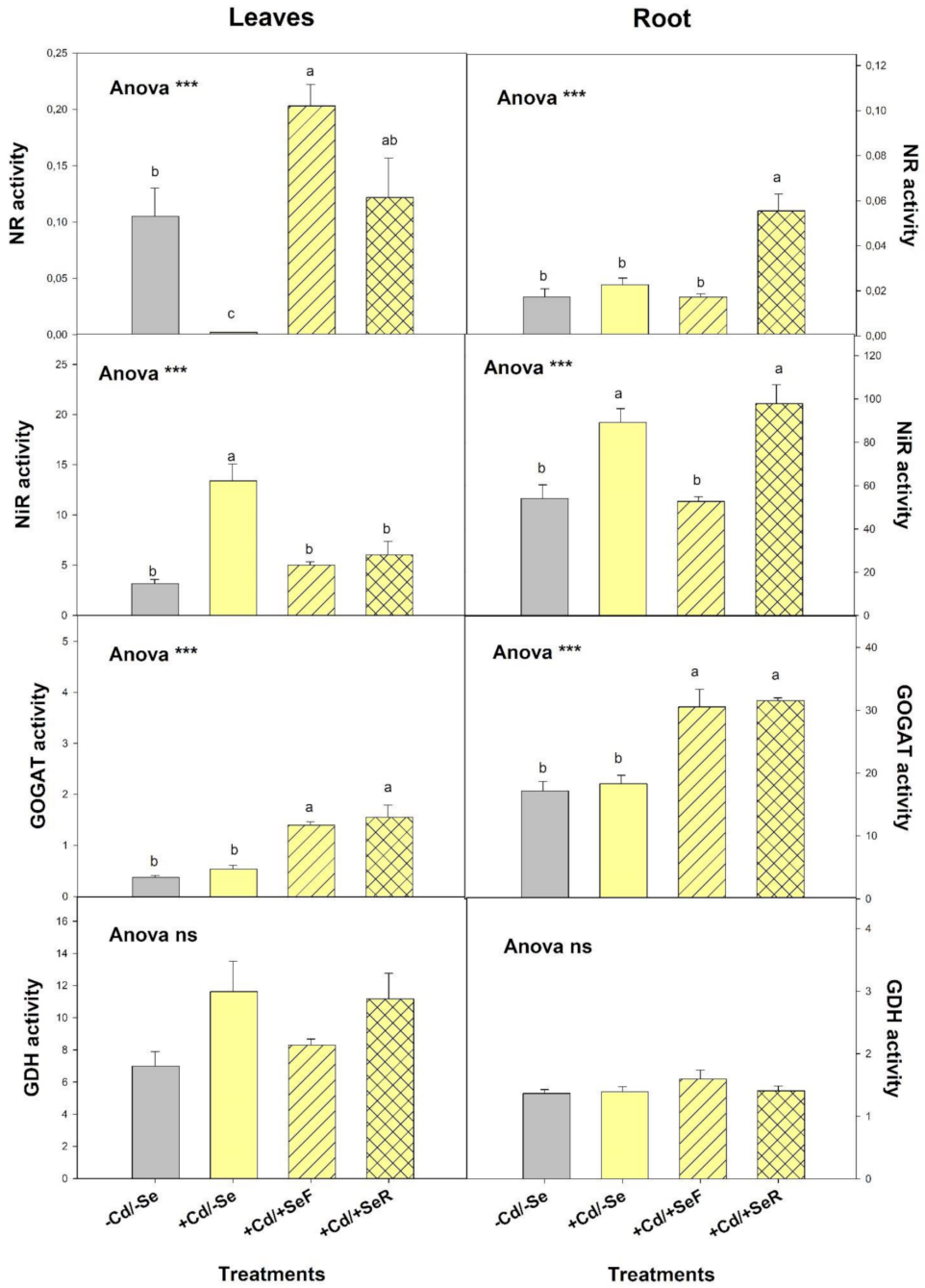


**Figure 2.** Activity of the enzymes nitrate reductase (NR activity; expressed in mM $NO_2^-$ formed $mg^{-1}$ protein $min^{-1}$), nitrite reductase (NiR activity; expressed in mM $NO_2^-$ transformed $mg^{-1}$ protein $min^{-1}$), glutamate synthase (GOGAT activity; expressed in mM NADH oxidized $mg^{-1}$ protein $min^{-1}$), and glutamate dehydrogenase (GDH activity; expressed in mM NADH oxidized $mg^{-1}$ protein $min^{-1}$) quantified in leaf and root tissue from pepper plants from the var. 'Cristal' grown in a hydroponic system under four different treatments which combined the application of Cd and Se (−Cd/−Se, +Cd/−Se; +Cd/+SeF and +Cd/+SeR). The results shown were obtained at the end of the experiment. In the ANOVA: 'ns' indicates non-significant differences for a 95% confidence interval, and *** indicate significant differences at $p < 0.001$. Lower case letters indicate significant differences between treatments at $p < 0.05$ established by Tukey's test. The bars indicate the SE of the mean ($n = 6$).

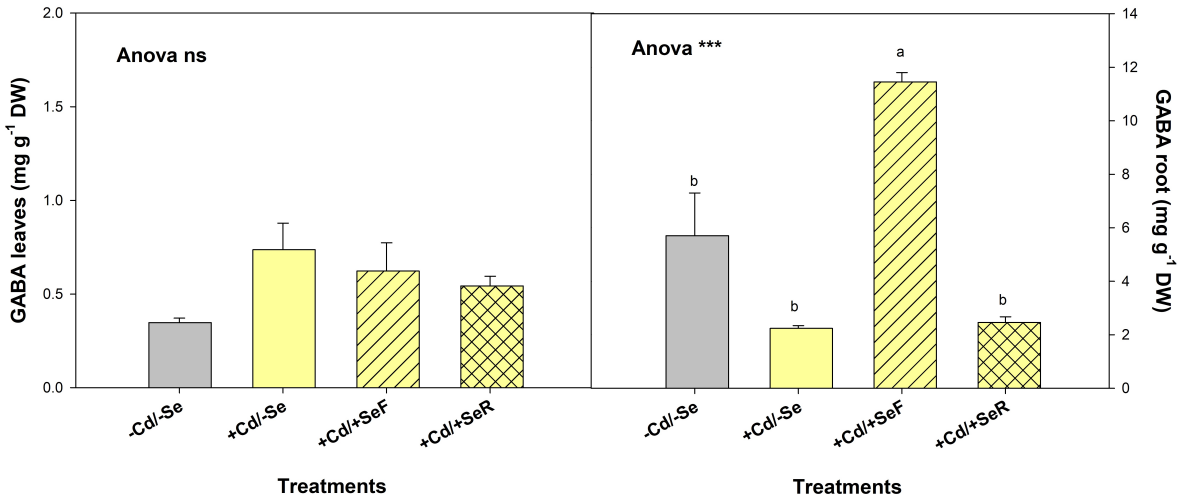


**Figure 3.** Concentration of total polyamines (spermine, spermidine, putrescine, and cadaverine) quantifies in leaf tissue from pepper plants of the var. 'Cristal' grown in a hydroponic system under four different treatments which combined the application of Cd and Se (−Cd/−Se, +Cd/−Se; +Cd/+SeF and +Cd/+SeR). The results shown were obtained at the end of the experiment. In the ANOVA: *** indicate significant differences at $p < 0.001$. Lower case letters indicate significant differences between treatments at $p < 0.05$ established by Tukey's test. The bars indicate the SE of the mean ($n = 6$).

As for the polyamine forms, free, soluble-conjugated, and insoluble, in the case of Pas Spm and Cad, the most common one was free, followed by soluble conjugated, with the insoluble one having the lowest concentration. While for Spd, the concentrations increased in the following order: soluble-conjugated = free > insoluble, and for Put, free > insoluble > soluble conjugated (Table 1). The +Cd/−Se treatment maintained this distribution in each of the polyamines, while the +Cd/+SeR treatment increased the percentage of the soluble-conjugate form at the expense of a decrease in the insoluble form in the case of Spd and Cad. In the case of +Cd/+SeF, an increase in the soluble-conjugated form was observed, to the expense of a decrease in the free form of Put. Table 2 shows the (Spm+Spd)/Put and Put/(Spm+Spmd) ratios. Significant differences were found in these ratios according to the treatments. Thus, the +Cd/+SeR treatment had the highest (Spm+Spd)/Put ratio and the lowest Put/(Spm+Spmd) ratio with respect to the rest of the treatments, which obtained very similar values.

**Table 1.** Polyamines quantified according to its form (free, soluble-conjugated, and insoluble-bound) in leaf and root tissue from pepper plants of the var. 'Cristal' grown in a hydroponic system under four different treatments which combined the application of Cd and Se (−Cd/−Se, +Cd/−Se; +Cd/+SeF and +Cd/+SeR). The results shown were obtained at the end of the experiment. In the ANOVA: *, **, and *** indicate significant differences at $p < 0.05$, 0.01, and 0.001, respectively. 'ns' indicates non-significant differences for a 95% confidence level. Lower case letters indicate significant differences between treatments at $p < 0.05$ established by Tukey's test. The bars indicate the SE of the mean ($n = 6$).

| Polyamines | Form (μmol $g^{-1}$ DW) | Treatments | | | | ANOVA |
|---|---|---|---|---|---|---|
| | | −Cd/−Se | +Cd/−Se | +Cd/+Se$_F$ | +Cd/+Se$_R$ | |
| Spermine | Free | 0.324 c | 0.508 c | 1.438 a | 1.098 b | *** |
| | Soluble-conjugated | 0.096 c | 0.152 c | 0.349 a | 0.226 b | *** |
| | Insoluble-bound | 0.033 c | 0.048 c | 0.072 a | 0.055 bc | *** |
| Spermidine | Free | 1.272 c | 1.408 c | 2.768 a | 2.000 b | *** |
| | Soluble-conjugated | 1.376 d | 1.610 c | 3.010 b | 4.210 a | *** |
| | Insoluble-bound | 0.190 c | 0.338 bc | 0.640 a | 0.480 ab | *** |
| Putrescine | Free | 1.958 c | 2.370 c | 3.746 a | 3.124 b | *** |
| | Soluble-conjugated | 0.154 c | 0.274 c | 1.218 a | 0.844 b | *** |
| | Insoluble-bound | 0.378 c | 0.496 bc | 0.784 a | 0.632 ab | *** |
| Cadaverine | Free | 0.360 c | 0.562 b | 0.732 a | 0.584 ab | *** |
| | Soluble-conjugated | 0.178 b | 0.184 b | 0.356 a | 0.234 b | *** |
| | Insoluble-bound | 0.025 c | 0.036 c | 0.081 a | 0.064 b | *** |
| **Polyamines** | **Polyamines (% total polyamines)** | | | | | |
| | **Form** | **−Cd/−Se** | **+Cd/−Se** | **+Cd/+SeF** | **+Cd/+SeR** | **ANOVA** |
| Spermine | Free | 71.3 b | 71.2 b | 77.4 ab | 79.7 a | * |
| | Soluble-conjugated | 21.4 | 22.0 | 18.7 | 16.4 | Ns |
| | Insoluble-bound | 7.4 a | 6.8 a | 3.9 b | 4.0 b | *** |
| Spermidine | Free | 44.8 a | 41.9 a | 43.1 a | 29.8 b | *** |
| | Soluble-conjugated | 48.6 b | 48.0 b | 46.9 b | 63.0 a | *** |
| | Insoluble-bound | 6.6 b | 10.1 a | 10.0 ab | 7.2 ab | * |
| Putrescine | Free | 78.6 a | 75.6 a | 65.1 b | 68.0 b | *** |
| | Soluble-conjugated | 6.2 b | 8.6 b | 21.3 a | 18.3 a | *** |
| | Insoluble-bound | 15.2 | 15.8 | 13.6 | 13.7 | Ns |
| Cadaverine | Free | 63.9 | 71.6 | 62.6 | 66.2 | Ns |
| | Soluble-conjugated | 31.6 | 23.7 | 30.4 | 26.5 | Ns |
| | Insoluble-bound | 4.5 c | 4.7 bc | 7.0 ab | 7.3 a | ** |

**Table 2.** (Spm+Spd)/Put and Put/(Spm+Spmd) ratios of the leaf concentration data of pepper plants from the 'Cristal' variety grown in a hydroponic system under four different treatments which combined the application of Cd and Se (−Cd/−Se, +Cd/−Se; +Cd/+SeF and +Cd/+SeR). The results shown were obtained at the end of the experiment. In the ANOVA: *** indicate significant differences at $p < 0.001$. Lower case letters indicate significant differences between treatments at $p < 0.05$ established by Tukey's multiple range test ($n = 6$).

| | −Cd/−Se | +Cd/−Se | +Cd/+SeF | +Cd/+SeR | ANOVA |
|---|---|---|---|---|---|
| (Spm+Spd)/Put | 1.33 b | 1.30 b | 1.45 b | 1.77 a | *** |
| Put/(Spm+Spmd) | 0.76 a | 0.77 a | 0.70 a | 0.57 b | *** |

### *3.3. Other Metabolites Identified and Quantified in Leaves and Root by RMN*

#### 3.3.1. Carbohydrates Concentration in the Different Plant Tissues

The presence of +Cd in the nutrient solution (+Cd/−Se) altered the concentration of sugars in the leaves and roots of the plants, as it decreased the concentration of sucrose in the leaves and the concentration of fructose, glucose, and sucrose in the root, as compared with the control treatment −Cd/−Se (Figure 4). This response was similar to the applica-

tion of the +Cd/+SeR, except that in this treatment, a decrease in the leaf concentration of glucose was also observed. In the case of the +Cd/+SeF treatment, the values of the concentration of these sugars were very similar to the control treatment plants, except for the concentration of glucose and sucrose in the root, in which an increase was observed in the former and a decrease in the latter, as compared to −Cd/−Se control plants.

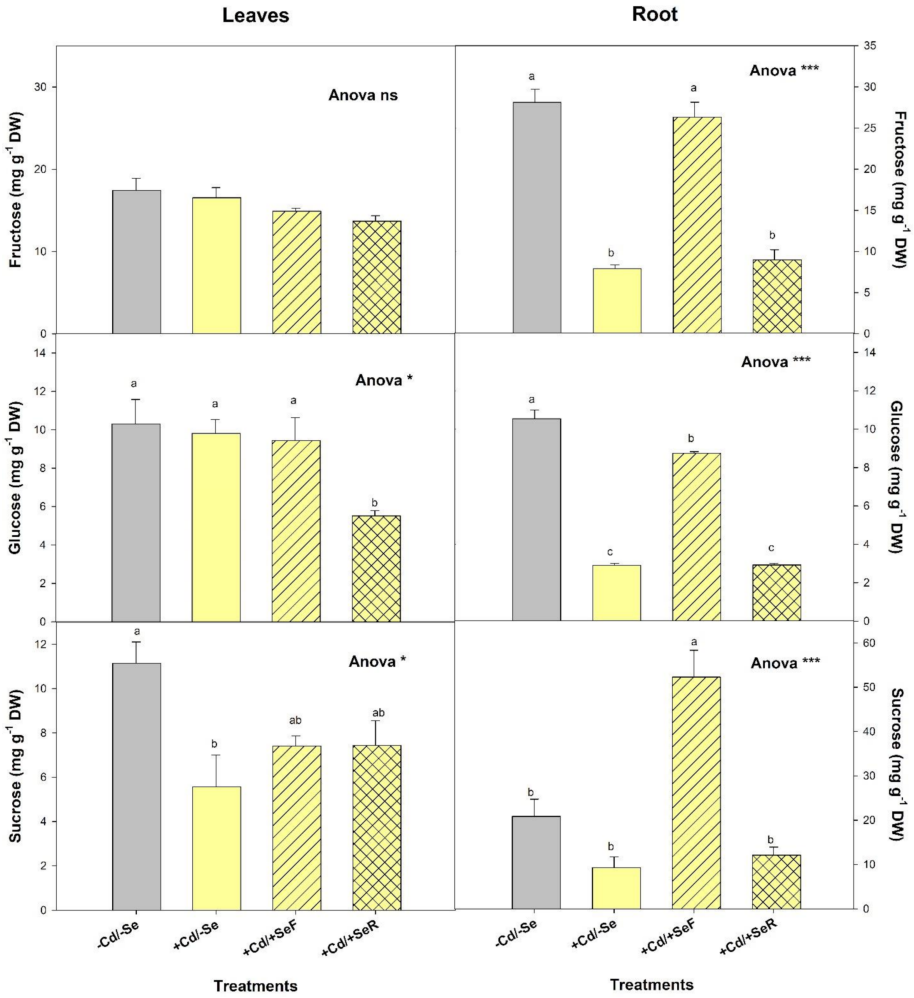


**Figure 4.** Concentration of fructose, glucose, and sucrose quantified in leaf and root tissue from pepper plants of the var. 'Cristal' grown in a hydroponic system under four different treatments, which combined the application of Cd and Se (−Cd/−Se, +Cd/−Se; +Cd/+SeF and +Cd/+SeR). The results shown were obtained at the end of the experiment. In the ANOVA: 'ns' indicates non-significant differences for a 95% confidence level, and * and *** indicate significant differences at $p < 0.05$, 0.01, and 0.001, respectively. Lower case letters indicate significant differences between treatments at $p < 0.05$ established by Tukey's test. The bars indicate the SE of the mean ($n = 6$).

### 3.3.2. GABA

The concentration of GABA (Figure 5) in the leaf tissue did not show significant differences between the treatments assayed. In the root, however, an increase was observed in the plants treated with +Cd/+SeF with respect to the rest of the treatments.

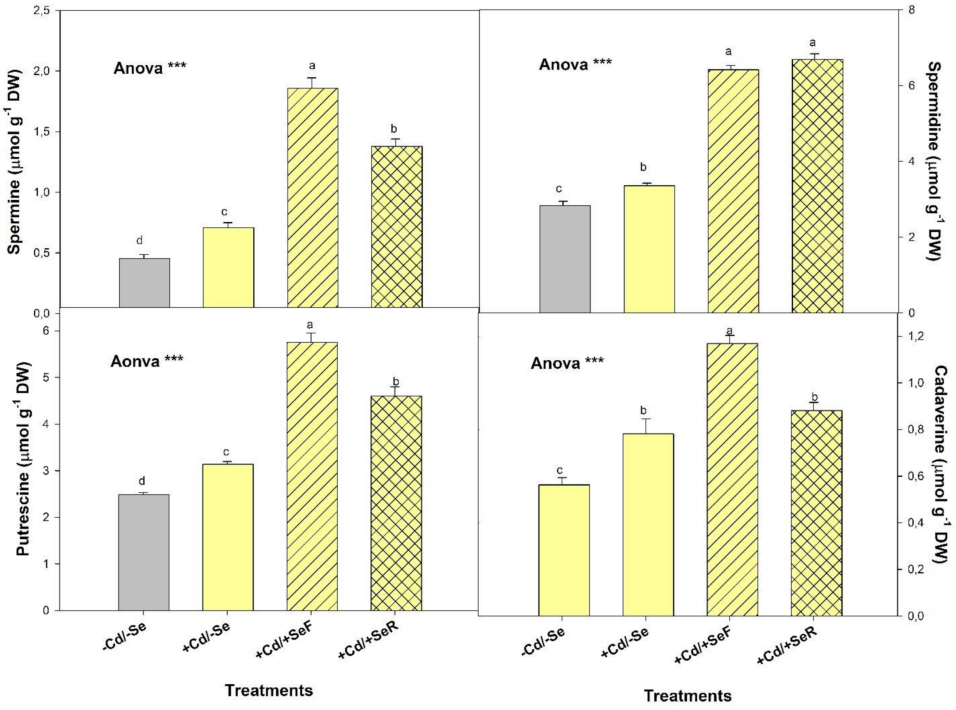


**Figure 5.** Concentration of GABA quantified in leaf and root tissue from pepper plants of the var. 'Cristal' grown in a hydroponic system under four different treatments, which combined the application of Cd and Se (−Cd/−Se, +Cd/−Se; +Cd/+SeF and +Cd/+SeR). The results shown were obtained at the end of the experiment. In the ANOVA: 'ns' indicates non-significant differences for a 95% confidence level, and *** indicate significant differences at $p < 0.001$. Lower case letters indicate significant differences between treatments at $p < 0.05$ established by Tukey's test. The bars indicate the SE of the mean ($n = 6$).

### 3.3.3. Amino Acids

The H-NMR analysis was able to detect and analyze the amino acids Tyr, Ala, Ile, Val, Phe, Pro, Glu, and Gln in the root. In leaves, these amino acids were also quantified, as well as Asp, but with the exception of Pro and Phe. In leaves, the most common amino acid was glutamate (65%), followed by glutamine (16%), and aspartate (8.4%), while in the root, the most common amino acids were proline (48%), followed by glutamate (21%) and Alanine (19%).

In leaves, the plants grown in the presence of Cd (+Cd/−Se, +Cd/+SeF, and +Cd/+SeR), increased their content of aspartate and glutamine with respect to −Cd/−Se, with this increase being significant in the +Cd/+SeR treatment for aspartate, and in the treatment +Cd/+SeR and +Cd/+SeF for glutamine (Table 3).

In the root, significant differences were found in the concentrations of all the amino acids quantified with the treatments (Table 2). With respect to treatment −Cd/−Se, the following responses were observed: (i) the concentrations of the amino acids Ile and Val decreased in treatments +Cd/−Se and +Cd/+SeR; (ii) the concentrations of Ala and Glu decreased in the +Cd/+SeR treatment, and only Ala for the +Cd/+SeR treatment; (iii) the concentrations of Phe and Pro increased in the plants from the +Cd/+SeF treatment, and Phe also decreased in the +Cd/+SeR treatment; iv) the concentration of Gln decreased in the +Cd/−Se and +Cd/+SeR treatments, and increased in the +Cd/+SeF one.

**Table 3.** Amino acids quantified in leaf and root tissue of pepper plants from the 'Cristal' variety grown in a hydroponic system under four different treatments which combined the application of Cd and Se (−Cd/−Se, +Cd/−Se; +Cd/+SeF and +Cd/+SeR). The results shown were obtained at the end of the experiment. In the ANOVA: 'ns' indicates non-significant differences for a 95% confidence level, and *, **, and *** indicate significant differences at $p < 0.05$, 0.01, and 0.001, respectively. Lower case letters indicate significant differences between treatments at $p < 0.05$ established by Tukey's multiple range test ($n = 6$). 'nd' indicates no detection with NMR.

| **Amino Acids Detected in Leaves by RMN (μmol $g^{-1}$ DW)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | **Asp** | **Tyr** | **Ala** | **Iso** | **Val** | **Phe** | **Pro** | **Glu** | **Gln** |
| −Cd/−Se | 7.06 b | 3.31 | 3.70 | 0.82 | 0.90 | nd | nd | 54.78 | 13.55 b |
| +Cd/−Se | 13.37 ab | 3.38 | 4.41 | 1.16 | 1.25 | nd | nd | 61.37 | 16.56 ab |
| +Cd/+SeF | 9.92 ab | 3.45 | 3.96 | 1.11 | 1.03 | nd | nd | 56.82 | 19.16 a |
| +Cd/+SeR | 13.52 a | 3.27 | 5.43 | 1.08 | 1.25 | nd | nd | 57.50 | 18.41 a |
| ANOVA | * | ns | ns | ns | ns | - | - | ns | ** |
| **Amino Acids Detected in Root by RMN (μmol $g^{-1}$ DW)** | | | | | | | | | |
| **Treatment** | **Asp** | **Tyr** | **Ala** | **Iso** | **Val** | **Phe** | **Pro** | **Glu** | **Gln** |
| −Cd/−Se | nd | 4.17 ab | 45.80 a | 2.64 a | 2.02 a | 1.92 b | 118.13 b | 50.30 a | 16.97 b |
| +Cd/−Se | nd | 4.08 ab | 21.89 bc | 1.12 b | 1.20 b | 1.91 b | 55.68 b | 37.79 a | 6.36 c |
| +Cd/+SeF | nd | 3.80 b | 38.95 ab | 2.21 a | 1.89 a | 2.50 a | 241.81 a | 46.35 a | 27.44 a |
| +Cd/+SeR | nd | 5.00 a | 14.93 c | 1.09 b | 1.25 b | 1.43 c | 56.20 b | 21.21 b | 4.17 c |
| ANOVA | - | * | ** | ** | *** | *** | *** | *** | *** |

### 3.3.4. Organic Acid Salts

The organic acid salts detected and quantified by H-NMR in the leaves and roots were citrate, malate, formate, fumarate, and chlorogenate (Table 4). Their concentration as a percentage in the leaves followed the order: malate > citrate > chlorogenate > fumarate > formate; while in the root, citrate was found in the highest percentage followed by malate.

**Table 4.** Organic acid salts citrate (Cit), formate (For), fumarate (Fum), malate (Mal), and chlorogenate (CGA) quantified in leaf and root tissue of pepper plants from the 'Cristal' variety grown in a hydroponic system under four different treatments, which combined the application of Cd and Se (−Cd/−Se, +Cd/−Se; +Cd/+SeF and +Cd/+SeR). The results shown were obtained at the end of the experiment. In the ANOVA: 'ns' indicates non-significant differences for a 95% confidence level, and *, **, and *** indicate significant differences at $p < 0.05$, 0.01, and 0.001, respectively. Lower case letters indicate significant differences between treatments at $p < 0.05$ established by Tukey's multiple range test ($n = 6$).

| | **Organic Acid Salts Detected by RMN (μmol $g^{-1}$ DW)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Leaves** | | | | | **Root** | | | | |
| **Treatment** | **Cit** | **Mal** | **For** | **Fum** | **CGA** | **Cit** | **Mal** | **For** | **Fum** | **CGA** |
| −Cd/−Se | 8.28 ab | 36.39 a | 0.67 | 0.84 | 1.13 bc | 29.56 b | 26.18 b | 0.63 ab | 0.44 b | nd |
| +Cd/−Se | 12.75 a | 39.45 a | 1.00 | 3.17 | 2.08 ab | 32.74 ab | 20.06 b | 0.46 b | 0.61 ab | nd |
| +Cd/+$Se_F$ | 6.40 b | 29.91 ab | 0.8 | 0.78 | 0.86 c | 53.77 a | 43.48 a | 0.72 a | 1.06 a | nd |
| +Cd/+$Se_R$ | 11.50 ab | 21.25 b | 0.83 | 1.25 | 2.37 a | 41.90 b | 31.62 ab | 0.56 ab | 0.41 b | nd |
| ANOVA | * | ** | ns | ns | ns | *** | ** | * | ** | - |

In the leaves, the treatment assays resulted in significant differences in the concentrations of citrate, malate, and chlorogenate. With respect to the control treatment, −Cd/−Se, the concentration of malate decreased, while the concentration of chlorogenate significantly increased for the +Cd/SeR plants. In the roots, an increase in the concentration of citrate, malate, and fumarate was observed in the +Cd/+SeF as compared to the −Cd/−Se treatment (Table 3).

## 4. Discussion

In the first part of the present publication [8], it was shown that the presence of Cd in the nutrient solution (+Cd/−Se, +Cd/+SeF and +Cd/+SeR) decreased the total dry biomass by 48%, 45%, and 38%, respectively, as compared to the control treatment (−Cd/−Se). Therefore, the application of Se through the root palliated the negative effect of Cd in the nutrient solution. It was also observed that the pepper plants could restrict the transport of Cd from the root to the leaves, at the expense of accumulating it in the root. Nevertheless, the low quantities that arrived at the leaf (30 ppm Cd) were very toxic, resulting in a reduction in growth. On the other hand, the application of Se through the leaves (foliarly) or the roots inhibited the transport of Cd to the leaves and deactivated its toxicity. Thus, in the present publication, we wanted to discover if the application of Se was able to inhibit Cd toxicity through the regulation/alteration of biochemical processes associated with C and N metabolism (sugars, organic acid salts, amino acids, polyamines, and nitrogen assimilation). The Cd concentrations reached in the plants from each of the treatments: +Cd/−Se, +Cd/−Se, +Cd/+SeF, and Cd/+SeR, are notable. In the leaves, these were 0.010, 26, 18, 18 ppm, respectively; and in the root, 2.15, 2400, 1000, and 2400 ppm, respectively [8].

### *4.1. Selenium in the Root Induced Changes in the Accumulation of Pas and Its Homeostasis, Favoring the Tolerance of Plants to Cd Excess*

Polyamines (Pas) are low molecular weight polycationic molecules [45], which are fundamental for the development and growth of eukaryotic and prokaryotic cells [46]. In plants cells, Put, Spd, and Spm are the most important, and can be found in three forms: (i) free, (ii) conjugated with phenolic acids and other low molecular weight compounds, and (iii) insoluble, associated to proteins and cell walls. These compounds are involved in a wide range of functions in the growth and development of plants, including senescence, responses to fungi and viruses, and environmental stresses, including toxicity due to heavy metals [11,47–52]. Their function in the tolerance to abiotic stresses are due, among other causes, to their capacity to avoid the oxidation of cell structures, and this is achieved because PAs (i) have an enormous capacity to bond with cations and anions of membrane phospholipids or nucleic acids, protecting them from oxidative stress, and (ii) can directly bond to heavy metals such as Co, Cu, Fe, or Ni, precisely avoiding the formation of these free radicals [53–55].

In our experiment, it was observed that pepper plants naturally increased the concentration of PAs when they were grown with a nutrient solution containing Cd (Figure 4). This response, however, was not sufficiently intense or efficient for protecting the plants from its toxicity. In other plants sensitive to Cd [56,57], the increase in PAs was accompanied by a greater increase of Put as compared to Spm and Spd, so that the Spm+Spd/Put ratio decreased as compared to conditions without Cd. This is due to the increase in the concentration of Cd and the increase in enzymatic activity and/or gene expression of Arg decarboxylase (ADC) and Orn decarboxylase (ODC, amino oxydases (DAO), and polyamine oxydases (PAO)) [56,57]. In our experiment with pepper plants, although they are very sensitive to Cd, the response was different, as the Spm+Spd/Put ratio was not altered (Table 2), so that the decrease in this ratio was not universal for all the plants sensitive to Cd toxicity.

The application of Se, either foliarly or through the root, increased the concentration of the polyamines Spm, Spd, and Put. Thus, this response could have helped the plants to protect themselves from the ROS compounds produced by Cd. In fact, in a previous publication [8], it was observed that plants treated with +Cd/−Se had am MDA content (a compound that indicates the level of lipid peroxidation) of 10 nm $g^{-1}$ fw, while the plants treated with Se, either foliarly or through the root, obtained an MDA value of 6 nm $g^{-1}$ fw, the same as the control plants. Thus, the application of Se to plants treated with Cd induced the accumulation of PAs, which helped in reducing oxidative stress. Of these three polyamines, Put was found at the highest concentration, due to the treatment with Se. Put

is a PA with a low molecular weight diamine structure, so that it can be rapidly translocated towards the cell membranes, bonding with them to offer a fast and safe protection, as compared to Spd and Spm, as they are triamine and tetramine molecules, respectively [50]. In the bibliography, we found evidence that PAs help in the tolerance of stress caused by heavy metals. Thus, Tiae et al., (2019) [58] applied PAs foliarly (Spm, Spd, or Put) to wheat plants treated with Cd, and observed that the foliar application of Put obtained good results in the growth of the plants, as it increased their antioxidant capacity to deal with the toxicity of this metal. As for PA homeostasis, differences were also found in the +Cd/+SeR treatment with respect to the rest of the treatments. The Spm+Spd/Put ratio increased in this treatment (1.77 vs. 1.36). For Spm and Spd, an increase was observed in their soluble-conjugated forms to the expense of a decrease in their free form. Some authors have related the tolerance to stresses with the increase in the Spm+Spd/Put ratio [59] and the conversion of the free forms of the PA to their conjugated ones [57]. Therefore, the changes in the polyamines, both in their concentration and the Spm+Spd/Put ratio, and their conjugated forms, are related with a greater tolerance to an excess of Cd with the application of Se to the roots.

### *4.2. Does the Foliar Application of Se Damage the Plants?*

The foliar application of Se (+Cd/+SeF), although it improved the vegetative growth and development of plants, did not avoid the phytotoxic damage caused by Cd, as observed with the application of Se to the root (+Cd/+SeR), despite the plants from both treatments accumulating the same concentration of Cd. An increase in the leaf concentration of $H_2O_2$ was observed in plants from the +Cd/+SeF treatment [8], with respect to (+Cd/+SeR), in parallel to an increase in the leaf concentration of Spm, Put, Cad, and the root concentration of GABA. In addition, a differential response was also observed in the root, with respect to the amino acids phenylalanine, proline, and glutamine, the organic acids salt citrate and malate, and the sugar sucrose, as their concentration was higher as compared to the other treatments (Table 3). This response could suggest that the application of Se to the leaves (+Cd/+SeF) could have activated a series of responses that were different from the root treatment (+Cd/SerR), which would make the +Cd/+SeF less effective, or cause some damages to the plant, although these may not be as severe as the control treatment (+Cd/−Se). Thus, the application of Se to the leaves could have increased the concentrations of Put and Cad in this organ, which are secreted in the apoplast and boost the activity of PAOs (flavin-containing polyamine oxidases) and CuAOs (copper-dependent amine oxidases), leading to a higher production of $H_2O_2$, thus threatening the redox homeostasis of cells [60]. This hypothesis could be justified by the close relationship observed between Put, Spm, Spd, GABA, and $H_2O_2$ (see Schematic representation of the biosynthesis, back-conversion, and terminal catabolism of Put, Spd, and Spm in plants in Sporman et al., 2020 [61]). From this point on, this excess of $H_2O_2$, although it could act as a signal for plants to respond to abiotic stresses, could have also affected other physiological processes, thus resulting in changes to C metabolism characterized by amino acids, organic acid salts, and sugars. It has been observed that damage can occur when the concentrations of PAO and $H_2O_2$ are high, as they oxidize Spd quickly, leading to a decreased regulation of survival genes, so that the protection pathway is not observed [61–63]. This very different response observed in the +Cd/+SeF treatment could be associated with the catabolism of the polyamines in which a Put-Spd-Spm conversion is observed, resulting in an accumulation of ammonium and $H_2O_2$, aside from the production of other molecules such as GABA [61].

### *4.3. Nitrogen Metabolism in Leaves and Root*

With respect to nitrogen metabolism, we found three differential responses in plants from the +Cd/−Se, +Cd/+SeF, and +Cd/+SeF treatments. What was common to these responses was the increase in concentration of ammonium in the leaves and roots, with this concentration being greater in the +Cd/+SeF and +Cd/+SeR leaves, and the roots from the +Cd/−Se plants. However, this accumulation of ammonium cannot be explained

only with the nitrate and nitrite concentration data, or the enzymatic activities of NR, NiR, GOGAT, and GDH, which determine the synthesis and catabolism of ammonium. Thus, other processes such as the absorption of nitrate by the roots, the anabolism/catabolism of the polyamines, and/or deamination of free amino acids and other N forms could affect the final ammonium concentration in the plants. The observation revealed that in the treatment with Cd without Se (+Cd/−Se), there was an inhibition of NR and an overstimulation of NiR, while in the treatments with Se, there was an overall stimulation of GOGAT. Other studies have shown that in zucchini plants, the presence of Cd in the nutrient solution led to an increase in nitrate and a decrease in ammonium, as the enzymatic activity of NR, GOCAT, GS and GDH was inhibited [64]. Similar results were found in potato plants, as Cd decreased the enzymatic activities of NR, NiR, GS, and GOGAT, with the subsequent reduction of nitrate and nitrite, and an increase in ammonium. However, these effects on zucchini and potato were reversed by the presence of Si and Se, for zucchini and potato plants, respectively [65]. However, it is not yet clear why heavy metals reduce these activities, although it could be due to the direct effects of the heavy metals due to indirect effects caused by alterations produced in other physiological processes, such as net photosynthesis, or chlorophyll biosynthesis [66].

### *4.4. Other Metabolites Identified and Quantified in Leaves and Root Related with the High Tolerance to Cd with the Application of +Cd/+SeR*

One of the differentiating responses produced by the +Cd/+SeR treatment with respect to the other treatments (−Cd/−Se, +Cd/+SeF) is the decrease in concentration of malate and the increase in chlorogenate in the leaves, and the decrease of phenylalanine and glutamate in roots (Figure 6). Malate is the only organic acid salts that restricts the accumulation of Cd in plants, as it blocks the anionic channels, which it can penetrate. In many plants, it has been observed that a segregation of malate to the root zone decreases the accumulation of Cd [67]. Phenylalanine intervenes in important metabolic processes such as protein biosynthesis and the phenylpropanoid route, producing a broad range of secondary metabolites such as flavonoids, anthocyanins, lignins, or phenylpropanoids, which can palliate the effects caused by toxicity to heavy metals [68,69]. Glutamate intervenes in nitrogen assimilation processes. More specifically, it reacts with ammonium to incorporate nitrogen to the plants [70]. In the present work, the decrease in phenylalanine in the +Cd/+SeR plants could indicate that this treatment activates the phenylpropanoid route to protect against Cd toxicity, while the decrease in glutamate could indicate an increase in the biochemical activity associated with the synthesis of elicitor molecules [68]. Both mechanisms could help the +Cd/+SeR plants to withstand Cd toxicity in the pepper plant tissues. This also occurs with chlorogenic acids, as it is a phenolic compound, a caffeic acid, and quinic acid ester, which acts as an intermediate in the biosynthesis of lignin [71]. In turn, the decrease in the concentration of malate suggests that these plants also put into place mechanisms to avoid the accumulation of the excess accumulation of Cd in the tissues. The segregation of malate to the root, to the expense of its decrease in the other tissues, could be blocking the entry of Cd, as we have observed in the study by Perez-Millan et al. (2021) [8]. However, in this work, it is not clear why root Se triggers all of these processes. It would be necessary to conduct molecular biology experiments to elucidate why Se is able to start all of these biochemical processes and changes. In fact, it has been observed in tomato plants that Se application produces changes in miR172, CRTISO, bZIP, DREB1A transcriptional factors, and gene expression of HQT1, HCT1, and PAL what could be related with abiotic stress tolerance [72,73].

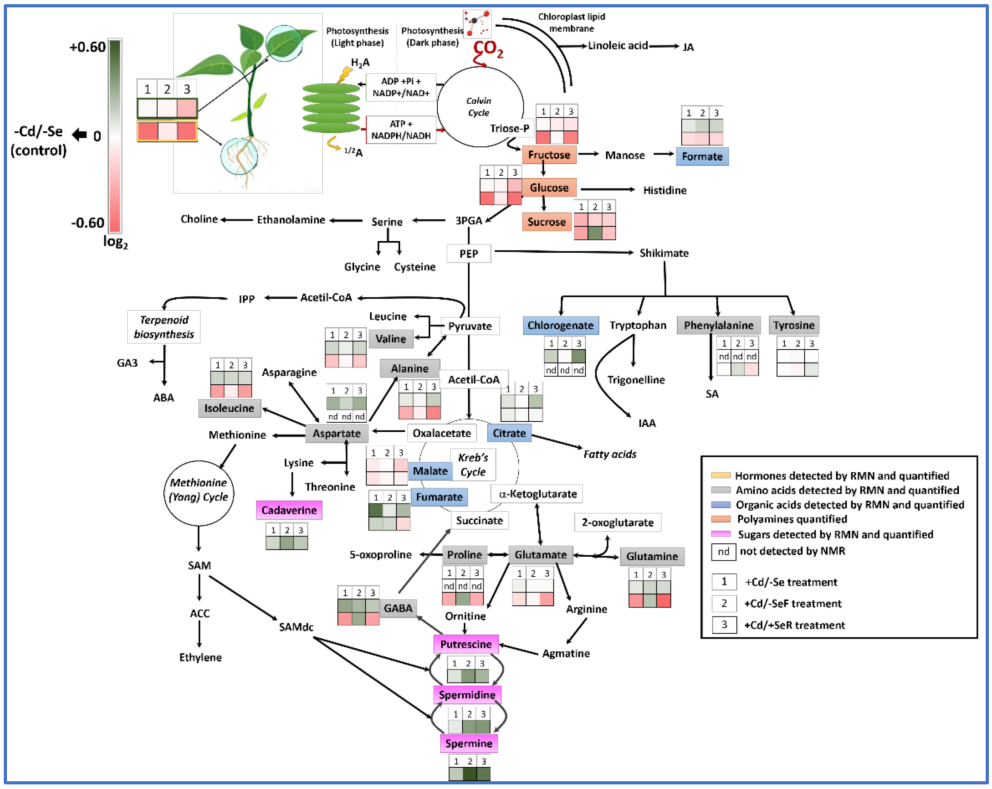


**Figure 6.** Proposed diagram of the distribution of the metabolites quantified with NMR ('nd' indicates that the metabolite was not quantified by NMR). For each metabolite quantified by NMR, a heat map is shown for leaf (top squares) and root (bottom squares) tissue for each Cd excess treatment (1: +Cd/−Se; 2: +Cd/+SeF; 3: +Cd/+SeR). In both tissues, the green color indicates a higher relative concentration, and the red color indicates a lower relative concentration as compared to the control treatment (−Cd/−Se). Scale is the log2 of the mean concentration values after normalization (n = 6).

## 5. Conclusions

The previous publication on this experiment highlighted that pepper plants were sensitive to Cd when it was found in high concentrations in the nutrient solution, and that the negative effects could be offset if the plants were provided with Se foliarly or through the root, with the latter providing greater benefits. In the present work, we deciphered the mechanisms through which the application of Se via the root could decrease the accumulation and toxicity of Cd with respect to C and N metabolism, in which polyamines played an important part. Thus, the greatest tolerance of the +Cd/SeR treatment, with respect to the other treatments, could be due to this treatment inducing an increase in the concentration of the polyamines Spm, Spd, Put, and Cad, so that the Spm+Spd/Put ratio and the conjugated forms are greater than the free or insoluble forms. This response makes the plants more tolerant to Cd. Furthermore, the study of the organic acid salts and amino acids revealed that these plants could put into play other protection pathways in the presence of Cd, such as the phenylpropanoid pathway, the activation of elicitors, and segregation of malic acid to block its entry into the plant. On the other hand, although the +Cd/+SeF treatment was also efficient against the presence of Cd, it was not as efficient as +Cd/+SeR. The data from the metabolic study indicated that the foliar treatment with Se caused some damages to the pepper plants or triggered a series metabolic response that was different from those observed in the SeR plants, which were less efficient against Cd toxicity.

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S.S.-G.; visualization, M.A.-S.; supervision, S.S.-G.; project administration, F.G.-S. All authors have read and agreed to the published version of the manuscript.

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