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# CdS Nanoparticles in Soil Induce Metabolic Reprogramming in Broad Bean (*Vicia faba* L.) Roots and Leaves



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The rapid development of nanotechnology has raised concern regarding the environmental toxicity of nanoparticles (NPs). However, little is known about the molecular mechanisms underlying NP toxicity in plants. Understanding toxic mechanisms in organisms at molecular level, especially in crop plants, is important for their sustainable use and development. In this study, although none of the phenotypic parameters of broad bean plants (photosynthetic pigments contents, biomass, and lipid peroxidation) were overtly impacted in response to CdS-NPs in soil during 28 d of exposure, metabolomics revealed marked and statistically significant alterations in the metabolite profiles of plant roots and leaves. The reprogramming of antioxidant metabolite production presumably reflected the molecular defense response of the plants to CdS-NPs stress. The sensitive responses of flavone, putrescine and noradrenaline in the leaves suggests the use of these compounds in legumes as biomarkers of oxidative stress induced by the presence of CdS-NPs in soil. Metabolomics might thus be a suitable approach for the early detection of soil contamination by Cd. In the plants, the reprogramming of carbon and nitrogen metabolism (including sugars, organic acids, amino acids, and N-containing compounds) alleviated the toxicity of CdS-NPs, which may have been caused by free $Cd^{2+}$ ions or perhaps by a particle-specific response. Importantly, this response might adversely impact crop yield and quality in plants under long-term exposure to CdS-NPs.

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# CdS Nanoparticles in Soil Induce Metabolic Reprogramming in Broad Bean (*Vicia faba* L.) Roots and Leaves

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## Abstract

The rapid development of nanotechnology has raised concern regarding the environmental toxicity of nanoparticles (NPs). However, little is known about the molecular mechanisms underlying NP toxicity in plants. Broad bean (*Vicia faba* L.) plants were cultivated in soil amended with 0, 10, and 100 mg cadmium sulfide (CdS)-NPs kg soil$^{-1}$ for 4 weeks and then the phenotypic, biochemical, and metabolic responses of the plants to CdS-NPs stress were evaluated. Metabolomics analysis revealed the significant up-regulation (1.2- to 39.2-fold) of several antioxidative metabolites, including *N*-acetyl-5-hydroxytryptamine, 2-hydroxybutanoic acid, putrescine, and flavone, upon CdS-NPs exposure, but no negative phenotypic effects were visible (plant biomass, photosynthetic pigment contents, and lipid peroxidation). This observation was in accordance with the observed regulation of antioxidative-defense-related metabolic pathways (tyrosine pathway and phenylpropanoid biosynthesis) that were identified by biological pathway analysis. Importantly, twice as many metabolites were modulated in leaves than in roots, including three nitrogen-related (purine metabolism; alanine, aspartate, and glutamate metabolism; β-alanine metabolism) and two carbon-related (pantothenate and CoA biosynthesis and carbon fixation) metabolic pathways. These results indicate that to alleviate the toxicity of CdS-NPs exposure in soil, plants significantly reprogram the metabolic profiles of leaves rather than of roots, which might subsequently impact both harvest and crop quality.

# Introduction

The wide-ranging applications of cadmium sulfide nanoparticles (CdS-NPs) include biological imaging, photovoltaic technology, glasses, plastics and ceramics.[1-5] However, during the fabrication, use, storage and disposal of CdS-NPs, these materials are eventually released into the environment by a number of routes, including wastewater irrigation, biosolid fertilizer application and atmospheric deposition.[6] Soil is the ultimate sink for most NPs and as such, terrestrial plants are likely to be highly exposed. Since plants are primary producers in terrestrial ecosystems, an understanding of the underlying mechanisms of NPs toxicity to these plant species is critical to the accurate assessment risk to humans and other species.

The uptake and toxicity of Cd-containing NPs (e.g., CdS, CdSe, and CdTe) in plants have been studied in hydroponic and sand cultures with ryegrass (*Lolium perenne*),[7] onion (*Allium cepa*),[7] rockcress (*Arabidopsis thaliana*),[8, 9] snow pea (*Pisum sativum* L.),[10] soybean (*Tohya* variety),[11] zucchini (*Cucurbita pepo* L.),[12] and a woody plant (*Kandelia obovata*).[13] In hydroponic cultures of *A. thaliana*, CdS-NPs (80 mg $kg^{-1}$) exposure strongly inhibited chlorophyll biosynthesis and the cellular respiration rate,[8] and CdSe-NPs (5.8 nM) exposure decreased content ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG).[9] In soil, the interaction of NPs with soil organic matter, clay minerals, microorganisms, and macrofauna often alters the fate and toxicity of these particles.[14-18] Therefore, the effects of CdS-NPs on crop plants will likely differ between plants grown in soil vs. hydroponic or sand cultures.

Most previous studies examining the exposure of NPs to plants have used phenotypic parameters as

end points,[19] but this approach does not provide information on the underlying mechanisms leading to the toxicity or the detoxification of NPs. More recently, transcriptomics has been used to investigate the mechanisms by which *A. thaliana* is able to tolerate 80 mg CdS-NPs $kg^{-1}$ under hydroponic culture. The results showed that of the 195 genes up-regulated, 32% were associated with detoxification, represented by reactive oxygen species (ROS) metabolism and the expression of lipid transfer proteins involved in defense.[8] Further studies of the effects of CdS-NPs (size $< 10$ nm) on soybean found that the plants were able to reduce the stress caused by CdS-NPs by increasing the amino acid content in their leaves.[11]. Importantly, plants contain > 200,000 different low-molecular-weight (LMW) metabolites,[20] including phenolic acids, carboxylic acids, carbohydrates and amino acids. These metabolites are the end products of gene expression and changes in their contents determine the ultimate biochemical phenotype of the plant.[21-25] Plant metabolism is highly dynamic series of processes and metabolomics can provide a snapshot of the in vivo metabolic profile.[26] By undertaking a quantitative analysis of metabolites, metabolomics can yield accurate information on the physiological mechanisms induced by NP exposure. In previous studies, gas chromatography-mass spectrometry (GC-MS)-based metabolomics was used to explore plant responses under various NP exposures, including $Cu(OH)_2$, CuO, $CeO_2$, and Ag NPs.[27-30] Different NPs were shown to induce distinctive metabolic changes in plants, mainly relating to pathways involved with lipids, energy and amino acids and the production of antioxidants.[28-32]

The objectives of the present study were (1) to investigate the phenotypic and biochemical responses of broad bean plants (*Vicia faba* L.) exposed to CdS-NPs in soil; (2) to analyze the changes in the metabolites of broad bean plants induced by CdS-NPs exposure and (3) to explore

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4 the defense mechanisms of broad bean plants against CdS-NPs stress at the molecular level. Broad bean (*Vicia faba* L.) is a leguminous plant and was chosen as it is easy to grow, has a low fertilizer requirement and serves as a forage crop for animals and a protein source for humans.[33] Moreover, broad bean is an excellent model legume, as demonstrated with a number studies of heavy metal pollution in the environment.[34] Specifically, broad bean was grown in soil amended with 0, 10, and 100 mg CdS-NPs $kg^{-1}$ and changes in several phenotypic parameters (plant biomass, photosynthetic pigments, lipid peroxidation and total phenolic compounds) and in the root and leaf metabolites were measured. The results provide a comprehensive picture of the effects of NPs on leguminous plants at both the physiological and the molecular level, which yields information critical to the accurate assessment of risk of these materials in the environment.

## Materials and Methods

**CdS-NPs Characterization.** CdS-NPs ($K_{sp}$ = 7.94 × $10^{-27}$) were purchased from Kela Co., Ltd., China. The hydrodynamic size and zeta potential of the particles were determined by mixing 1 and 10 mg CdS-NPs in 100 mL of nanopure water and then sonicating the suspensions for 10 min at 25°C using a flat-tip probe sonicator (Ymnl-1000Y, Nanjing Immanuel Instrument Equipment Co. Ltd., Nanjing, PR China) at a power of 65 W in cycles of 5 s of sonication and a 5-s pause. The hydrodynamic diameter of CdS-NPs in nanopure water was 140−615 nm with an average 294 nm, and the zeta potential was −26.3 ± 0.7 mV, as measured by dynamic light scattering (Malvernseries ZEN 3500, Malvern Instruments Ltd., Worcester, UK). The morphology of CdS-NPs was examined by scanning electron microscopy (S-3400N II, Hitachi, Tokyo, Japan) (**Figure S1A** in the Supporting Information (SI)). The CdS-NPs ranged in size from 10 to 100 nm as determined by

transmission electron microscopy (JEM-200CX, JEOL, Japan) (SI **Figure S1B and C**). This kind of CdS-NPs is sold to industry use and could represent the contamination of CdS-NPs in the environment.

**Broad Bean Exposure.** Field soil was collected from Harbin city, Heilongjiang Province, China. The soil samples were air-dried for 2 weeks and then sieved through a 2-mm mesh. The physiochemical properties of the soil, including total organic matter, total phosphorous and cation exchange capacities (CEC), are described in SI **Table S1**. Broad bean (*Vicia faba* L.) seeds were purchased from Luwang Seed Co. Ltd. (Sichuan Province, China). Three treatments were established: control (no CdS-NPs), 10 mg CdS-NPs $kg^{-1}$, and 100 mg CdS-NPs $kg^{-1}$. A treatment with free $Cd^{2+}$ was not established because the concentration of dissolved $Cd^{2+}$ ions from CdS-NPs in deionized water at 25 °C was low ($1.28 \times 10^{-8}$ mg $L^{-1}$) according to the solubility products ($K_{sp}$) of CdS ($7.94 \times 10^{-27}$).[35] Black plastic boxes (each 7 cm × 5 cm × 8 cm) contained 30 holes with a diameter of 0.2 cm at the bottom were filled with 100 g of soil containing 0, 10, or 100 mg CdS-NPs (kg soil dry weight) $^{-1}$, hereafter expressed as mg CdS-NPs kg $soil^{-1}$. After 24 h, broad bean seeds were sown at a depth of 1 cm. Six replicate plants (one plant/pot) were grown for each treatment. The plants were cultivated for 28 days in a greenhouse under a 25°C/20°C day/night cycle. The relative humidity in the greenhouse was 65−70%. During the 16-h photoperiod, active radiation was set at 180 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The pots were watered three times (each 4 mL) daily to maintain 60% of field water capacity.

**Physiological Responses.**

*Photosynthetic Pigment Measurement.* The total chlorophyll (Chl) content in broad bean shoots was measured as described by Lichtenthaler et al.[36] Briefly, 0.1 g of fresh leaves were extracted at room temperature with 5 mL of a mixture of ethanol and 80% acetone/20% deionized water (1/1, by volume). All samples were kept in the dark for 12 h to avoid Chl degradation. The absorbance of Chl in the extracts was measured at 663, 645, and 470 nm using a UV–Vis spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan).

*Lipid Peroxidation.* A thiobarbituric acid reactive substances assay was used to measure lipid peroxidation in the plant leaves.[37] Briefly, 0.1 g of fresh leaves and roots were weighed and then mixed for 30 min with 4 mL of 0.1% trichloroacetic acid (TCA). After the mixture was centrifuged at 10,000*g* for 15 min, 0.1 mL of the supernatant was mixed with 2 mL of 20% TCA and 2 mL of 0.5% thiobarbituric acid. This mixture was heated in a water bath at 95°C for 30 min and then allowed to cool to room temperature. The UV absorbance was measured at 450, 532 and 600 nm. Lipid peroxidation was expressed as μmol malondialdehyde (MDA) equivalents (g fresh weight) $^{-1}$.[37]

*Measurement of Total Phenolics.* The total phenolics content was determined spectrophotometrically following the method of [38] Briefly, 0.1 g of fresh leaves and roots were extracted with 5 mL of an ethanol/acetone/water mixture (5/4/1) for 12 h at room temperature. After centrifugation of the extract at 10,000 *g* for 15 min, 0.1 mL of the supernatant was mixed with Folin-Ciocalteu phenol reagent (0.25 mL) and then after 1 min, with 3 mL of 5% $Na_2CO_3$ solution. The sample was allowed to stand for 1 h in the dark, after which the absorbance at 760 nm was

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4 measured. Total phenolics content of the sample was expressed as mg of gallic acid equivalents
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7 (GAE) (g fresh weight)$^{-1}$.

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11 **Element Analyses.** At harvest, the plants were washed first with water and then with deionized
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13 water to remove residual particles. After removal of the surface water, the leaves, stems and roots
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15 were weighed. A portion of the tissues was dried at 80 °C for 72 h and ground into powder for
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17 metal content analysis. Briefly, 0.01 g of dried tissue was digested in a mixture of 6 mL $H_2O_2$ and 4
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19 mL $HNO_3$ at 160°C for 40 min using a microwave digester (Milestone Ethos Up, Italy). The
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21 resulting suspension was filtered through a membrane with 0.22-μm pore size. Inductively coupled
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23 plasma-mass spectrometry (ICP-MS, NexION-300, PerkinElmer, USA) was used to measure the Cd
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25 and Zn concentrations of the extracts. K, Ca, Mg, and Fe concentrations were quantified using ICP
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27 with optical emission spectrometry (ICP-OES, Optima 5300, PerkinElmer, USA). The standard
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29 reference material (SRM 1573a, tomato leaves purchased from NIST, Gaithersburg, USA) was
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31 digested and measured using the same procedures. The recoveries of Cd and Zn were in the range of
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33 93−106%. To determine carbon, nitrogen, and sulfur concentrations in plant biomass, dried plant
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35 samples were ground in an agate mortar and passed through a 35-mesh (0.5 mm) sieve. Two mg of
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37 the ground plant tissue was analyzed using an elemental analyzer (Vario EL II Element Analyzer,
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39 Elementar, Germany) (SI **Table S2**). After removal of the plants, the soil remaining in the pot was
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41 collected, mixed thoroughly and water-extracted. The levels of water-extractable Cd and
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43 micronutrients (Zn, K, Ca, Mg, and Fe) in the soil was quantified using ICP-MS and ICP-OES (SI
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47 **Table S3**).
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**Metabolites Analysis in Leaves and Roots.** At harvest, a portion of fresh tissues was ground into powder in liquid $N_2$ and stored at −80°C for metabolite analysis by GC-MS.[29] Briefly, 60 mg of ground roots and leaves were extracted with 360 μL of cold methanol. Forty μL of 2-chloro-L-phenylalanine in methanol (0.3 mg $mL^{-1}$) was added as an internal standard and the mixture was then ultrasonicated for 30 min at room temperature. After the addition of 200 μL of chloroform, the mixture was vortexed and 400 μL of water was added. The mixture was again vortexed, ultrasonicated at room temperature for 30 min and centrifuged at 14,000 *g* for 10 min at 4°C. A quality control (QC) sample was prepared by mixing aliquots of all treatment samples based on the assumption that the QC sample contained a mean concentration of all components present in the samples under investigation. An aliquot (300 μL) of the supernatant was transferred to a glass vial and dried using a centrifugal vacuum concentrator at room temperature. Eighty μL of methoxyamine hydrochloride in pyridine solution (15 mg $mL^{-1}$) was subsequently added. The resultant mixture was vortexed for 2 min and incubated at 37°C for 90 min. Eighty μL of trifluoroacetamide containing 1% trimethylchlorosilane and 20 μL of *n*-hexane were added to the mixture, which was then vortexed for 2 min and heated at 70°C for 60 min. The mixture was allowed to stand at room temperature for 30 min before analysis by GC-MS (for details, see the SI).

**Statistical Analysis**. The relative abundances of the metabolites were analyzed using a supervised partial least squares discriminant analysis (PLS-DA) and a multivariate analysis, both run using MetaboAnalyst 4.0 (https://www.metaboanalyst.ca/). These analyses were conducted to normalize (by sum) the data by log transformation, such that individual features were more comparable.[39] A variable importance in projection (VIP) value was obtained by an unbiased estimate of the

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4 classification error of the results of the PLS-DA analysis. A VIP value > 1.5 was considered to
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7 indicate a significant impact on the metabolite of interest.[40] Biological pathways were analyzed on
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10 the basis of GC-MS data using MetaboAnalyst 4.0. Significant differences in mean plant biomass,
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12 stem length, number of leaves, photosynthetic pigments, MDA, total phenolic compounds, as well
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15 as Cd and mineral nutrient concentrations, in broad bean and soil between treatments were
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18 evaluated in a one-way ANOVA followed by a Tukey-Kramer post-hoc test, performed using SPSS
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20 24.0 software (SPSS, Chicago, USA) and plotted using GraphPad® Prism 8.0.1 (GraphPad
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23 Software Inc., USA). Differences were considered statistically significant at $p < 0.05$.
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27 **Results and Discussion**
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30 **Cd Uptake in Broad Bean.** Exposure to CdS-NPs increased the Cd content of all broad bean
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33 tissues in a dose-dependent fashion ($p < 0.05$) (SI **Figure S2A**), with roots > stems > leaves. The Cd
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35 content in leaves (1.0 ± 0.1 mg $kg^{-1}$), stems (2.3 ± 0.4 mg $kg^{-1}$), and roots (35.1 ± 7.0 mg
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38 $kg^{-1}$) in the high-dose (100 mg $kg^{-1}$) CdS-NPs treatment was nearly 27-, 30-, and 100-fold higher
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41 than in the control, respectively. Since CdSe-NPs with the size used in our study could not
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44 translocated to the leaves of plants.[9] As such, Cd detected in the leaves was mostly from dissolved
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46 $Cd^{2+}$ from CdS particles, which could be changed by the environment of the rhizosphere, such as
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49 pH and root exudates,[41] whereas Cd in the roots could be attributed to both $Cd^{2+}$ and CdS particles.
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52 Previous study has showed that CdS-NPs (< 10 nm) in aqueous medium could be translocated to
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54 xylem/phloem of root of woody plants by endocytosis.[13] The concentration of water-extractable Cd
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57 in soil was significantly higher in 100 mg $kg^{-1}$ dose of CdS-NPs than in the control soil ($p < 0.05$)
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60 (SI **Table S3**). Because water-soluble Cd represents the readily bioavailable proportion of Cd, the

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4 higher bioaccessibility of Cd indicated a greater accumulation of Cd in broad bean tissues of the
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7 100 mg CdS-NPs $kg^{-1}$ treatment.
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11 **Photosynthetic Pigments and Biomass of Broad Bean.** The Chl *a*, Chl *b* and carotenoid content
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14 of plants exposed to different doses of CdS-NPs are shown in SI **Figure S4A**. Chl *b* levels were
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17 unchanged by either dose of CdS-NPs. Chl *a* and carotenoid concentrations were significantly
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19 increased in plants exposed to 10 mg CdS-NPs $kg^{-1}$ ($p < 0.05$) whereas 100 mg CdS-NPs $kg^{-1}$ had
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22 no significant effect on either parameter (SI **Figure S4A**). A previous study reported the complete
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25 inhibition of Chl biosynthesis in *A. thaliana* growing in hydroponic culture with exposure to 80 mg
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27 $L^{-1}$ CdS-NPs.[8] The mechanism by which the lower (10 mg $kg^{-1}$) but not the higher (100 mg $kg^{-1}$)
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30 dose of CdS-NPs increased the Chl *a* and carotenoids contents of the plants is unclear but it may
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33 involve hormesis; this phenomena was previously demonstrated for wheat plants (*Triticum aestivum*
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35 L.) grown in soil contaminated with Cd at 3.3 mg $kg^{-1}$.[42] However, broad bean phenotype was
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38 unaffected by CdS-NPs in the current study; neither tissue biomass nor stem length was changed by
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41 particle exposure (SI **Figure S3**).
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45 **Lipid Peroxidation and Total Phenolic Compounds.** MDA is a byproduct of lipid peroxidation.[43]
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48 As shown in SI **Figure S4B**, the MDA content in leaves and roots was not significantly affected by
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50 exposure to CdS-NPs, indicating that neither dose induced measurable lipid peroxidation after 28 d
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53 of exposure. Phenolic compounds are non-enzymatic antioxidants in plants that play a key role in
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56 antioxidative defense.[44] CdS-NPs exposure had no effect on the content of total phenolic
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59 compounds in plant roots relative to controls (SI **Figure S4C**). However, exposure of leaves to 100
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mg CdS-NPs $kg^{-1}$ resulted in a significant decrease in phenolic content as compared to the 10 mg CdS-NPs $kg^{-1}$ treatment ($p < 0.05$) but no significant difference as compared to the control. The latter could be attributed to hormetic effects of plant responses to pollutants,[45] which is a concentration–response phenomenon characterized by low-dose stimulation and has been observed in a wide range of taxonomic groups (including microbes, plants, and animals), i.e., in our study, lower dose of CdS-NPs had a positive effect to induce production of phenolic compounds in leaves of broad bean, The induction of oxidative stress by the higher dose could be due to $Cd^{2+}$ ions released from the particles, as supported by the Cd tissue content data above, because a decrease in the content of phenolic compounds at the $Cd^{2+}$ concentration of 7.0 mg $L^{-1}$ was also observed in tea leaf calli.[46] A long-term exposure to CdS-NPs would have visible effects on lipid peroxidation and total phenolic compounds, because of the continuous dissolution of $Cd^{2+}$ from CdS-NPs, which needs further investigation.

**Macro- and Micro-nutrients in Broad Bean.** Mineral nutrients are essential for plant metabolism. While CdS-NPs did not significantly affect the content of K, Ca, Mg, and Fe in the leaf, stem or root tissues (SI **Table S2**), the Zn content was significantly increased in roots and leaves by 12.5% and 16.2%, respectively ($p < 0.05$) upon exposure to 100 mg $kg^{-1}$ CdS-NPs (SI **Figure S2B**). Zn and Cd exert antagonistic effects in terms of their accumulation in plants,[47] and plants have been shown to alleviate Cd-induced toxicity by preferentially taking up Zn.[48] This may explain the accumulation of Zn in the broad bean plants, particularly given that the amount of water-extractable Zn in the soil of plants exposed to 10 and 100 mg CdS-NPs $kg^{-1}$ decreased significantly (SI **Table S3**) by 26.2% and 57.8%, respectively. In addition, Zn has a role in stabilizing and protecting

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biomembranes against oxidative and peroxidative damage.[49, 50] In fact, the accumulation of Zn in plant tissues to alleviate Cd-induced stress may explain why plant biomass was unchanged (SI **Figure S3**) and lipid peroxidation was not induced (SI **Figure S4B**), in spite of the increase in the Cd content of the plants' tissues (SI **Figure S2A**).

**Metabolomics of Broad Bean Roots.** A GC-MS-based non-target metabolomics analysis identified and semi-quantified 242 metabolites in the roots of broad bean plants. The PLS-DA loading plot shows that the metabolites of plants under the two CdS-NPs treatments were clearly separated from those of the control (**Figure 1**), indicating that CdS-NPs altered the plant root metabolite profile. The PLS-DA model provides VIP score for all metabolites, with a VIP > 1.5 indicative of compounds most responsible for the separation.[51] SI **Figure S5A** lists the 31 metabolites with a VIP score >1.5. The levels of metabolites in roots that were significantly changed by CdS-NPs were identified with a univariate (independent *t*-test) and subsequent multivariate analysis (**Table 1**).

The levels of a number of ROS scavenging root metabolites were significantly affected by both doses of CdS-NPs, including *N*-acetyl-5-hydroxytryptamine, noradrenaline and 2-hydroxybutanoic acid. *N*-acetyl-5-hydroxytryptamine levels increased 2.7-fold and 1.5-fold in the roots of broad bean plants exposed to 10 and 100 mg CdS-NPs $kg^{-1}$, respectively, compared to the control (**Table 1**). *N*-acetyl-5-hydroxytryptamine is the precursor of *N*-acetyl-5-methoxytryptamine, a compound that directly scavenges the ROS induced by various stress conditions.[52, 53] Therefore, the up-regulation of *N*-acetyl-5-hydroxytryptamine suggests that CdS-NPs triggered the overproduction of ROS in broad bean roots. By contrast, 10 and 100 mg CdS-NPs $kg^{-1}$ decreased the noradrenaline content of

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4 the roots significantly by 50% and 70%, respectively (**Table 1)**. Noradrenaline, the downstream
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7 product of dopamine, is a scavenger of free radicals in plants and may thus cause a decrease in the
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10 photosynthetic reduction of oxygen.[54] A down-regulation of noradrenaline may therefore reflect an
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12 imbalance of ROS production and scavenging. 2-Hydroxybutanoic acid stimulates the production of
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15 4-diaminobutanoic acid (GABA), a powerful antioxidant that also scavenges ROS.[55, 56] While
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18 2-hydroxybutanoic acid was not detected in the control, its levels in the roots of broad bean plants
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20 were markedly increased (**Table 1**). Taken together, the up-regulation of
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23 *N*-acetyl-5-hydroxytryptamine and 2-hydroxybutanoic acid and the down-regulation of
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26 noradrenaline appears to be part of a systematic defense strategy employed by broad bean against
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29 CdS-NPs-induced oxidative stress. The up-regulation of antioxidants may have contributed to the
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31 unchanged MDA content of the roots, as well as the overall lack of effect on biomass.
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35 High-dose CdS-NPs exposure also caused significant changes in the levels of a number of amino
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38 acids and their derivatives. For example, *O*-acetylserine, cysteinylglycine, L-glutamic acid,
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41 *N*-methyl-L-glutamic acid and glutamine increased by 10−160%, and *N*-ethylglycine,
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44 *N*-methyltryptophan, glycine-proline, and *N*-methyl-DL-alanine were decreased by 30−100% (**Table**
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46 **1**). Amino acids are not only the building blocks of proteins but also play key roles in a number of
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49 physiological pathways. Thus, the up- and down-regulation of amino acids and their derivatives
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52 may signal the alteration of protein biosynthesis and catabolism. Cysteinylglycine is a degradation
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55 product of glutathione (GSH), a soluble antioxidant that removes ROS in plants.[57] Thus, an increase
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57 in this amino acid derivative in the exposed plants suggests that CdS-NPs induced oxidative stress
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60 and therefore, a degradation of GSH. Glutamic acid and glutamine are related to primary nitrogen

metabolism. Their up-regulation in broad bean treated with high-dose CdS-NPs suggests that primary nitrogen metabolism in the plants was not inhibited by the particles. In addition to amino acids and their derivatives, the N-containing compounds urea, uracil, maleimide, adipamide and thioctamide were significantly increased in broad bean roots exposed to a high dose of CdS-NPs (**Table 1**). Together, these results indicate that CdS-NPs disrupted nitrogen metabolism in the exposed plants. Urea is a nitrogen source for bacteria, algae, fungi, and plants and is involved in nitrogen fixation in legumes.[58] Accordingly, the impact of CdS-NPs on nitrogen fixation merits further study.

The levels of metabolites involved in carbohydrate metabolism were also significantly changed by CdS-NPs exposure. In plants exposed to 100 mg CdS-NPs $kg^{-1}$ (**Table 1)**, glucose, ribose, and threitol levels decreased by 40−80%, whereas palatinitol, sedoheptulose, and turanose levels were significantly up-regulated (**Table 1**). Sugars play an important role in signal transduction, stress defenses, and energy metabolism,[59, 60] while sugar alcohols serve as energy conservation compounds.[61] Thus, the down-regulation of sugar levels and the up-regulation of sugar alcohol levels indicate that the plants use the analyzed sugars as an energy source in their stress defense responses, resulting in an accumulation of the respective high-energy compounds. Similarly, the levels of succinic acid, an intermediate of the TCA cycle that provides the carbon skeleton for carbohydrate biosynthesis, increased significantly by 50% and 20% in plants exposed to 10 and 100 mg $kg^{-1}$ CdS-NPs, respectively (**Table 1**). In summary, CdS exposure impacted both nitrogen and carbon metabolisms in the roots of broad bean plants cultivated in soil.

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**Metabolomics of Broad Bean Leaves.** In total, 275 metabolites in broad bean leaves were identified and semi-quantified by GC-MS. Similar to the roots, the PLS-DA loading plot shows that both CdS-NPs treatments were clearly separated from the control along PC1, which explained 14.7% of the total variance (**Figure 1**). Interestingly, this result suggests that although the leaves were not directly exposed to CdS-NPs, the particles significantly altered the metabolite profile of bean leaves. The 87 metabolites that changed significantly are listed in SI **Table S4**, and the 28 metabolites with a VIP score > 1.5 are shown in SI **Figure S5B**. Flavone, putrescine, and noradrenaline are three metabolites associated with ROS removal and antioxidative defense and their levels increased significantly in response to both doses of CdS-NPs (**Figure 2**). Levels of the flavonoid flavone, which has a high ROS-scavenging ability,[62] were increased 13- and 39-fold, respectively, following exposure of the plants to 10 and 100 mg CdS-NPs $kg^{-1}$ (**Figure 2**). Flavonoids play different roles in the ecology of plants, including as stress protectants and signaling molecules.[17, 63-65] In addition, the levels of the common polyamine putrescine increased 30% in leaves exposed to the high dose of CdS-NPs compared to the control ($p > 0.05$) (**Figure 2**). Putrescine scavenges ROS, activates antioxidants and protects biomembranes and biomolecules, by inducing metal chelation.[66] Noradrenaline levels in leaves were also significantly increased (SI **Table S4**). As discussed above, noradrenaline exhibits antioxidative activity, in addition to its participation in many cellular processes.[54] Taken together, the up-regulation of three antioxidants in broad bean leaves indicate active detoxification by the plants to achieve oxidative equilibrium and thus alleviate the oxidative stress induced by CdS-NPs. This high level of response explains the absence of oxidative damage and phenotypic changes in the leaves of exposed plants.

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4 Similar to roots, significant changes in amino acids and their derivatives were also observed in the leaves. The levels of alanine, glutamic acid, norleucine, cycloleucine, ornithine and oxoproline increased significantly in response to 100 mg CdS-NPs $kg^{-1}$ as compared to the control ($p < 0.05$) (**Figure 2**). An increase in amino acid levels in soybean leaves exposed to CdS-NPs (size < 10 nm) as determined by liquid chromatography coupled to MS was previously reported.[11] The amino acid glutamic acid is involved in ammonia assimilation,[67] and its levels increased 1.8-fold under high-dose CdS-NPs exposure (**Figure 2**). The levels of ornithine, the major nitrogenous compound in nodulated and non-nodulated plants,[68] increased 6.6-fold under high-dose CdS-NPs exposure (**Figure 2**). Allantoic acid is a component of the soluble nitrogen pool of nodulated plants,[68] and its levels increased 4.2-fold under high-dose CdS-NPs exposure (**Figure 2**). These data suggested a positive impact of CdS-NPs on plant nitrogen metabolism, and although the mechanisms are unclear, the increased synthesis of storage proteins may provide a means of detoxifying these particles.

High-dose CdS-NPs also significantly increased the levels of 2,4-diaminobutanoic acid and 3-aminobutanoic acid in broad bean leaves (**Figure 2**). These two compounds are derivatives of GABA, a non-protein amino acid. In addition, the levels of both a GABA degradation product (4-hydroxybutanoic acid) and a GABA precursor (putrescine) increased significantly ($p < 0.05$) in leaves exposed to CdS-NPs (**Figure 2**). The up-regulation of these four GABA-related compounds has previously been shown to be part of an active plant response to biotic and abiotic stresses.[64, 69, 70] Cysteinylglycine in leaves (SI **Table S4**) increased by 12% in response to 100 mg CdS-NPs $kg^{-1}$, very similar to the results in the roots (**Table 1**). As mentioned above, cysteinylglycine is the

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4 degradation product of the antioxidant GSH. Thus, it's up-regulation suggests that CdS-NPs
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7 activated GSH to remove excess amounts of ROS in both the roots and leaves of broad bean plants.
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11 Nitrogen and carbon metabolism are linked through the TCA cycle. High-dose CdS-NPs
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14 up-regulated several TCA cycle intermediates in the leaves, including isocitric acid, fumaric acid,
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16 itaconic acid and maleic acid (**Figure 2**),[71-73] indicating enhanced activation of the TCA cycle in the
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19 exposed plants.
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23 Nicotinic acid is a key component of plant pathways involved in redox homeostasis and stress
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26 signaling.[74] The up-regulation of nicotinic acid in broad bean plants exposed to 100 mg CdS-NPs
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29 $kg^{-1}$ (**Figure 2**) suggests a defense response of the leaves to oxidative stress induced by the
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32 particles. This conclusion is supported by the significant increase of the antioxidant
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34 4-hydroxybenzoic acid in the leaves of exposed plants (**Figure 2**).
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38 **Pathway Disruption in Broad Bean**. A pathway enrichment analysis was performed using the
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41 online tool MetaboAnalyst 4.0. The results are shown in **Figure 3**. In the roots, two biological
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44 pathways, glyoxylate/dicarboxylate metabolism and tyrosine metabolism, were significantly
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46 changed in plants exposed to the higher dose of CdS-NPs ($p < 0.05$) (**Figure 3A**). This finding is
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49 consistent with the above-discussed disruption of carbon and nitrogen metabolism in the roots of
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52 treated plants (summarized in **Figure 4A**).
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56 In the leaves, pathway analysis revealed that six metabolic pathways were significantly ($p < 0.05$)
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59 disrupted (**Figure 3B**): phenylpropanoid biosynthesis; pantothenate and CoA biosynthesis; purine
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metabolism; alanine, aspartate, and glutamate metabolism; β-alanine metabolism; and carbon fixation. The larger number of disturbed metabolic pathways in the leaves than in the roots was unexpected, given that the leaves were not directly exposed to the CdS-NPs. Thus, low-molecular-weight metabolites would seem to be a more sensitive endpoint than phenotypic parameters in toxicity assessments.[28] Among these six biological pathways, the phenylpropanoid pathway is involved in all plant responses to biotic and abiotic stresses and in the activation of plant defense systems,[75] which in this study included the production of a variety of antioxidative metabolites in leaves stressed by CdS-NPs exposure (**Figure 4B**). Three pathways related to nitrogen metabolism (purine metabolism, alanine, aspartate, and glutamate metabolism and β-alanine metabolism) and two related to carbon metabolism (pantothenate and CoA biosynthesis and carbon fixation) were also activated. Taken together, the analysis shows that CdS-NPs, especially at the higher dose, significantly activated the antioxidative defense system of broad bean plants and also impacted carbon and nitrogen metabolism in their leaves, which markedly more sensitive than the roots to CdS-NPs-induced stress.

**Environmental Implications.** Although none of the phenotypic parameters of broad bean plants (photosynthetic pigments contents, biomass, and lipid peroxidation) were overtly impacted in response to CdS-NPs in soil during 28 d of exposure, metabolomics revealed marked and statistically significant alterations in the metabolite profiles of plant roots and leaves. The reprogramming of antioxidant metabolite production presumably reflected the molecular defense response of the plants to CdS-NPs stress. The sensitive responses of flavone, putrescine and noradrenaline in the leaves suggests the use of these compounds in legumes as biomarkers of

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4 oxidative stress induced by the presence of CdS-NPs in soil. Metabolomics might thus be a suitable
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7 approach for the early detection of soil contamination by Cd.
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11 In the plants, the reprogramming of carbon and nitrogen metabolism (including sugars, organic
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14 acids, amino acids, and N-containing compounds) alleviated the toxicity of CdS-NPs, which may
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16 have been caused by free $Cd^{2+}$ ions or perhaps by a particle-specific response. Importantly, this
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19 response might adversely impact crop yield and quality in plants under long-term exposure to
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22 CdS-NPs.
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41 ## Notes
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44 The authors have no conflicts of interest to declare.
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48 ## Supporting Information
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50 Details on the methods and discussion. Tables showing the physiochemical properties and metal
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53 concentration of the experimental soil (Table S1), the concentration of mineral nutrients in plant
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56 tissues (Table S2), the concentration of water-extractable metals and nutrient elements in soil (Table
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59 S3), and the regulated metabolites in leaves in plants exposed to CdS-NPs-amended soil for 28 days
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4 (Table S4). Figures showing the characterization of CdS-NPs (Figure S1), tissue concentrations of
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7 Cd and Zn (Figure S2), the fresh biomass of tissues, stem length, number of leaves (Figure S3), the
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10 levels of photosynthetic pigments, MDA and total phenolic compounds in tissues (Figure S4) and
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12 VIP scores from the PLS-DA analysis of metabolites in roots and leaves (Figure S5).
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**Table 1.** Selected metabolites in roots of broad bean plants exposed for 28 days to soil containing10 and 100 mg CdS-NPs $kg^{-1}$. Data are reported as the mean and standard deviation ($n = 4$).

| Metabolite | VIP | CdS-NPs concentration (mg $kg^{-1}$ soil) | | | |
|---|---|---|---|---|---|
| | | 10 | 100 | 10 | 100 |
| | | *p* | | Fold change[a] | |
| **Amino acids and derivatives** | | | | | |
| *O*-Acetylserine | 0.26 | 0.004 | 0.186 | 1.6 | 1.2 |
| Cysteinylglycine | 1.91 | 0.751 | 0.620 | 0.8 | 1.2 |
| *N*-Ethylglycine | 1.60 | 0.046 | 0.112 | 0.7 | 0.6 |
| Glycine-proline | 3.31 | 0.941 | 0.192 | 0.9 | 0.5 |
| Glutamine | 0.08 | 0.014 | 0.799 | 1.5 | 1.1 |
| L-Glutamic acid | 1.95 | 0.986 | 0.252 | 1.0 | 2.6 |
| *N*-Methyl-DL-alanine | 1.90 | 0.258 | 0.243 | 0.8 | 0.7 |
| *N*-Methyl-L-glutamic acid | 1.52 | 0.524 | 0.469 | 2.0 | 2.4 |
| *N*-Methyltryptophan | 2.20 | 0.799 | - | 1.3 | ND[b] |
| **N-containing compounds** | | | | | |
| Adipamide | 3.54 | 0.643 | 0.035 | 0.6 | 3.2 |
| *N*-Acetyl-5-hydroxytryptamine | 2.79 | 0.039 | 0.141 | 2.7 | 1.5 |
| 2'-Deoxyadenosine | 2.55 | 0.519 | 0.043 | 1.1 | 0.7 |
| 2,4-Diaminobutanoic acid | 1.87 | 0.909 | - | 0.9 | ND |
| DL-Dihydrosphingosine | 1.55 | - | - | NM[c] | NM |
| 3-Hydroxypyridine | 3.26 | 0.268 | 0.071 | 0.7 | 0.4 |
| Isoxanthopterin | 0.43 | 0.371 | 0.036 | 0.8 | 0.6 |
| Maleimide | 0.26 | 0.055 | 0.002 | 0.8 | 1.4 |
| 6-Methylmercaptopurine | 0.82 | 0.015 | 0.467 | 0.3 | 1.1 |
| Noradrenaline | 2.16 | 0.020 | 0.008 | 0.5 | 0.3 |
| Thioctamide | 3.69 | 0.707 | 0.017 | 1.5 | 4.4 |
| Uracil | 0.34 | 0.998 | 0.008 | 1.0 | 1.6 |
| Urea | 1.70 | 0.806 | 0.654 | 1.2 | 1.3 |
| **Sugars and sugar alcohols** | | | | | |
| Dihydrocarvone | 0.42 | 0.694 | 0.037 | 0.8 | 1.7 |
| Glucose | 1.79 | 0.589 | 0.259 | 1.1 | 0.6 |
| Palatinitol | 0.49 | 0.066 | 0.008 | 1.3 | 1.9 |
| Ribose | 0.35 | 0.256 | 0.046 | 0.8 | 0.6 |
| Sedoheptulose | 2.08 | 0.536 | 0.209 | 1.4 | 2.2 |
| Threitol | 5.00 | 0.709 | 0.018 | 1.0 | 0.2 |
| Turanose | 1.63 | 0.514 | 0.804 | 1.4 | 1.1 |
| **Organic acids** | | | | | |
| Benzoylformic acid | 1.84 | - | - | NM | NM |
| Digalacturonic acid | 0.15 | 0.022 | 0.246 | 1.2 | 1.2 |
| 2-Hydroxybutanoic acid | 1.44 | - | - | NM | NM |
| 3-Hydroxypropionic acid | 1.51 | 0.380 | 0.273 | 1.4 | 1.5 |
| 2-Ketovaleric acid | 1.49 | 0.175 | 0.108 | 1.2 | 0.6 |
| Toluenesulfonic acid | 1.84 | - | 0.724 | ND | 1.5 |
| Succinic acid | 0.19 | 0.021 | 0.054 | 1.5 | 1.2 |
| **Others** | | | | | |
| Benzoin | 1.61 | 0.944 | 0.464 | 1.0 | 1.3 |
| 1-Hydroxyanthraquinone | 0.09 | 0.022 | 0.607 | 0.6 | 0.9 |
| 21-Hydroxypregnenolone | 2.33 | - | - | NM | NM |
| 3-Methylamino-1,2-propandiol | 0.05 | 0.024 | 0.018 | 0.8 | 0.8 |

| | | | | | |
|---|---|---|---|---|---|
| Naphthalene | 2.44 | 0.299 | 0.131 | 4.0 | 0.2 |
| Octanal | 1.72 | - | - | NM | NM |
| Phosphate | 2.21 | 0.273 | 0.568 | 0.9 | 0.8 |
| 2,4,6-Trihydroxybenzophenone | 3.31 | 0.261 | 0.173 | 4.6 | 4.4 |

[a]Relative abundance of metabolites in roots in the 10 and 100 mg CdS-NPs $kg^{-1}$ treatments compared with the control.

[b]New metabolites appeared in both CdS-NPs treatments.

[c]Not detected.

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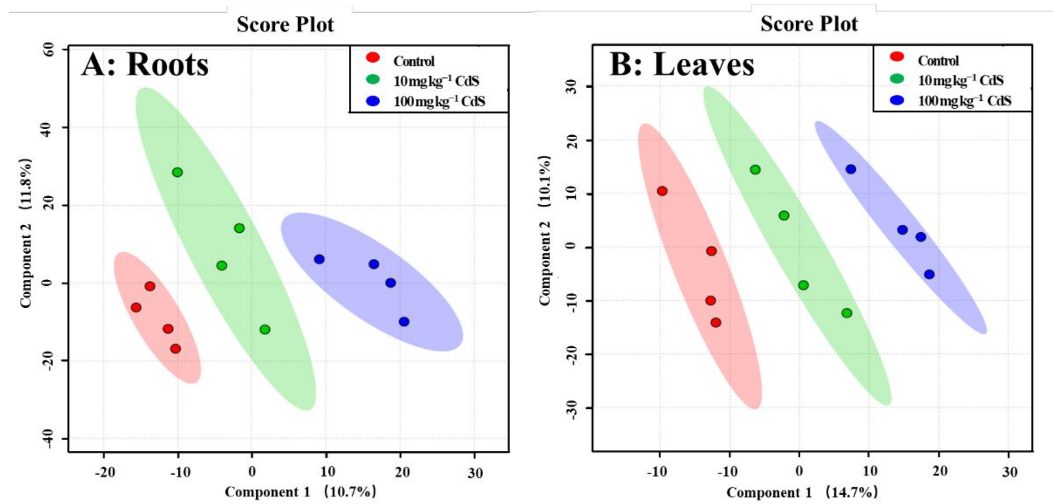


**Figure 1.** Score plot of partial least squares discriminant analysis (PLS-DA) of metabolites in roots (A) and leaves (B) of broad bean plants exposed for 28 days to two different concentrations of CdS-NPs. Data are reported as the mean and standard deviation ($n$ = 4).

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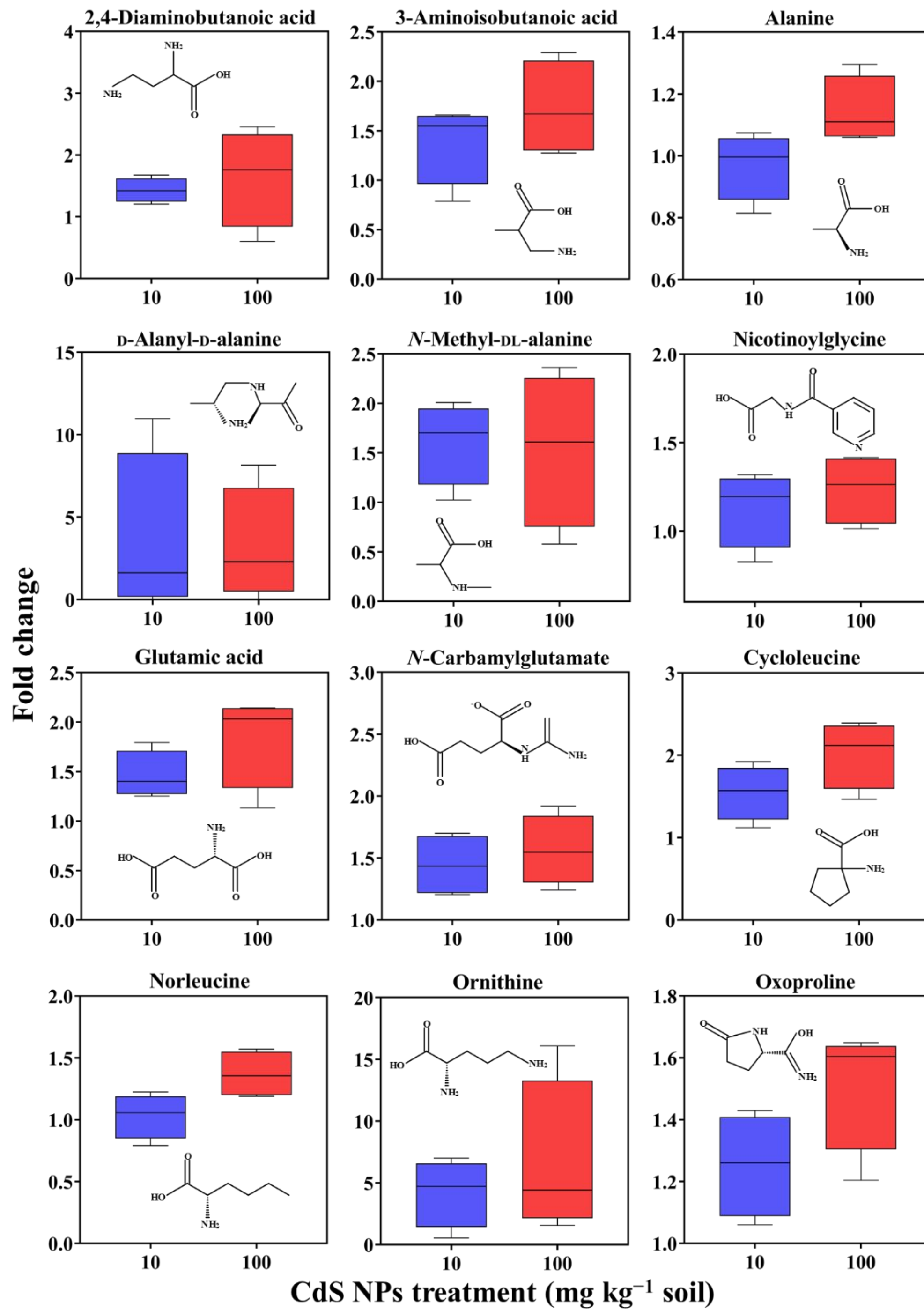

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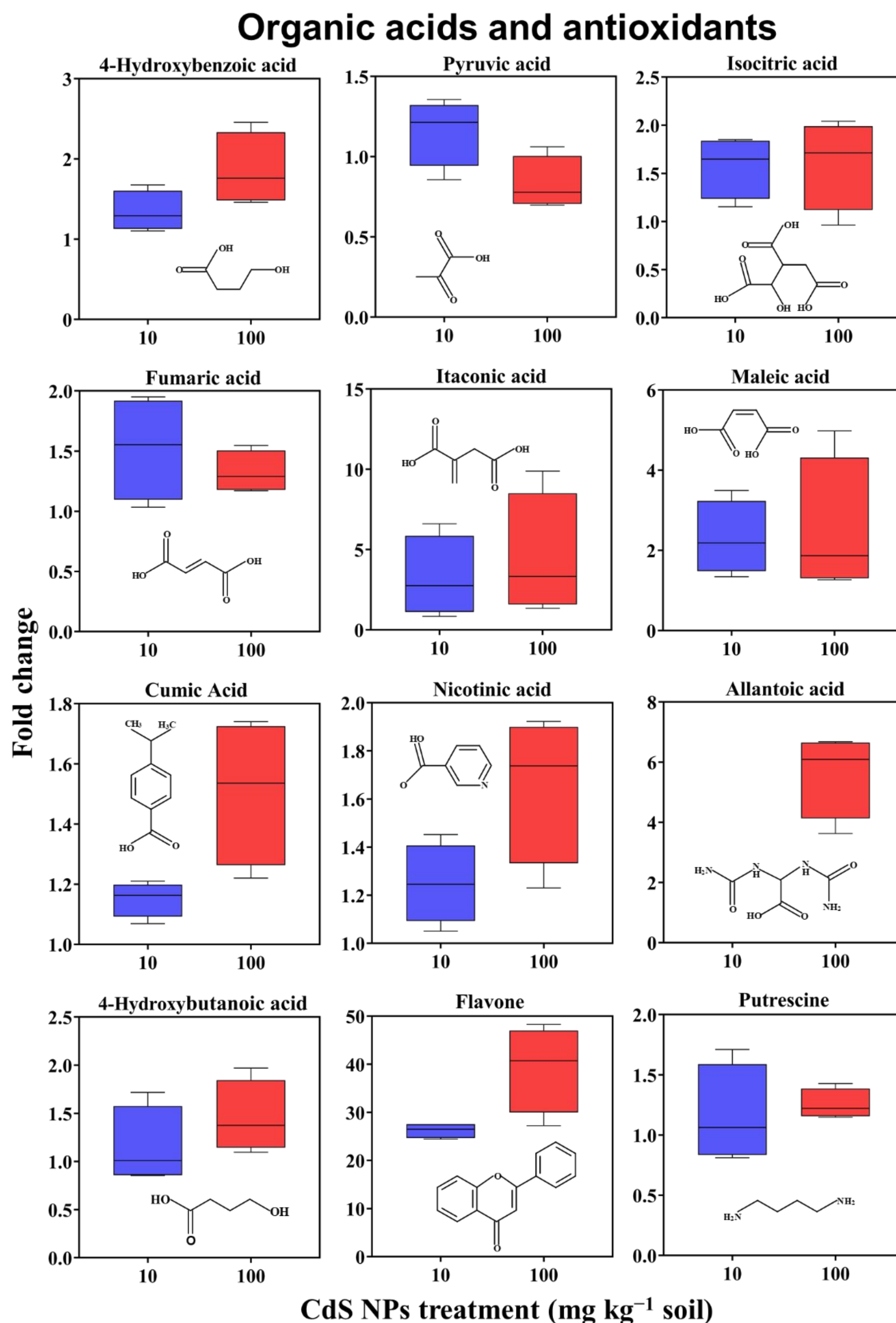


**Figure 2.** Relative changes in the abundances of metabolites in the leaves of broad bean plants exposed for 28 days to soil containing 10 and 100 mg CdS-NPs kg$^{-1}$. The results are expressed as the fold change compared to the control treatment. Data are reported as the mean and standard deviation ($n$ = 4).

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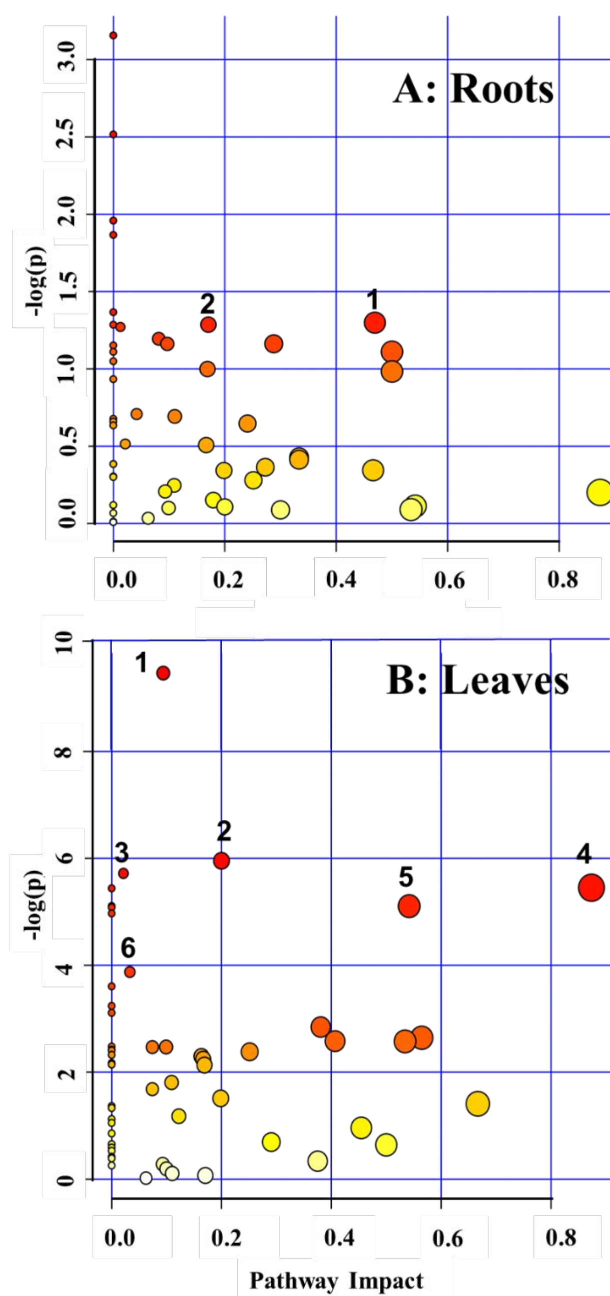


**Figure 3.** Pathway analysis using MetaboAnalyst 4.0 for broad bean roots (A) and leaves (B) exposed to 100 mg CdS-NPs $kg^{-1}$ soil. All detected metabolites were considered in the pathway analysis. Altered pathways in roots (A): glyoxylate and dicarboxylate metabolism (1) and tyrosine metabolism (2). Altered pathways in leaves (B): phenylpropanoid biosynthesis (1), pantothenate and CoA biosynthesis (2), purine metabolism (3), alanine, aspartate, and glutamate metabolism (4), β-alanine metabolism (5) and carbon fixation (6).

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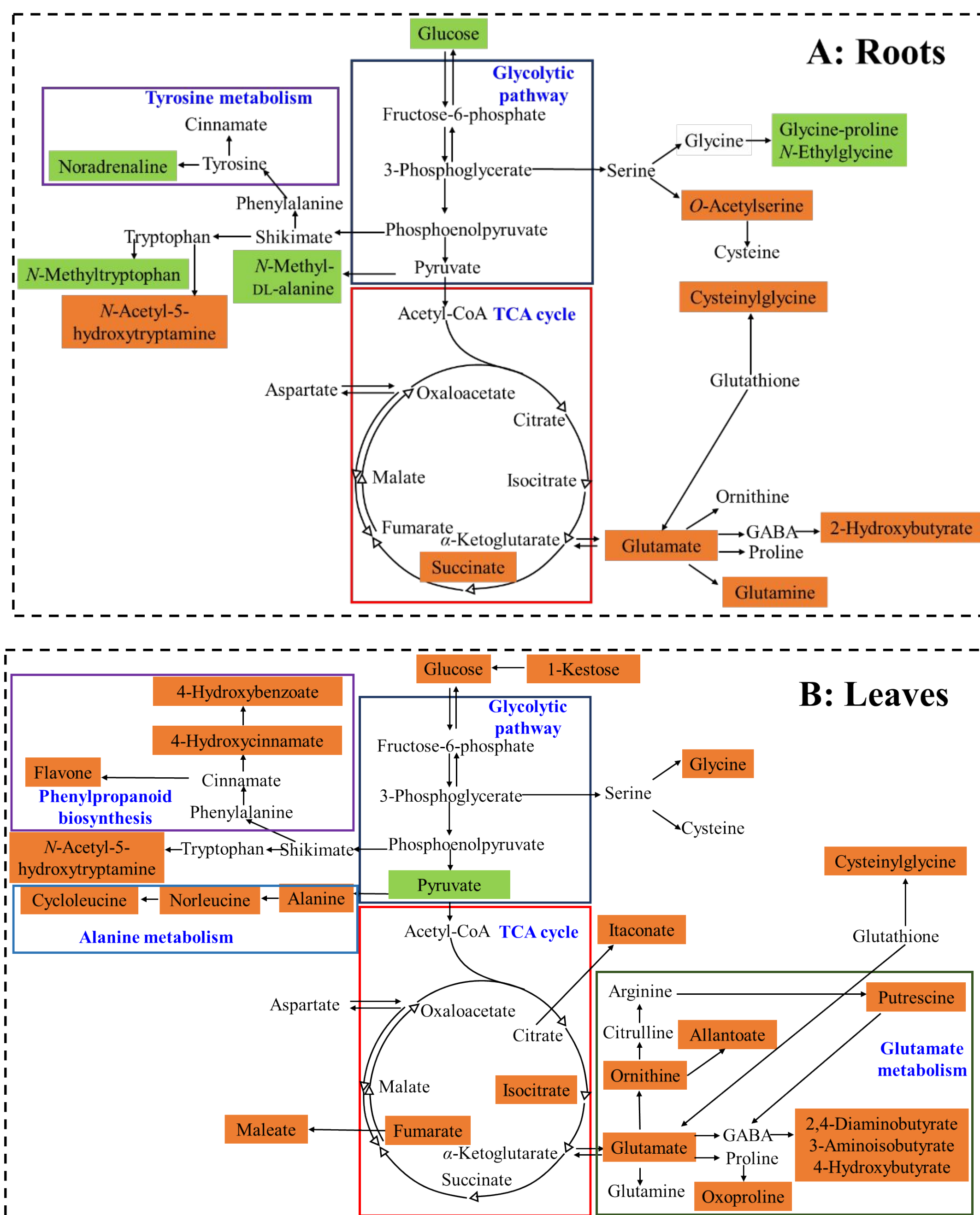


**Figure 4.** Proposed metabolic changes in the roots (A) and leaves (B) of broad bean plants exposed to **soil containing 100 mg** CdS-NPs **kg$^{-1}$**. Orange- and green-shadings represent up-regulated and down-regulated metabolites, respectively.

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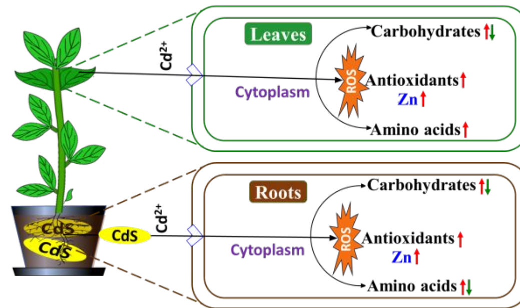


**TOC**

## Novelty:

Plants reprogram metabolite profiles of antioxidative defense system to alleviate CdS nanoparticles-induced toxicity, at the cost of carbon and nitrogen assimilation.