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Effect of Adaptation to High Concentrations of Cadmium on Soil Phytoremediation Potential of the Middle European Ecotype of a Cosmopolitan Cadmium Hyperaccumulator *Solanum nigrum* L.

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Featured Application: The Middle European ecotype of Cd hyperaccumulator *Solanum nigrum* L. ssp. nigrum was found to show extraordinarily strong tolerance to high contents of Cd in soil (over 50 mg kg^{-1} Cd) and high Cd accumulation capacity at this concentration range. Its adapted A50 variety obtained from the seeds of first-generation plants grown in soil with 50 mg kg^{-1} Cd appeared to display further considerable enhancement of resistance to Cd stress, accumulation capacity, and healthy state. This makes the Middle European ecotype and its adapted variety A50 particularly useful to sustainable decontamination of heavily polluted "hot spots" in degraded post-industrial areas.

Abstract: The Cd hyperaccumulator *Solanum nigrum* L. exhibits a cosmopolitan character and proven high and differentiated efficiency. This suggests the possibility of optimizing its Cd phytoremediation capacity and applicability through searching among remote ecotypes/genotypes. However, the extensive studies on this hyperaccumulator have been limited to Far East (Asian) regions. Pioneer pot experiments on the Middle European ecotype of *S. nigrum* within a concentration range of 0–50 mg kg^{-1} Cd in soil revealed its Cd phytoremediation capacity to be comparable to Asian ecotypes but with a fundamentally different Cd tolerance threshold. While biomass of the Asian ecotypes declined sharply at $C_{soil} \approx 10$ mg kg^{-1} Cd, in the Middle European ecotype, a gradual mild biomass decrease occurred within the whole $C_{soil} \approx 0$–50 mg kg^{-1} Cd range with no toxic symptoms. Its adapted A50 variety was obtained from the seeds of first-generation plants grown in soil with $C_{soil} \approx 50$ mg kg^{-1} Cd. In this variety, Cd tolerance, accumulation performance, and all physiological parameters (chlorophyll, carotenoids, RuBisCO, and first- and second-line defense anti-oxidant activity) were significantly enhanced, while cell damage by ROS was considerably lesser. This makes the Middle European ecotype and its adapted variety A50 particularly useful to sustainable decontamination of heavily polluted "hot spots" in degraded post-industrial areas.

Keywords: cadmium phytoremediation; *Solanum nigrum* L.; Middle European vs. Asian ecotypes; Cd hyperaccumulative properties; hyperaccumulation mechanism differentiation; adapted variety efficiency; physiological parameter response; ecotype stress tolerance; heavily polluted soil decontamination

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1. Introduction

Cadmium (Cd) is one of the most widespread problematic non-essential priority pollutants of soils due to a multitude of historic and contemporary geogenic and anthropogenic diffuse pollution sources. These include air emissions and waste from smelting/mining

industries and fossil fuel or biomass-fired power plants, fertilizers, agrochemicals and manure, sewage sludge, waste-based soil amendments applied to soil within circular economy implementation, and often contaminated irrigation water [1]. High mobility in the environment and the long-range transmission of pollutants (LRTP) with air fluxes have caused the occurrence of high diffuse soil pollution with Cd far away from emission sources [2]. High susceptibility to accumulation in tissues of some basic agricultural products (e.g., rice and root and leafy vegetables) may increase daily Cd intake by consumers to a level exceeding daily tolerable limits, thus particularly strongly endangering human health. Widespread Cd pollution of soils has induced searching for non-invasive, efficient, and cost-effective methods for its reduction to a level ensuring food and environmental security. In farmland, this is dictated mostly by cultivated crops and their ability to accumulate Cd in the edible parts. The development in recent decades of phytoremediation methods with the use of plants that hyperaccumulate pollutants creates promising prospects for non-invasive, low-cost, operationally simple, and environmentally acceptable reductions in soil pollution. However, due to the non-essentiality and strong toxicity of Cd to plants, only scarce species display tolerance to Cd and hyperaccumulation properties. In a hyperaccumulator database from 2017, only seven species were registered as Cd hyperaccumulators [3], and few species, mostly endemic or exotic, have been added to this list in the following years. Among them, only *Solanum nigrum* L. that has been recently discovered as an efficient Cd hyperaccumulator [4] occurs as a common wild plant worldwide. Moreover, it appeared that different ecotypes of *S. nigrum* grown far away from each other display significantly different Cd tolerance and hyperaccumulation capacity, thus creating the possibility of selecting the most efficient hyperaccumulator by comparing different ecotypes [5]. Surprisingly enough, despite high interest to phytoremediation of soils polluted with Cd and hundreds of studies on *S. nigrum,* they are concentrated mostly in the Far East region, while reports originating from other parts of the world are scarce and often not related to Cd accumulation [6–14]. Simultaneously, since the beginning of the industrial revolution at the end of 18th century, Cd remains one of the most problematic pollutants in the Northern Hemisphere, and specifically in Europe, due to it having historically the longest and strongest impact of agrochemicals and emissions from heavy industry and power production. Long-range transport with LRTP strongly contributes to the diffuse pollution of agricultural lands, and Poland and Germany are currently responsible for the biggest Cd emission and deposition in many European countries [15]. In surface soils of mining areas, heavy industrial regions, urban areas, and farmlands with extensive agriculture in Europe, the USA, and Canada, Cd occurs in concentrations at the level of 10 mg kg^{-1} up to roughly 100–300 mg kg^{-1}, occasionally reaching even bigger values [1]. This leads to excessive levels of Cd in edible plants, which is dangerous to human health. Such areas, mostly thickly populated (e.g., in the Upper Silesia industrial region in Poland or in Ruhr in Germany), must be efficiently remediated. Phytoremediation appears to be the best non-invasive, cost-effective, and sustainable option, and *S. nigrum* as a common cosmopolitan weed plant with well confirmed Cd hyperaccumulative properties seems to be a perfect candidate for this purpose.

Solanum nigrum L. ssp. *nigrum* is an annual herb belonging to the large Solanaceae family, of the section *Solanum,* and is identified as a Cd hyperaccumulator. It appears to have a naturally high Cd hyperaccumulation capacity independent of soil properties and Cd content at the site of the plant's origin [4,16–18]. Simultaneously, a recent study [5] revealed that the ecotypes of the same species *Solanum nigrum* L. ssp. *nigrum* grown in different remote sites isolated from each other may show distinctly differentiated Cd hyperaccumulation capacities. This is also reflected by the different tolerance of studied *ecotypes* to Cd stress. Notwithstanding this, all different ecotypes of *S. nigrum* show diverse but strong tolerance to Cd stress and could effectively protect themselves from Cd toxicity.

The aim of this pioneer study was to assess Cd hyperaccumulating capacity of the common Middle European ecotype of *S. nigrum* that has never been studied before. The study contributes to the search for the most efficient variety of the only cosmopolitan Cd

hyperaccumulator among a very limited number of species able to hyperaccumulate this extremely toxic element. The adaptability to Cd stress of this ecotype originated from unpolluted soil was also a point of interest. It was hypothesized that its natural tolerance to Cd stress could be enhanced already in the first generation of plants growing in soil with high concentrations of Cd due to the inherited adaptation ability. This would allow to increase Cd phytoremediation capacity of *S. nigrum* without application of additional measures, e.g., ligands. This way the adapted native plants may have been used for effective phytoremediation of highly Cd-polluted areas occurring in thickly populated industrial and post-industrial regions and contribute to revival of their environmental status and sustainability.

2. Materials and Methods

2.1. Seed Selection, Soil Characteristics and Experimental Design

For the experiment, seeds of the Middle European ecotype of *Solanum nigrum* L. ssp. *nigrum* were collected in the City Botanical Garden in Zabrze (Upper Silesia, Poland) (50°17′45.81″ N 18°45′52.37″ E). These seeds, originated from plants grown freely in the natural unpolluted soil (C_{soil} = 0.22 mg kg^{-1} Cd) as weeds, were used as a primary experimental material (N0). Besides, the seeds from the same plants but adapted to high Cd pollution through growing in the soil treated with 50 mg kg^{-1} Cd in the previous year (first-generation adapted variety A50) were selected for a comparative study.

The experiment was carried out at the Institute of Environmental Engineering, Polish Academy of Sciences in Zabrze. The topsoil (0–20 cm) was collected from the area of seeds origin and analyzed according to the standard methods of soil analysis [19]. The soil was slightly acidic (pH 6.57), total organic carbon TOC 16.73 g kg^{-1}, total N 0.73 g kg^{-1}, available P 15.33 mg kg^{-1}, available K 189.05 mg kg^{-1}, Cd 0.22 mg kg^{-1}, Pb 17.15 mg kg^{-1}, and Zn 37.88 mg kg^{-1}. Cadmium (Cd) and other PTEs—Potentially Toxic Elements (Pb, Zn) represented a low natural level. The tested soil was brown soil according to the World Reference Base for Soil Resources WRB (IUSS Working Group WRB 2022) [20] classification, of a loamy texture.

After sieving through a 1 mm sieve and homogenizing, the soil naturally dried in the open air was divided into 20 kg DW portions, next spiked with $CdCl_2$ 2.5 H_2O (added as a solution of superior pure reagent >99.99%, Sigma-Aldrich, St. Louis, MO, USA) and equilibrated for three months. In total, an untreated control sample (T0, 0.22 mg kg^{-1} Cd) and four Cd treatments (with nominal concentrations 10, 20, 30, 50 mg kg^{-1} Cd) were applied. The actual measured Cd concentrations in the homogenized and equilibrated soil are given in Table 1. The research was conducted as a pot experiment, in three independent biological replicates.

Table 1. Effects of different Cd treatments on the biomass of the Middle-European ecotype of *S. nigrum* L., non-adapted (N0) and adapted to Cd stress (A50).

T-Treatments mg Cd kg^{-1}	**0**	**10**	**20**	**30**	**50**
Cd_{soil} mg Cd kg^{-1}	**0.22 ± 0.08**	**10.52 ± 0.87**	**20.44 ± 1.96**	**30.09 ± 1.52**	**50.49 ± 2.29**
			Bm_R–Biomass of root (g pot^{-1} DW)		
N0	2.06 ± 0.41 aA	2.02 ± 0.62 aA	1.59 ± 0.48 aA	1.34 ± 0.85 aA	1.31 ± 0.15 aA
A50	2.00 ± 0.28 aA	1.89 ± 0.08 aA	1.43 ± 0.37 aA	1.38 ± 0.70 aA	1.41 ± 0.33 aA
			Bm_S–Biomass of shoot (g pot^{-1} DW)		
N0	13.00 ± 3.51 aA	11.90 ± 2.11 aA	11.00 ± 1.29 aA	8.26 ± 3.96 aA	9.65 ± 0.68 aA
A50	12.01 ± 1.80 aA	10.86 ± 3.69 aA	9.98 ± 1.51 aA	9.08 ± 4.40 aA	11.38 ± 1.01 aA

Note: (1) T (0 = 50) mean soil treatments with Cd (0, 10, 20, 30, 40, 50 mg Cd kg^{-1}, respectively; (2) Data in each line marked with the same capital letters are not significantly different at $p < 0.05$; Data in each column/section marked with the same lowercase letters are not significantly different at $p < 0.05$.

The homogenized and equilibrated soil of each Cd treatment was put into pots φ = 25 cm and H = 20 cm. (2.5 kg per pot, with 3 g $(NH_4)_2SO_4$ Sigma-Aldrich, St. Lois, MO, USA, added as fertilizer). For the experiment, *S. nigrum* seeds collected from plants grown in the natural unpolluted soil (N0) and in the soil treated with 50 mg kg^{-1} Cd in the previous year (A50), were sterilized with 0.1% $HgCl_2$ (with purity ≥ 99.5%, Sigma-Aldrich, St. Louis, MO, USA) for 10 min., soaked in tap water for 2 days, and sown into each pot, 20–30 seeds per pot. After sowing and moisturizing the soil up to 50% of water capacity, the pots were placed into the foliar garden tunnel. During the experiment, the pots were randomly replaced several times. The soil moisture in the pots was maintained at approximately 50–60% of the water capacity using tap water. After reaching approximately 5 cm in height, plants in each pot were thinned to 10 uniform seedlings. The experiment was ended when about 70% of the plants were in flowering stage. During the plant growth, humidity and temperature in the tunnel were continuously automatically detected (for 90 days, from June until the end of August). The *S. nigrum* plants were harvested after 90 days at the flowering stage and directed for analysis.

2.2. Experimental Methods and Sample Analysis

2.2.1. Cd Uptake, Accumulation and Translocation in *S. nigrum*

The methods of experimental procedure and sample analysis were, in general, similar to those applied in another study [5]. The harvested *S. nigrum* plants were washed three times with tap water, next with deionized water, and separated into root (underground part) and shoot (aboveground part consisting of stem, leaf and flowers). The plant material was then oven dried at 105 °C for 30 min, followed by drying at 75 °C to constant weight (with 0.0001 g accuracy). For drying, a laboratory oven (SML, ZALMED, Warsaw, Poland) was used. Biomass (Bm_n) was measured as dry weight (DW) of underground and aboveground plant parts, where n-index means root (R) and shoot (S).

The Cd concentrations in the plant material (root and shoot) were determined after grinding into fine powder in GRINDOMIX GM 200 high-speed grinder (Retsch, Düsseldorf, Germany) and open digestion of about 0.5 g of the powdered material with a mixture of concentrated nitric and perchloric acids (Merck, Darmstadt, Germany) in proportion 15:5, until the mineralization the next day, followed by quantitative filtration and Cd determination with ICP-OES (OPTIMA 2000 DV, Perkin-Elmer, Waltham, MA, USA). For quality assurance/quality control (QA/QC), the standard reference material (NIST SRM 1547, peach leaves) was used.

Soil samples were analyzed for Cd content following a similar procedure with the use. of microwave-assisted digestion (Anton Paar Multiwave 3000 SOL, Gratz, Austria). The digestion was carried out at 1400W, IR = 240 °C and pressure values p = 60 bar. After mineralization and quantitative filtration, Cd contents in soil were determined with the use of the same ICP-OES (OPTIMA 2000 DV, Perkin-Elmer, Waltham, MA, USA).

On the basis of Cd concentrations (C_n) and biomass (Bm_n) determined in the specific parts *n* of the plants, where *n*-index means root (R) and shoot (S), Cd enrichment factors of the related parts of the plants $EF_n = C_n/C_{soil}$, translocation factors $TF = C_S/C_R$, accumulated Cd loads $L_n = C_n\ Bm_n$, and load translocation factors $LTF = L_S/L_R$—from root to shoot were calculated according to Dai et al. [5,21]. In these equations, C_n is Cd concentrations in root and shoot, respectively (mg kg^{-1}), L_n is accumulated loads of Cd in root and shoot, respectively (μg pot^{-1}), Bm_n is biomass of root and shoot, respectively (g pot^{-1}).

2.2.2. Impact of Cd on the Physiological Parameters of *S. nigrum*

To assess the mechanisms of plant defense and adaptability to Cd stress, the physiological parameters were determined in fresh leaves of all *S. nigrum* samples from each pot, i.e., as the results of experiment conducted in three independent biological replicates. The analysis was performed at the Institute of Biology, University of Szczecin (Poland). In this research, a considerably broader scope of physiological parameters was investigated than that presented in other research on *Solanum nigrum* L. as Cd hyperaccumulator

(e.g., [5,21–23]). Except photosynthetic pigments, the applied methods for determination of other physiological parameters differed in some significant details (mostly in the units used) with respect to these presented in the relevant publications. This did not allow direct comparison of results, but clearly presented trends. To avoid misinterpretation, methods used for assessment of physiological parameters are presented here in more detail.

Concentrations of photosynthetic pigments were determined using Lichtenthaler and Buschmann protocol [24]. To estimate chlorophyll a, chlorophyll b and carotenoids, leaves were homogenized and extracted in 80% (v/v) acetone (Merck, Darmstadt, Germany) (leaves, FW:acetone, 1:10, w/v). After rotation and centrifugation, the supernatant was used for measurements of absorbance values at 470, 645, and 665 nm by UV-Vis spectrophotometer (ThermoFisher Scientific, Madison, WI, USA) and subsequent calculations of chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoids. The results were expressed as mg g^{-1} FW.

Superoxide anion and hydrogen peroxide (ROS) content was assessed according to the procedure described by Cembrowska-Lech [25] using dihydroethidium (DHE) (ThermoFisher, Waltham, MA, USA) for $O^{2\bullet-}$, and CDCDHFDA-AM (6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate) (ThermoFisher, Waltham, MA, USA) for H_2O_2 analysis. The labelled cells were analyzed using flow cytometer (Partec, Goerlitz, Germany) with an air-cooled 20 mV argon-ion laser. The relative $O^{2\bullet-}$ and H_2O_2 level was expressed as the mean fluorescence intensity (percentage of the control).

Enzyme extraction and western blot analysis were performed after fine grinding plant samples in liquid N_2 with the use of a Retsch MM200 (Retsch, Haan, Germany) laboratory ball mill, homogenization in the lysis buffer, and subsequent boiling and centrifugation. Samples containing 50 µg protein were loaded per line and separated on 12% SDS-PAGE gel [26]. After electrophoresis, the gels were electroblotted onto PVDF membranes (Amersham™ Protran™ 0.2 µm NC, Amersham, UK). Following triple washing in TBST (Merck, Darmstadt, Germany), the blotting membranes were incubated in a blocking solution and probed with the polyclonal antibody (Agrisera, Vännäs, Sweden): Cu/ZnSOD (AS10 652), MnSOD (AS09 524), FeSOD (AS06 125), CAT (AS09 501), APX (AS08 368), GR (AS06 181), GPX (AS06 183), RbcL (AS03 037), and RbcS (AS07 259). The membranes were then washed three times in TBST and probed with peroxidase conjugated secondary antibody (AS09 602 or AS09 603, Agrisera, Vännäs, Sweden). The immunoblots were incubated with a detection solution containing acetate buffer, diaminobenzidine (Merck, Darmstadt, Germany) and H_2O_2 (Merck, Darmstadt, Germany). The data were as immunoblot band visualization and the band intensities were determined using the Fiji ImageJ software v2.9.0 (LOCI, University of Wisconsin, Madison, WI, USA) [27].

Superoxide dismutase (EC 1.15.1.1) activity was tested according to Giannopolitis and Ries [28] by the inhibition of NBT chloride photoreduction. The assay was carried out using the following reaction mixture: 0.1 M potassium phosphate buffer (pH 7.8), 1.3 µM riboflavin, 13 mM methionine), 63 µM NBT, 0.1 mM EDTA (all reagents from Merck, Darmstadt, Germany) and 100 µL of the enzymatic extract. The reaction mixture was illuminated (50 µmol m^{-2} s^{-1}) at 25 °C for 10 min and the absorbance measured at 560 nm. One unit of SOD activity was defined as the amount of the enzyme required to inhibit the reduction of NBT by 50% under the specified conditions. SOD activity of the extracts was expressed as U mg^{-1} protein.

Catalase (EC 1.11.1.6) activity was measured according to Rao et al. [29]. The enzyme activity was monitored spectrophotometrically at 240 nm for 60 s using the following mixture: 50 mM potassium phosphate buffer (pH 7.0), 14.3 mM H_2O_2 (both reagents from Merck, Darmstadt, Germany) and 100 µL of enzymatic extract. Purified CAT (Merck, Darmstadt, Germany) was used as a calibration standard. CAT activity was expressed as U mg^{-1} protein. Data for both enzyme activities were expressed as means of independent biological replicates ± SD.

Glutathione reductase (EC 1.8.1.7) activity was analyzed as described by Esterbauer and Grill [30] by following the rate of NADPH oxidation at 340 nm for 3 min. The as-

say mixture contained: 0.1 mM potassium phosphate buffer (pH 7.8), 0.5 mM NADPH, 10 mM oxidized glutathione (GSSG), 10 mM EDTA (all reagents from Merck, Darmstadt, Germany) and 100 µL of enzyme extract. The GR activity was expressed as nmol NADPH min^{-1} mg^{-1} protein.

Glutathione peroxidase (EC 1.11.1.9) activity was assessed as described by Nagalakshmi and Prasad [31] by following the rate of NADPH oxidation at 340 nm for 5 min. The reaction mixture contained: 0.5 M potassium phosphate buffer (pH 8.2), 10 mM EDTA, 1.14 M NaCl, 10 mM GSH, 2 mM NADPH, and 2.5 mM H_2O_2 (all reagents from Merck, Darmstadt, Germany), and 100 µL of enzyme extract. The reaction was started by adding 2 U of GR (Merck, Darmstadt, Germany). The GS activity was expressed as nmol NADPH min^{-1} mg^{-1} protein.

The protein content in the enzymatic extracts was assayed by Bradford's method [32], using bovine serum albumin (BSA) (Merck, Darmstadt, Germany) as a standard. Glutathione in the reduced (GSH) and oxidized (GSSG) form were assayed following procedure described by Smith [33], which comprised grinding plant samples in liquid N_2, extraction in sulphosalicylic acid (1:10, *w*/*v*) (Merck, Darmstadt, Germany), centrifugation, and measuring absorbance values in the neutralized supernatant for total glutathione (GSH + GSSG) and in GSSG alone after GSH masking, in double-extracted and suitably incubated samples at 412 nm and 25 °C with the use of UV-Vis spectrophotometer (Thermo Fisher Scientific, Madison, USA). The results were expressed as molar concentrations of GSH (in nmol GSH g^{-1} FW) and GSSG (in nmol GSSG g^{-1} FW).

The ascorbic acid (AsA) contents were determined as described by Kampfenkel et al. [34] in supernatants of adequately prepared extracts from plant samples, by measuring absorbance values at 525 nm on UV-Vis spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA) and subsequent calculations of molar concentrations of AsA (mmol AsA g^{-1} FW).

Lipid peroxidation was estimated by determination of malondialdehyde (MDA) contents according to Bailly et al. [35], by measuring absorbance values at 532 and 600 nm with the use of extinction coefficient 155 mM^{-1} cm^{-1}. Results were expressed as µmol g^{-1}.

2.3. Data Processing and Statistical Analysis

The experiments were conducted in three biological replicates and the results were expressed as mean ± sd. Data processing, averages, standard deviation values (sd), accumulated Cd loads L_n, enrichment factor (EF) and translocation factors (TF, LTF) calculations were performed using Microsoft Excel 365. The means were analyzed for significance using one–way analysis of variance, ANOVA (Statistica for Windows v. 13.0, Stat–Soft Inc., Tulsa, OK, USA). Data were checked for normality and homogeneity of variance. Tukey HSD (Honestly Significant Difference) multiple comparisons test was used to check the significance of differences ($p < 0.05$). When the data did not conform to the normal distribution or failed to pass the variance homogeneity test, non-parametric Kruskal-Wallis Test followed by post-hoc Dunn's test was used to analyze the differences between groups.

3. Results and Discussion

3.1. Effect of Adaptation to Cd Stress on Biomass of the Middle European Ecotype of S. nigrum

There were no distinct morphological differences in the state of roots and shoots between both investigated varieties of the Middle European ecotype of *S. nigrum* L. ssp. *nigrum*, i.e., between the naturally non-adapted one (N0) and that adapted in the first-generation to high Cd concentration in the soil (A50). Both N0 and A50 varieties retained similar growth and healthy appearance within the entire range of Cd treatments of the soil (from 0 to 50 mg kg^{-1}DW, which corresponded to the actual measured mean concentrations of Cd in the soil from 0.22 mg kg^{-1} to 50.49 mg kg^{-1}) (Table 1). However, more intense green color of leaves and analytical data clearly show trends related to adaptation of *S. nigrum* to high Cd concentrations in soil.

The non-adapted variety N0 displayed a mild downward trend in root biomass with increasing Cd contents in the soil, scarcely by 36% at T50 compared to T0. Shoot biomass showed an even weaker decline (by 26%), at several-fold bigger biomass of shoot than that of root. Shoot to root (S/R) biomass ratio ranged from 5.7 to 7.3 (mean 6.5) throughout the entire soil treatment range, i.e., from T0 to T50 (C_{soil} = 0.22–50.49 mg kg^{-1} Cd, DW).

In turn, declining trends of biomass of the adapted variety A50 with increase of Cd stress from T0 to T50 appeared to be distinctly weaker. It reached 30% for roots and only 5% for shoots, at S/R biomass ratio varying from 6.0 to 8.4 (mean 7.2). The increase of S/R ratio at T50 was mostly due to a relatively smaller impact of Cd stress on shoot biomass and increasing resilience mechanisms, particularly in A50.

The mean root biomass of the adapted variety A50 in T0–T20 range (C_{soil} = 0.22–20.44 mg kg^{-1} Cd, DW) was roughly from 3 to 10% lower than that of the non-adapted one N0, while the mean shoot biomass of A50 was more uniformly, from 8 to 9% lower, respectively. However, at higher Cd concentrations in the soil (T30–T50, C_{soil} = 30.09–50.49 mg kg^{-1} Cd, DW), the mean biomass both of root and shoot of the adapted (A50) variety surpassed that of the non-adapted (N0) one. The differences in A50 vs. N0 biomass showed clearly increasing trend (from 3% to 29%, and from 10% to 18% in root and shoot, respectively), although there was no statistical significance (Table 1). This evidently resulted from the generally high resistance of the Middle European *S. nigrum* ecotype to Cd, but a weaker impact of high Cd concentrations in the soil on the adapted plant growth.

There are no data on other ecotypes of S. *nigrum* grown under the same soil/climate conditions. However, identical comparative pot experiments performed on cinnamon soil type (Xantic Ali-Udic Cambisols) [20], with the use of remote Asian ecotypes, two Chinese (Shenyang SY and Hanzhong HZ) and one Japanese ones (Kyoto KT) [5], showed very close root and shoot biomass values of the Middle European and the Asian ecotypes at T0 treatment, but considerably lesser resistance of the Asian ecotypes to growing Cd content in the soil. Already at C_{soil} > 10 mg kg^{-1} Cd, fast decrease of biomass (DW) of the Asian ecotypes was observed. The reported reduction of root biomass was up to 2.5–4-fold (by 62–76%), and that of shoot up to over 3-fold (by 69–72%) at $C_{soil} \approx$ 50 mg kg^{-1} Cd compared to control (T0 treatment). In contrast, at the same Cd treatment range, the Middle European ecotype showed a decline scarcely up to 36% and 26% of root biomass, and by 26% and 5% of shoot biomass in the N0 and A50 varieties, respectively. Much lesser S/R ratio (3.4–3.8) of the Asian ecotypes than of the Middle European N0 and A50 varieties (S/R = 7.3 and 8.1, respectively) at $C_{soil} \approx$ 50 mg kg^{-1} Cd indicated a considerably stronger adverse impact of high Cd stress on the Asian ecotype growth.

Pot experiments on the Korean ecotype *S. nigrum* L. from Daegu (35°88′ N, 128°59′ E) cultivated in a growth chamber at 25 °C, 14 h photoperiod and 60% relative humidity in sand soaked with half-strength Hoagland nutrient solution, also indicated a decrease of biomass (DW) of leaves, stems and roots by 79, 75.6, and 75%, respectively, at exposure of plants to 50–80 mg kg^{-1} Cd [36]. The Korean ecotype grown in untreated medium, at shoot biomass comparable to Chinese and Japanese ecotypes, and about two-fold bigger root biomass, displayed its very sharp decline in the treatment range from 10 to 50 mg kg^{-1} Cd, but no significant changes at further increase of Cd concentrations to 80 mg kg^{-1}. This indicates the launch of a new defense mechanism under the deep stress conditions. The sensitivity of plants to Cd in this experiment may have been additionally affected with the growth medium close to hydroponic conditions. This usually results in bigger Cd accumulation in plants, but in adequately higher stress than at Cd uptake by plants from soil.

One more comparative pot experiment conducted at a glasshouse on the Australian (Melbourne) and Chinese (SY) ecotypes grown in burozem soil from the La Trobe University campus (Melbourne) at 20 ± 5 °C and natural light, showed about 30% lower biomass of the Ausralian ecotype, and similar tolerance to Cd content in the soil [16]. Concentration of 20 mg^{-1} kg Cd in the soil was a threshold content assuring plant resilience. A sharp decline of biomass by 85–90% at 40 mg^{-1} kg Cd and no further significant changes up to 80 mg^{-1} kg Cd in the soil indicated similar reaction of studied Chinese (SY) and Australian

S. nigrum ecotypes to stress as it was observed with respect to Korean ecotype. It appears that under very high stress, new defense mechanisms start to act, however after a very serious damage to plant physiology.

In contrast, such breakdown of defense mechanisms has not been observed in the Middle European ecotype of *S. nigrum*, in both non-adapted N0 and adapted A50 varieties. In these varieties, a decline in biomass occurred gradually and smoothly, and on a relatively small scale, in particular compared to Asian/Australian ecotypes. There is no other evidence of different, much weaker reactions of European ecotypes to Cd stress. However, a reported reduction in shoot biomass (DW) scarcely by 64% compared to control in the Provence (France) ecotype of *S. nigrum* in the pot experiment at 100 mg^{-1} kg Cd in the clayey loam soil [12] suggests considerably higher natural resistance of European ecotypes.

3.2. Cd Accumulation, Enrichment and Translocation in the Natural (N0) and Adapted (A50) Plants Exposed to Growing Cd Stress

Increasing contents of Cd in the soil resulted in growing concentrations of Cd in roots and shoots of the Middle European ecotype of *S. nigrum*, both of non-adapted (N0) and adapted to high concentrations of Cd (A50). However, Cd uptake by both varieties differed substantially. The non-adapted variety appeared to accumulate somewhat bigger contents of Cd in the root system over the entire range of Cd concentrations in the soil, evidently due to its lesser transport with xylem sap to shoots (Table 2). This manifested itself in noticeably bigger Cd concentrations, and even more visible accumulation of Cd loads in shoots of the adapted variety A50 within the entire range of elevated Cd concentrations in the soil (T10–T50).

Table 2. Cd accumulation, enrichment and translocation of Cd in the non-adapted (N0) and adapted (A50) Middle European ecotype of *S. nigrum* L. at different Cd concentrations in soil.

T	C_{soil} mg kg^{-1}	C_n mg kg^{-1}		EF_n		TF		L_n µg pot^{-1}		LTF	
	Symbol	N0	A50	N0	A50	N0	A50	N0	A50	N0	A50
						Root					
0	0.22 ± 0.08	0.57 ± 0.14 dA	0.24 ± 0.02 dB	2.59 ± 0.63 bA	1.09 ± 0.08 bB			1.20 ± 0.49 cA	0.48 ± 0.06 cB		
10	10.52 ± 0.87	55.49 ± 7.96 cA	50.83 ± 11.66 bcA	5.27 ± 0.76 aA	4.83 ± 1.11 aA			108.6 ± 21.9 bcA	95.90 ± 21.58 bcA		
20	20.44 ± 1.96	127.20 ± 11.95 bA	104.21 ± 21.42 bA	6.22 ± 0.56 aA	5.10 ± 1.04 aA			201.8 ± 68.6 bA	150.1 ± 58.2 bAB		
30	30.09 ± 1.52	166.74 ± 28.40 bA	110.16 ± 25.99 bB	5.54 ± 0.94 aA	3.66 ± 0.86 aB			210.4 ± 124.5 bA	157.6 ± 92.6 bAB		
50	50.49 ± 2.29	324.07 ± 31.20 aA	279.91 ± 49.01 aA	6.42 ± 0.62 aA	5.54 ± 0.97 aA			420.8 ± 17.5 aA	401.7 ± 142.1 aA		
						Shoot					
0	0.22 ± 0.08	0.82 ± 0.34 dA	0.49 ± 0.08 dA	3.72 ± 1.53 bA	2.23 ± 0.34 cA	1.40 ± 0.23 aB	2.06 ± 0.27 aA	10.9 ± 5.92 cA	5.85 ± 0.81 cB	8.77 ± 1.63 aB	12.26 ± 0.86 aA
10	10.52 ± 0.87	81.05 ± 7.81 cA	90.67 ± 7.61 cA	7.70 ± 0.74 aA	8.62 ± 0.72 aA	1.48 ± 0.27 aA	1.83 ± 0.28 aA	957.9 ± 127.2 bA	988.8 ± 377.7 bA	8.91 ± 0.71 aA	10.51 ± 3.71 abA
20	20.44 ± 1.96	111.05 ± 2.15 bA	129.27 ± 34.06 bA	5.43 ± 0.10 bA	6.32 ± 1.67 bA	0.88 ± 0.10 bB	1.23 ± 0.11 bA	1222.0 ± 157.7 abA	1269.1 ± 289.9 abA	6.44 ± 1.82 abA	8.79 ± 1.46 abA
30	30.09 ± 1.52	103.03 ± 7.81 bA	117.01 ± 16.04 bA	3.42 ± 0.26 bcA	3.89 ± 0.53 cA	0.62 ± 0.06 cB	1.11 ± 0.33 bcA	835.69 ± 377.0 bA	1015.2 ± 403.3 bA	4.41 ± 1.11 bB	7.43 ± 2.55 abA
50	50.49 ± 2.29	174.65 ± 9.92 aA	164.55 ± 13.52 aA	3.46 ± 0.20 bcA	3.26 ± 0.27 cA	0.54 ± 0.04 cA	0.60 ± 0.15 cA	1685.51 ± 154.6 aA	1867.6 ± 151.6 aA	4.00 ± 0.20 bA	5.11 ± 1.96 bA
						Total					
0	0.22 ± 0.08							12.15 ± 6.40 cA	6.33 ± 0.86 cB		
10	10.52 ± 0.87							1066.6 ± 148.9 bA	1084.7 ± 386.1 bA		

Table 2. *Cont.*

T	C_{soil} mg kg^{-1}	C_n mg kg^{-1}		EF_n		TF		L_n µg pot^{-1}		LTF	
	Symbol	N0	A50	N0	A50	N0	A50	N0	A50	N0	A50
20	20.44 ± 1.96							1423.8 ± 183.9 abA	1419.2 ± 344.7 abA		
30	30.09 ± 1.52							1046.1 ± 500.7 bA	1172.8 ± 490.5 bA		
50	50.49 ± 2.29							2106.3 ± 85.9 aA	2269.3 ± 240.6 aA		

C_{soil} (mg kg^{-1})—measured actual Cd concentrations in soil at T (treatments): 0, 10, 20, 30, 50 mg kg^{-1}, respectively; N0—*S. nigrum* not adapted and A50—*S. nigrum* adapted to high concentrations of Cd (50 mg kg^{-1} Cd) in soil; Enrichment factors $EF_n = C_n/C_{soil}$, Translocation factors $TF = C_{shoot}/C_{root}$ where C_n—concentrations in root and shoot, respectively; Accumulated loads L_n (µg pot^{-1}) = C_n Bm_n, where C_n—concentrations in root and shoot, respectively (µg g^{-1}); Bm_n—biomass of root and shoot, respectively (g pot^{-1}); $L_{total} = L_{root} + L_{shoot}$—total accumulated load in root and shoot; Accumulated load translocation factor LTF—load translocation factor from root to shoot; Data in each section/column marked by the same lowercase letters are not significantly different at $p < 0.05$; Data in each section/line marked by the same capital letters are not significantly different at $p < 0.05$.

The values of enrichment and translocation factors justified high Cd-hyperaccumulative properties of the non-adapted Middle European ecotype of *S. nigrum* (N0). Enrichment factor EF for roots ranged roughly from 2.6.to 6.4, and for shoots from 3.4 to 7.7, at translocation factor TF exceeding or close to 1, up to T20 (C_{soil} = 20.44 mg kg^{-1} Cd). At higher Cd contents in the soil, TF was <1, but still high, while Cd concentrations in shoot were well above 100 mg kg^{-1} Cd, thus meeting the requirements for a hyperaccumulator.

The adapted variety (A50) under the same growing conditions displayed somewhat lesser range of EF for roots (from 1.1 to 5.5) and bigger for shoots (from 2.2 to 8.6), at TF > 1 up to T30 (C_{soil} = 30.09 mg kg^{-1} Cd). All Cd concentrations in shoot of A50 at C_{soil} > 10 mg kg^{-1} Cd were above 100 mg kg^{-1} Cd, and bigger than in N0, thus confirming somewhat stronger Cd hyperaccumulative properties measured by its accumulation in shoot.

Actual accumulated Cd loads in the investigated soil concentration range highlighted lesser Cd accumulation in the A50 root system than in that of N0 due to greater translocation of Cd loads from root to shoot (LTF). It showed a regular declining trend with the increase of Cd concentrations in the soil (LTF = 8.8 to 4.0 for N0 vs. LTF = 12.3 to 5.2 for A50). Remarkably, total Cd loads accumulated in roots and shoots of both N0 and A50 at increasing concentrations of Cd in the soil appeared to be similar to each other (Table 2). This means that the total amounts of Cd accumulated in the adapted A50 and non-adapted N0 varieties are basically similar, while long-range xylem sap transport from roots to leave vacuoles appeared to be more intense in the adapted variety A50.

The pattern of Cd accumulation in root and shoot of both varieties of *S. nigrum* as a hyperaccumulator clearly illustrates the difference in Cd uptake and translocation between Cd excluders and hyperaccumulators. Namely, in Cd excluders, such as e.g., *S. torvum*, reduced concentrations of Cd in roots of a plant growing in Cd-contaminated soil is mainly due to Cd-induced drought stress in the root system. The transcriptional regulation of dehydration-related genes that inhibit water transport in aquaporins thus causing a drought effect, constitutes defensive mechanism against Cd penetration into plant tissues [21,37]. It results also in reduced Cd flow from roots through stem to leaves. In contrast, in A50, reduced Cd content in root compared to N0, was caused solely by increased xylem sap long range flow rate at the lack of barriers to Cd uptake from the soil. Compared to the Asian ecotypes, both Middle European varieties showed similar Cd accumulation capacity within the tolerable to the Asian ecotypes Cd_{soil} range, not causing biomass reduction (e.g., C_R = 57.8–81.4 mg kg^{-1}, C_S = 78.7–102 mg kg^{-1}, and L_T = 856–1348 µg pot^{-1} for SY, HZ and KT [5] vs. C_R = 50.8–55.5 mg kg^{-1}, C_S = 81.1–90.7 mg kg^{-1}, and L_T = 1067–1085 µg pot^{-1} at C_{soil} = 10 mg kg^{-1} Cd for N0 and A50 Table 2).

However, sensitivity to Cd stress at C_{soil} > 10 mg kg^{-1} Cd results in declining biomass and total Cd accumulation capacity, at deteriorating physiological parameters but increased

Cd concentrations in tissues of the Asian ecotypes. Opposite, the Middle Europan varieties showed high resistance to Cd stress over the entire range of studied concentrations in the soil, up to C_{soil} = 50 mg kg^{-1} Cd. They displayed lesser Cd concentrations in plant tissues, but higher total accumulated loads of Cd and their overall good physiological status (e.g., C_R = 301–342 mg kg^{-1}, C_S = 318–387 mg kg^{-1}, and L_T = 1083–1781 µg pot^{-1} for SY, HZ and KT [5] vs. C_R = 324–280 mg kg^{-1}, C_S = 175–165 mg kg^{-1}, and L_T = 2106–2269 µg pot^{-1} at C_{soil} = 50 mg kg^{-1} Cd for N0 and A50—Table 2).

Within this study, no limit of Cd tolerance of the Middle European ecotype of *S. nigrum*, either non-adapted, or adapted to Cd stress was reached. Although, among very scarce European studies on *S. nigrum*, one study on the exposure to C_{soil} = 100 mg kg^{-1} Cd, i.e., to twice as big concentration as the maximum studied here, of the local South-West European ecotype, revealed still high Cd accumulation capacity and declining tolerance [12]. Concentrations of Cd in root and shoot (DW) of this ecotype appeared to be comparable to that assessed for the Middle European ecotype and reached C_R = 377 mg kg^{-1}, C_S = 107 mg kg^{-1} at C_{soil} = 100 mg kg^{-1} Cd vs. C_R = 324–280 mg kg^{-1}, and C_S = 175–165 mg kg^{-1} at C_{soil} = 50 mg kg^{-1} Cd assessed for the Middle-European ecotype at two times lesser Cd concentration in soil. This indicates further gradual decrease by about 39% of Cd accumulation in shoot provided that these European ecotypes indeed react similarly to Cd stress.

These data show the need of conducting further comparative experiments on different remote eco/genotypes under the same soil/climate conditions and extended range of Cd treatments to unbiasedly select the most appropriate hyperaccumulator and assess Cd tolerance thresholds for each eco/genotype. Apart from the Cd contents in soil, a variety of external factors like soil type and composition, moisture content and temperatures may affect Cd hyperaccumulation capacity and overall phytoremediation efficiency of eco/genotypes of *S. nigrum*. Comparing the results obtained in this study with other data available so far, in addition to different tolerance to Cd stress of various remote Asian/Far East and European ecotypes of *S. nigrum*, two diverse defense mechanisms were indicated. One, observed in Asian/Far East ecotypes is more conservative. It displays almost unrestricted translocation of Cd with xylem sap from root to shoot until deep breakdown of physiological functions of a plant under critical level of Cd stress, and further partial adaptation of a plant to functioning under excessive stress. This manifests itself in constantly high accumulation of Cd in shoot at poor plant condition and deep biomass reduction [5,16]. Another mechanism was displayed in this study by both N0 and A50 varieties of the Middle European ecotype of *S. nigrum*, but in particular by the adapted variety A50. It is aimed at protecting the physiological functions of a plant through active molecular mechanisms in roots, which control Cd loading capacity into the xylem sap for long-distance transport from root to shoot and storage in leaf vacuoles [5,38]. This resulted in no Cd stress-induced breakdown in the Middle European ecotype, but also in lower concentrations of Cd accumulated both in root and shoot tissues at critical contents of this metal in the soil (Table 2). Cd accumulation in root was somewhat lower, and in shoot somewhat higher in the adapted A50 than in the non-adapted N0 variety practically in all Cd-fortified soils, however at a non-significant level ($p < 0.05$). Consequently, compared to N0, A50 displayed lower enrichment factors EF in root, but higher in shoot, and higher translocation factor TF. In T0–T30 range it was >1, while the non-adapted N0 variety complied with this requirement only within T0-T10 range.

Therefore, the overall effect of adaptation of the Middle European ecotype to high Cd contents in the soil manifested itself in higher tolerance of the A50 variety to Cd stress. This resulted in bigger shoot biomass and either higher Cd concentrations in xylem sap at the loading stage from roots, or greater long-range xylem sap flow rate from roots to leave vacuoles. Much lesser Cd accumulation in stem than in leaves observed in the South-West European [12] and also in the Asian (SY) [5] ecotypes suggests rather this last mechanism. Moreover, despite considerably higher Cd concentrations in tissues of the Asian *S. nigrum* ecotypes SY, NZ and KT [5], they display evidently weaker protection mechanisms against stress and strong reduction of biomass preceded by a physiological breakdown. This

caused that the total Cd accumulation capacity of the Middle European ecotype at critical Cd contents in the soil appeared to be visibly higher (by 49%, 26% and 15% in N0, and by 52%, 32% and 22% in A50, respectively).

3.3. Effect of Adaptation to High Cd Contents in Soil on Physiological Properties of the Middle European Ecotype of Solanum nigrum

3.3.1. Photosynthetic Pigments

Tends in the contents of photosynthetic pigments in leaves of the non-adapted N0 variety of the studied Middle European ecotype of *S. nigrum* L., despite lack of visual changes in the plant/leaves status, showed general similarity to these observed in the Asian ecotypes [5]. Chlorophyll a, chlorophyll b and carotenoid contents displayed no significant changes only at T10, while at higher Cd concentrations in soil, a significant decline of all pigment contents occurred (Figure 1), However, at T30 the declining trend ceased and no further significant ($p < 0.05$) changes were observed in chlorophyll a and carotene content, while in chlorophyll b a mild decline occurred already at T $\geq$ 20. In contrast, in the A50 variety contents of all photosynthetic pigments were considerably higher than in N0 at all treatments (by 24%, 23% and 14% at T0 and by 59%, 57% and 18% at T50, respectively). At bigger concentrations of Cd in the soil, relatively mild decline of photosynthetic pigments in the A50 variety occurred (by 34%, 28% and 45% for chlorophyll a, chlorophyl b and carotenoids vs. 64% for chlorophyll a and b and 43% for carotenoids in N0 at T50).

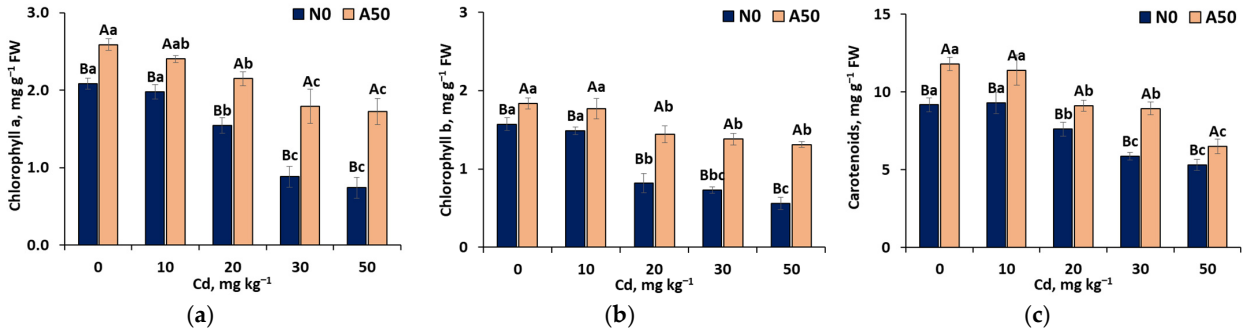

Figure 1. Effects of different Cd treatments on (**a**) chlorophyll *a*, (**b**) chlorophyll *b*, (**c**) carotenoids content in the non-adapted N0 and adapted A50 varieties of *S. nigrum* L. (1) 0 to 50 means control and soil treatments with Cd (mg kg^{-1}); (2) Data for the same treatments marked by the same capital letters over bars are not significantly different at $p < 0.05$. Data for different treatments marked by the same lowercase letters over bars are not significantly different at $p < 0.05$.

The adapted A50 variety of the Middle European *S. nigrum* ecotype appeared to be more enriched in photosynthetic pigments in leaves and more resistant to Cd stress than the non-adapted variety N0. These data showed that both varieties are tolerant to Cd, in particular A50 that showed mild decline and retained relatively high photosynthetic parameters in the entire range of Cd treatments. The photosynthetic performance of leaves of the non-adapted N0 variety of the studied Middle European ecotype of *S. nigrum* L., despite the lack of visible changes in the plant/leaves status, showed greater sensitivity to Cd stress.

3.3.2. RuBisCO Activities

Activities of RuBisCO proteins representing both large RbcL and small RbcS subunits (Figure 2a,b) responsible for converting atmospheric CO_2 into organic carbon forms, shoved different response to oxidative stress in the non-adapted N0 and adapted A50 varieties. Opposite to photosynthetic pigments, no difference in the activity of both subunits at T0 (control) was observed. Increase of abiotic oxidative stress caused by the growing content of Cd in the soil resulted in the regular decline of RbcL and RbcS in N0. It was more pronounced with respect to RbcS that showed complete disappearance at T50 (Figure 2b).

It is believed that expression of RbcS regulates the size of the RuBisCO pool and therefore affects the overall catalytic efficiency of the RuBisCO complex mostly located in the RbcL This well correlates with regularly declining RbcL activity in the N0 variety, up to 78% at T50 compared to T0 (Figure 2a).

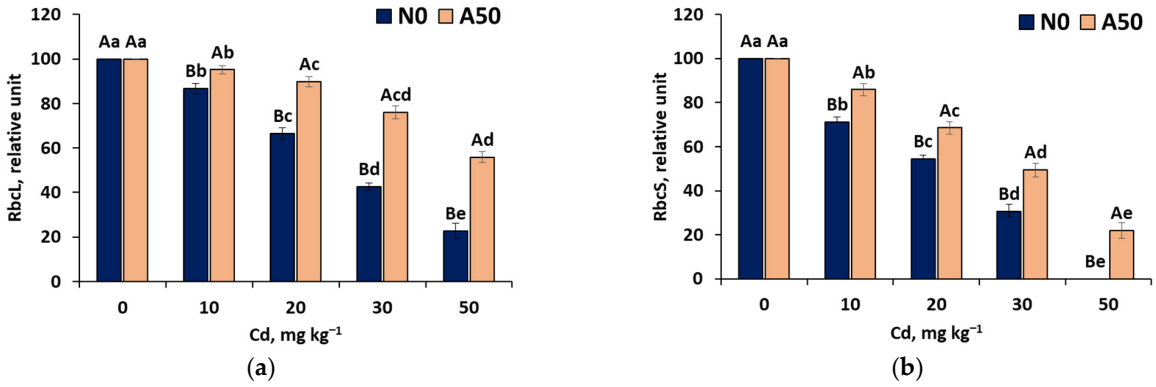

Figure 2. Effects of different Cd treatments on (**a**) RbcL and (**b**) RbcS content in the non-adapted N0 and adapted A50 varieties of *S. nigrum* L. (1) 0 to 50 means control and soil treatments with Cd (mg kg^{-1}); (2) Data for the same treatments marked by the same capital letters over bars are not significantly different at $p < 0.05$. Data for different treatments marked by the same lowercase letters over bars are not significantly different at $p < 0.05$.

In contrast, in the A50 variety both RbcS and RbcL activities display much bigger resistance to Cd stress (Figure 2a,b), showing decline of RbcS by 78%, and RbcL by 44% only at T50, while at T30 the RbcL decline did not exceed 24% (Figure 2a,b).

3.3.3. Reacrtive Oxygen Species (ROS)

Reactive oxygen species (ROS), the superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) generated in excess, are involved in physiological stress and pathological processes in the apoplast of plant cells. They displayed a distinct increase in the leaves of both non-adapted N0 and adapted A50 varieties in parallel to the increase in Cd content in the soil, indicating growing oxidative stress (Figure 3). However, no signs of crisis and deep cell damage with respect to superoxide anion content was observed. The increase for N0 and A50 was regular, by 105% and 69%, respectively, at Cd treatments over the entire range between T0 and T50. The $O_2^{\bullet}$ level was nonetheless considerably (by 31%) bigger in leaves of N0 than of A50 (Figure 3a) indicating lesser oxidative stress of the adapted variety.

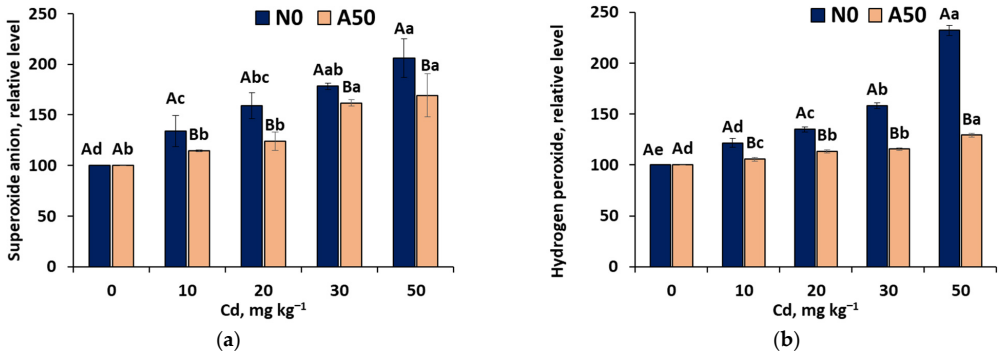

Figure 3. Effects of different Cd treatments on ROS contents: (**a**) superoxide anion and (**b**) hydrogen peroxide in in non-adapted N0 and adapted A50 varieties of *S. nigrum* L. (1) 0 to 50 means control and soil treatments with Cd (mg kg^{-1}); (2) Data for the same treatments marked by the same capital letters over bars are not significantly different at $p < 0.05$. Data for different treatments marked by the same lowercase letters over bars are not significantly different at $p < 0.05$.

Oxidative stress caused by hydrogen peroxide (H_2O_2) also appeared to be more pronounced for the N0 variety. A substantially higher level of this ROS was detected in its leaves at T50 compared to T30 treatment (by 74%), while increase of H_2O_2 in the treatment range T0–T30 was weaker (by 58%). In contrast, the adapted A50 variety did not show much growth of H_2O_2 level over the entire Cd concentration range (scarcely by 29% compared to T0). This indicated its high resistance to Cd stress practically up to the highest T50 treatment, at a distinctly lower ROS level in the leaves of the adapted A50 variety. Inversely, sharp up-regulation of H_2O_2 at T30–T50 range in N0 showed symptoms of greater stress at T50.

3.3.4. First Line Defense Antioxidant Activities

Among antioxidant defense systems in plants, key antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) are considered the most important and efficient antioxidants that prevent or suppress the formation of free radicals and ROS in plant cells. Specifically, CAT degrades H_2O_2 into H_2O and O_2. SOD catalyzes the dismutation of two superoxide anions ($O_2^{\bullet -}$) and water into H_2O_2 and O_2 [39–41].

Activities of these first line antioxidant enzymes in the leaves of non-adapted N0 and adapted A50 varieties of the Middle-European ecotype of *S. nigrum* showed Cd stress-related changes following specific patterns. In N0 leaves, up-regulation of all key enzymes (CAT, SOD, GPX) indicating growing defense mechanism was observed up to T10 (with respect to SOD up to T20). This was followed by a down-regulation due to the weakening of the defense system (Figure 4). The strongest relative up-regulation trends were observed in SOD contents (up to 32% at T20), and the weakest in GPX activities (up to 12% at T10). The strongest relative down-regulation occurred in CAT activities (by 60% at T50 compared to T10 treatment).

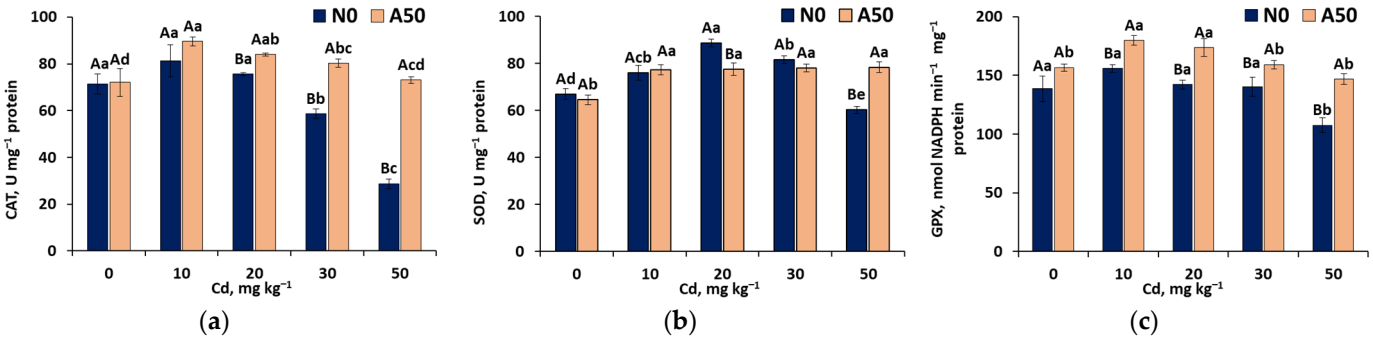

Figure 4. Response of the first-line defense antioxidant (**a**) CAT, (**b**) SOD and (**c**) GPX) activity in the leaves of non-adapted N0 and adapted A50 varieties of *S. nigrum* to the growing Cd stress. (1) 0 to 50 means control and soil treatments with Cd (mg kg^{-1}); (2) Data for the same treatments marked by the same capital letters over bars are not significantly different at $p < 0.05$. Data for different treatments marked by the same lowercase letters over bars are not significantly different at $p < 0.05$.

In contrast, in the adapted variety A50, the first line antioxidant activities, after reaching the highest level at T10 (by 24% for CAT, by 20% for SOD and by 15% for GPX), remained virtually unchanged, showing only a slight decrease above the initial level at T0 (CAT, GPX), while SOD activity kept a weak upward trend up to T50 (Figure 4).

Worth mentioning that SOD activities were always bigger in N0 than in A50 at all Cd treatments, except that at T50, when decline of SOD in N0 reversed the relationship. Of three SOD isozymes distinguished by covalently linked metal ions and cellular locations (Cu/ZnSOD, MnSOD and FeSOD) [39], Cu/ZnSOD isozyme showed the same N0/A50 relations as SOD enzyme, indicating its predominance in both varieties (Figure 5a). In the adapted A50 variety, activities of all three isozymes showed increase at T10 (by ~19%, 13% and 21%, respectively) and stable level ($p < 0.05$) in the Cd range T10-T50, for MnSOD and FeSOD considerably higher than in N0 (Figure 5a–c). Opposite, in the non-adapted variety N0 activities of MnSOD isozyme showed a slight increase at T10 (by 7% compared to T0),

followed by a decrease (by 32%) in the range T10-T30 and stabilization at T30-T50 ($p < 0.05$) (Figure 5b). Activities of FeSOD in N0 remained at the same lower level compared to T0 over the entire Cd treatment range (by19%, $p < 0.05$). Differential expression of antioxidant isozymes in the leaves of *S. nigrum* exposed to Cd stress indicated substantial differences in production superoxide anions in specific cellular locations. Since Cu/ZnSOD isozyme, which shows the biggest affinity to overall SOD pattern and expression dynamics, is found in cytoplasm and in chloroplasts, this indicates that superoxide anions requiring deactivation are generated mostly in these cellular locations. Lesser activity changes showed MnSOD located in mitochondria, while the most stable activity displayed FeSOD found in chloroplasts.

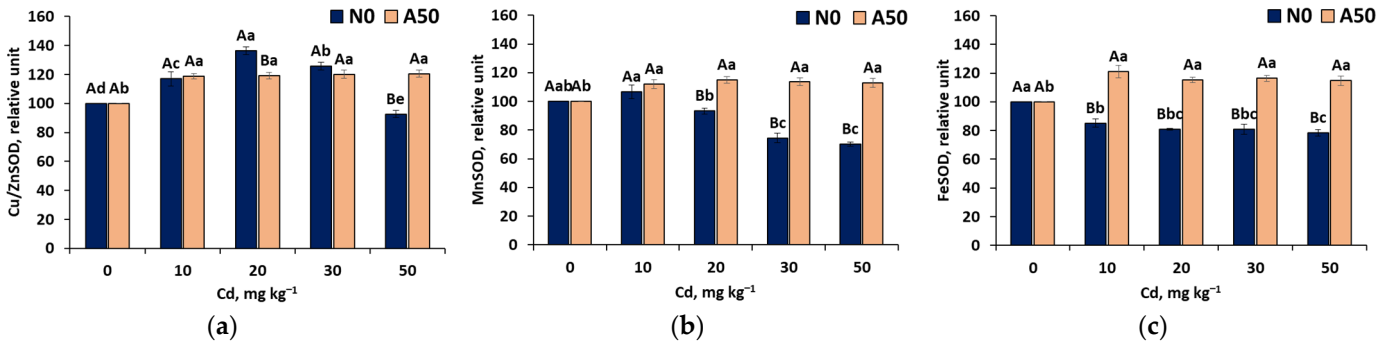

Figure 5. Response of SOD isozyme (**a**) Cu/ZnSOD, (**b**) MnSOD (**c**) FeSOD activities in the leaves of non-adapted N0 and adapted A50 varieties of *S. nigrum* to the growing Cd stress. (1) 0 to 50 means control and soil treatments with Cd (mg kg^{-1}); (2) Data for the same treatments marked by the same capital letters are not significantly different at $p < 0.05$. Data for different treatments marked by the same lowercase letters over bars are not significantly different at $p < 0.05$.

3.3.5. Second Line Defence Antioxidant Activities

In reducing Cd stress, a group of scavenging antioxidants also plays an important role. This second line defense antioxidants [41] scavenge and neutralize free radicals by donating electron to them, thus converting themselves into free radicals, but of lesser damaging effect. They, in turn, are easily neutralized by other antioxidants of this group.

The prominent representatives of this group are ascorbate and glutathione enzymatic (APX, AsA, GR) and non-enzymatic compounds (GSH, GSSG). Besides scavenging ROS directly, they perform other functions that enhance antioxidant capacity and reduce oxidative stress [42]. Of these antioxidants, a very strong reaction to increasing Cd stress in *S. nigrum* showed ascorbate antioxidant compounds: ascorbate peroxidase APX and ascorbate AsA (Figure 6). APX occurs in cytosol and organelles (chloroplast, mitochondria and peroxisome). Ascorbate AsA is present also in nucleus and vacuoles of leaves cells [40].

With respect to APX in the leaves of the non-adapted N0 variety of *S. nigrum*, increase of its activity (by 14% in T10 related to T0), typical for this ecotype occurred, indicating activation of its defense functions. However, it was followed by the breakdown of defense capacity and fast decline of APX activity up to its thorough extinction over the T10–T50 range of Cd treatments. An enzymatic co-factor ascorbate (AsA) was affected even more, showing sharp decrease (by 33% compared to T0) until complete extinction at T30.

Enrichment of ascorbate compounds in the leaves of the adapted variety A50 appeared to be considerably bigger and showed much higher defense capacity. After an increase of APX at T10 by 28% compared to T0, its defense activity permanently exceeded control up to T30, while at T50, APX activity declined scarcely by 37% related to T0 (Figure 6a). Also, AsA contents in the adapted A50 variety appeared to be much less responsive to Cd stress than in N0 in the range T0–T30 and was extinct only at T50 (Figure 6b).

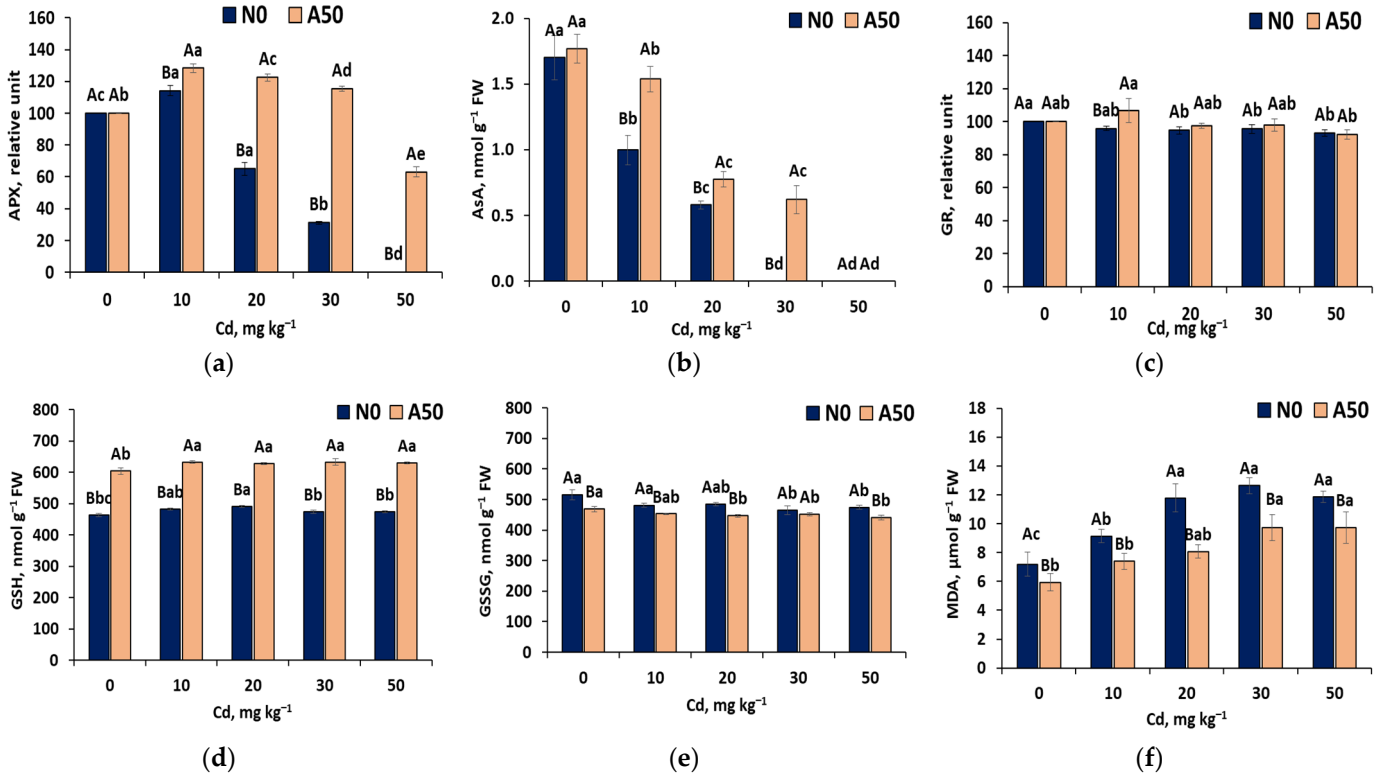

Figure 6. Response of the second-line defense antioxidants ascorbate: (**a**) APX, (**b**) AsA and glutathione compounds: (**c**) GR, (**d**) GSH, (**e**) GSSG activity and (**f**) MDA content in the leaves of non-adapted N0 and adapted A50 varieties of *S. nigrum* L. to the growing Cd stress. (1) 0 to 50 means control and soil treatments with Cd (mg kg^{-1}); (2) Data for the same treatments marked by the same capital letters over bars are not significantly different at $p < 0.05$. Data for different treatments marked by the same lowercase letters over bars are not significantly different at $p < 0.05$.

Simultaneously, the levels of second line glutathione antioxidant compounds present in plant cells (GR, GSH) and the level of disulfide GSSG generation in both *S. nigrum* varieties suggest much weaker response of these compounds to increasing Cd stress (Figure 6c–e). The level of glutathione reductase (GR) appeared to be similar in both varieties and did not show significant ($p < 0.05$) changes over the entire T0–T50 range of Cd treatments (Figure 6c). In turn, the contents of non-enzymatic antioxidant glutathione (GSH) were also stable in both varieties. Differences in AsA (Figure 6b) and GSH trends (Figure 6d) in response to Cd stress suggest also other mechanisms besides regulation of Ascorbate-Glutathione (AsA-GSH) pathway [42] in detoxifying ROS in this Middle-European ecotype of *S. nigrum*. While AsA showed a fast decline, GSH contents were basically constant, in A50 about 30% bigger than in N0. GSH displayed a very slight general increasing trend at T10 followed by the same slight (above T0 level) decrease in the T10–T50 range in the N0 variety and no significant changes in the adapted A50 variety (Figure 6d). Inversely, the levels of oxidized GSH (GSSG) were in the non-adapted variety N0 somewhat higher (by 7–10%) than in the adapted one A50, at the similar slight decreasing trend (Figure 6e). This resulted in the basically constant ratio GSH/GSSG at T10–T50, higher than at T0 in both varieties (1.0 > 0.9 in N0 and 1.40 > 1.29 in A50), and significantly lower in N0 than in A50 (1.0 vs. 1.40). This indicated an increase of defensive capacity in both varieties at Cd increase in the soil and lesser oxidative stress in the A50 variety, with a generally weak response of second line glutathione antioxidant compounds to oxidative stress. Along with moderate reduction of the first line defense antioxidant GPX in N0 at the highest Cd treatment T50 in the soil, and its higher activity in N50 at T50 than at T0 treatment, this points to the strong glutathione-based ROS resistance mechanism on the one hand,

and lesser involvement of glutathione compounds into Cd-induced ROS detoxification in comparison with CAT, SOD and ascorbate on the other hand.

High resilience of the Middle European ecotype of *S. nigrum* to Cd stress was confirmed by trends in the generation of the plant cell damage indicator malondialdehyde MDA in the leaves of studied non-adapted N0 and adapted A50 varieties (Figure 6f). In the range from T0 to T20 treatments, MDA contents in N0 increased, reflecting growing oxidative damage. However, in the range T20–T30, MDA remained at the constant level. Formation of MDA in A50 was significantly smaller than in N0 (by 21–46%), stabilizing in the T30–T50 range of Cd treatment. Such trends in MDA generation indicate high tolerance of both varieties of the Middle European ecotype of *S. nigrum* to abiotic stress caused by the growing contents of Cd in the soil, and significantly lesser oxidative damage in the A50 variety. Remarkable, that both varieties of the Middle European ecotype, non-adapted N0 and adapted A50, opposite to the Asian ecotypes [5] showed strong resistance to similarly high-levels of Cd stress (T30–T50) and differed mostly by the level of oxidative damage (by 46–22% lesser in A50).

4. Conclusions

The study in its entirety confirmed the assumption that different ecotypes of cosmopolitan Cd hyperaccumulator *S. nigrum* L. ssp. *nigrum*, which originate from remote geographical locations, may significantly differ with respect to Cd hyperaccumulative capacity, tolerance and regulation mechanisms. It appeared that the studied Middle European ecotype displayed much bigger tolerance to abiotic stress compared to the Asian ecotypes [5]. In addition, this tolerance can be significantly enhanced by adaptation through using seeds from the first generation of plants grown in the soil with high content of Cd (50 mg kg^{-1}). This manifested itself in the mild root and shoot biomass reduction at T50 and much higher shoot to root biomass ratio (S/R), in both the non-adapted N0, but particularly in the adapted A50 variety compared to the Asian ecotypes. These ecotypes, exposed to the same contents of Cd in the soil (in the range from 0 to 50 mg kg^{-1} Cd), show fast reduction of biomass already at C_{soil} = 10 mg kg^{-1} Cd.

Substantial differences between the Asian and Middle European ecotypes, and N0 and A50 varieties with respect to Cd enrichment and translocation in plant tissues was also observed. While the Asian ecotypes continued to accumulate growing contents of Cd under the critical stress conditions and showed fast declining biomass, the Middle European ecotype retained good health status and strong resistance to Cd stress over the entire range of the studied Cd concentrations in the soil, up to C_{soil} = 50 mg kg^{-1} Cd. Simultaneously, it showed lesser Cd concentrations in plant tissues, but similar or higher accumulation of Cd loads, both in shoots and total, in shoots and roots, compared to the Asian ecotypes.

Adaptation to Cd stress in the A50 variety resulted in enhancing all physiological parameters/factors crucial for plant development, growth and tolerance to abiotic stress. These parameters comprise chlorophyll a and b, carotenoids, RuBisCO, first line defense (neutralizing) antioxidants (CAT, SOD and its isozymes, GPX), and second line defense (scavenging) antioxidants (APX, AsA, GR, GSH). It was admittedly confirmed by clearly lower Cd stress indicated bylower MDA generation and higher GSH/GSSG ratio for the adapted A50 variety compared to the non-adapted N0 one.

Overall, the reported Asian ecotypes of *S. nigrum* ssp. *nigrum* appear to show somewhat better Cd phytoremediation performance than the Middle European ecotype within Cd tolerance range of Asian ecotypes, mostly at the moderate soil contamination level up to 10 mg kg^{-1} Cd. On the other hand, the Middle European ecotype stands out in much higher threshold of tolerance to Cd stress, reaching beyond the highest tested Cd content in soil (50 mg kg^{-1} Cd), probably close to 100 mg kg^{-1} Cd. Moreover, the adapted A50 variety of the Middle European ecotype shows further considerable enhancement of the plant resistance to Cd stress and the highest Cd accumulation capacity and healthy state of the plants at the biggest tested Cd concentrations in the soil. This makes the Middle European

ecotype and its adapted A50 variety particularly useful to sustainable decontamination of heavily polluted "hot spots" in the degraded post-industrial areas.

It should be added that significant differences between remote ecotypes of Cd hyperaccumulator *Solanum nigrum* L. ssp. *nigrum* in Cd accumulation capacity and tolerance to Cd stress suggest possible considerable differentiation of molecular mechanisms responsible for these properties in the multitude of ecotypes and genotypes growing worldwide. Some recent research indicates occurrence of differential responsive mechanisms to low and high Cd exposure in the same plant [43], thus even bigger differentiation at the molecular level can be expected in the remote ecotypes/genotypes. Selection of the most efficient Cd hyperaccumulating ecotypes/genotypes of *S. nigrum* through the extensive worldwide search, and elucidation of hyperaccumulative mechanisms may optimize phytoremediation of contaminated lands without using any supportive chemicals or other environmentally problematic or expensive measures.

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