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Evaluation of Cadmium Effects on Six *Solanum melongena* L. Cultivars from the Mediterranean Basin

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Abstract: Cadmium (Cd) contamination is a severe problem in the environment and produces detrimental effects on crop productivity and quality. Characterization of crop performance at different Cd concentrations is crucial to identify pollution-safe cultivars with low translocation efficiency to aboveground organs to be used for food safety. Here, we estimated germination, survival, growth, photosynthetic pigments, Cd bioaccumulation, among-organs translocation, and ionic balance in six *Solanum melongena* L. (eggplant) cultivars from the central Mediterranean basin. On two cultivars, we also analyzed expression of genes involved in Cd uptake, i.e., heavy metal ATPases (*HMA*s) and metal tolerance proteins (*MTP*s). We found that Cd has a negative effect on all the investigated parameters but with relevant among-cultivar differences. Cd-treated plants showed a decrease in germination rate and survival. Photosynthetic pigments showed opposite trends, i.e., with increasing Cd contents, we observed a decrease in chlorophylls and an increase in carotenoids. The investigated cultivars showed high ability of sequestrating Cd in roots but a low translocation efficiency to the aboveground organs, suggesting a good potential for food safety. The response of plants to Cd was mediated by a different expression of the *MTP* and *HMA* gene families. Our study represents the first comprehensive investigation of Cd tolerance in eggplant varieties from the Mediterranean basin and highlights the importance of comparative studies to identify Cd-tolerant cultivars.

Keywords: bioaccumulation; eggplant; physiological responses; tolerance; trace metals

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1. Introduction

Soil contamination by heavy metals is a major environmental problem which causes tremendous effects to natural and agricultural ecosystems [1,2]. Among different soil contaminants, Cadmium (Cd) is among the most concerning, as it is ubiquitous and can be absorbed by plants [3]. It occurs naturally in soil of volcanic areas, but is becoming abundant in many territories as a result of uncontrolled industrialization, unsustainable urbanization, and intensive agricultural practices [4–7]. Independently from its origin, Cd contamination is among the most serious threats for agriculture worldwide [8]. Indeed,

Cd has no biological function in plants, but exerts an extremely high toxicity [9–12] and it might accumulate in the edible parts of crops, causing health problems [13–15].

Cd is absorbed by root, reaches the vascular tissues through the apoplastic and the symplastic pathways [16] and can be then easily transported to different organs [17]. The mechanisms of Cd absorption and transport within plants has been investigated in recent years and several gene families, such as heavy metal ATPases (*HMA*s) and metal tolerance proteins (*MTP*s) have been identified as being involved [18,19]. Inside cells, Cd interferes with many metabolic processes, such as the interaction with carbon and nitrogen metabolism and the interference with sulfur assimilation and glutathione metabolism. In addition, Cd induces a complex array of physiological effects, causing oxidative damage and decreasing photosynthetic pigments content (see [8] and references therein) that affect plant growth and development, causing death even at low concentrations [3,8]. Uptake and transport of Cd in plants can impact the uptake of other elements, thus modifying plant ionic content balance [10,20–22].

Despite all these detrimental effects, plants are sometimes very well equipped with different defensive mechanisms to cope with heavy metal toxicity, including Cd [23]. Within different plant species, indeed, a variation in Cd tolerance has been reported and heavy metal-tolerant genotypes have been discovered in several important crops (e.g., [24–26]). In particular, due to differences in Cd uptake and accumulation, not only among plant species but also among cultivars, a pollution-safe cultivar strategy has recently been employed to reduce Cd toxicity by identifying low-Cd cultivars (i.e., cultivars with low Cd accumulation in the epigean tissues) [27,28]. However, the Cd tolerance issues in crops is still far from being resolved for many important crops.

Solanum melongena, the eggplant, is an economically important crop species cultivated worldwide for its edible fruit, which is well known for its nutritional value and health-promoting properties. According to the Food and Agriculture Organization (www.faostat.fao.com, accessed on 12 December 2020), global eggplant production reached 90.8 million tons in 2019 and 0.3% of this amount is produced in Italy. A high number of varieties has been selected both in the original domestication center and in several secondary diversification areas so this genetic variability represents a profitable source of potentially improved cultivars' traits [29,30]. In the Mediterranean basin, the eggplant has an ample genetic variability due to its high cultural and economic value [31]. Whilst information on Cd tolerance and identification of Cd pollution-safe cultivars from the primary diversification center is available [32,33], little is known about cultivars from secondary diversification centers such as the Mediterranean basin. Due to the extremely high toxicity of Cd and the elevated consumption of eggplant, implementing strategies to identify pollution-safe cultivars is crucial.

Here, we evaluate six eggplant cultivars to identify mechanisms involved in Cd accumulation and tolerance by assaying the effect of different Cd concentrations on seed germination, seedling growth, photosynthetic pigments content, among organs translocation, ionic content balance and expression of some gene members of *MTP* and *HMA* transporter families involved in the Cd metabolism. The main aim of this study was to perform a rapid screening of pollution-safe cultivars with low translocation efficiency to aboveground organs to be potentially used for food safety (i.e., low-Cd eggplant cultivars).

2. Materials and Methods

2.1. Plant Materials

In this study, we used six eggplant cultivars, namely "Bellezza Nera" (hereafter BN), "Cima Viola" (hereafter CV), Hybrid F1 "Purple Queen" (hereafter PQ), "Rotonda Bianca Sfumata di Rosa" (hereafter RB), "Violetta di Firenze" (hereafter VF), and "Violetta Lunga" (hereafter VL). Seeds of the six cultivars were provided by "La Semiorto Sementi" seed company (Sarno, SA, Italy).

2.2. Germination Assays at Different Cd Concentrations

Seeds from the six investigated cultivars were assayed for germination at different $CdSO_4$ concentrations. Seeds were surface sterilized with $NaOCl_4$ 1% active chlorine (v/v) for 5 min and then washed three times with sterilized Milli Q water. For each cultivar, seeds were placed in different Petri dishes with three disks of Whatman 3MM paper and were irrigated with 5 mL of $CdSO_4$ (Sigma_Aldrich, hereafter Cd) solution at different concentrations: 0, 5, 10, 25, 50, and 100 µM. The experiment was conducted in three replicates for each treatment, using 180 seeds per cultivar. Nutritive solution was replaced in all the treatments every week. For each treatment three replicates were performed. Petri dishes were closed with parafilm and placed in a growth chamber at 24 ± 2 °C. After ten days, germination was evaluated by tallying the seeds with emerged radicles (>1 mm).

2.3. Hydroponic Culture of Plantlets at Different Cd Concentrations

Seeds from the six investigated cultivars were sown in sterile perlite and kept in the dark until radicle emergence. Then, plantlets were exposed to light and placed in a growth chamber with controlled temperature (24 ± 2 °C), photoperiod (16 h light/8 h dark), irradiance (300 µmoles m^{-2} s^{-1}) and relative humidity (70%).

Seedlings at two fully expanded leaf stage were transplanted in nutritive hydroponic culture as reported in [34]. To allow plantlets to adapt to the hydroponic culture the Cd treatments at 0, 5, 10, 25, 50 and 100 µM began one week after plant relocation. For each Cd concentration, at least 20 plants for each cultivar were treated and then the survival of plants was estimated. After 15 days, roots and stem of each plant were measured using a caliper and all the plant organs were harvested and stored at −80°. Treated plants were weighted, and a growth index was calculated as follows:

$$\text{The growth index} = (\text{fresh weight } T_1 - \text{fresh weight } T_0)/\text{fresh weight } T_0$$

The tolerance index (T_i) was calculated as follows [35]:

$$T_i = \text{root length of plants grown in Cd solution/}$$

$$\text{root length of plants grown in control solution}$$

2.4. Ionome Analysis, Bioaccumulation Coefficients and the Translocation Index

From each cultivar, control (untreated) plantlets and plantlets treated with the sub-lethal Cd solution at 5 µM were used to characterize the content of different elements in the three plant organs (root, stem and leaf). Plantlets were placed in an oven at 50 °C until their weight was stabilized. Dried samples were transferred in a Teflon vessel with 4 mL of HNO_3 67% superpure (Merck) and 1 mL of H_2O_2 superpure (Merck). Vessels were placed in a microwave system (Multiwave Anton Paar). The mineralization program comprised three steps: (1) 5 min from 100 W power to 500 W; (2) 15 min at 800 W; (3) 10 min at 0 W for sample cooling. Measurements were performed as reported in [36], using an Agilent 7700 ICP-MS (Agilent Technologies Santa Clara, CA, USA) equipped with a frequency-matching radio frequency (RF) generator and 3rd generation Octapole Reaction System (ORS3) operating with helium gas in ORF.

Using ionome data, for each cultivar, we calculated and compared Cd concentration in the three plant organs (root, stem and leaf). We then calculated the bioaccumulation coefficient and the translocation indices. Plant Cd bioaccumulation coefficient (Cdbio) was estimated as the ratio between the Cd concentration in root [*Cd root*] [and its concentration in the nutritive solution [*Cd sol*] [37]:

$$\text{Cdbio} = [Cd\ root]/[Cd\ sol]$$

Then, we determined the Cd translocation coefficients (Cdt) from roots to shoots (Cdtrs):

$$\text{Cdtrs} = [\text{Cd shoot}]/[\text{Cd root}]$$

2.5. Photosynthetic Pigments Analysis

The determination of photosynthetic pigments (chlorophyll *a* and *b* and carotenoids) content was carried out on leaves of the six investigated cultivars treated at the different Cd concentrations that allowed plant survival (0, 5, 10, 25, 50 µM). We followed the procedure described in [38]: 50 mg of plant material were finely ground and then 1 volume of 80% (*v*/*v*) acetone solution was added. After centrifugation for 5′ at 14,000 rpm and 4 °C the absorbance was determined by spectrophotometric analysis (Perkin Elmer UV/Vis Spectrophotometer Lambda 25) at the following wavelengths: 470, 646.6, 646.8, 663.2 and 720 nm. The content of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophyll (Chl tot) and carotenoid (Car) was quantified by the following equations:

$$\text{Chl } a = (12.25 \times \text{Abs}_{663.2\text{nm}}) - (2.79 \times \text{Abs}_{646.8\text{nm}})$$

$$\text{Chl } b = (21.50 \times \text{Abs}_{646.8\text{nm}}) - (5.10 \times \text{Abs}_{663.2\text{nm}})$$

$$\text{Chl Tot} = (7.15 \times \text{Abs}_{663.2\text{nm}}) + (18.71 \times \text{Abs}_{646.8\text{nm}})$$

$$\text{Car} = [(1000 \times \text{Abs}_{470\text{nm}}) - (1.82 \times \text{Chl } a) - (85.02 \times \text{Chl } b)]/198$$

2.6. In Silico Identification of Genes Belonging to MTP and HMA Family

For eggplant, a draft genome sequence (SME_r2.5.1) is available and derives from sequencing of purebred cultivar [39]. To identify *MTP* and *HMA* genes in eggplant, we thus used an in silico procedure employing TBLASTP searches against the tomato genome (SL4), implemented in Sol Genomics Network database (SGN; https://solgenomics.net, accessed on 12 December 2020). We then searched the identified tomato sequences against the draft eggplant genome in Eggplant Genome Database (http://eggplant.kazusa.or.jp/, accessed on 9 January 2021) using BLAST. The Exonerate software [40] was used to determine the exon-intron organization and chromosomal gene localizations. The candidate genes were recognized as proteins with InterProScan (http://www.ebi.ac.uk/interpro/search/sequence-search accessed on 9 January 2021).

We analyzed the phylogenetic relationship of MTP and HMA proteins in eggplant and other Solanaceae using *Arabidopsis thaliana* as outgroup (see Appendix A for the employed methodology).

For amino acid properties, we predicted the transmembrane domains (TMDs) number, MW, pI, GRAVY, subcellular localization, and the putative transmembrane regions of *MTP* and *HMA* proteins with the ProParam tool (https://web.expasy.org/protparam/, accessed on 11 January 2021), Plant-mPLoc Server (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/, accessed on 14 January 2021), and TMHMM Server V.2.0 (http://www.cbs.dtu.dk/services/TMHMM/, accessed on 14 January 2021).

2.7. Quantitative PCR (qPCR) Analysis of Gene Expression

Total RNA was isolated from young leaves of two different cultivars: PQ and RB using the Rneasy Plant Mini Kit (Qiagen) and following the manufacturer's protocol. Analyses were performed on 5 plants for each cultivar. The extracted total RNA was stored at −80 °C until use. Total RNA concentration was determined by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, NC, USA), whilst its integrity was verified using a bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). To avoid genomic DNA contamination, total RNA samples were treated with RQ1 Rnase-Free Dnase I (Promega) and cDNA synthesis was performed using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Wilmington, NC, USA).

Quantitative analysis of gene expression was then performed using Corbett Life Science Rotor-Gene™ 6000. All amplification reactions were carried out in a final volume of

10 µL containing: 2 µL of H_2O Rnase-free, 2 µL of cDNA (1:20), 1 µL of primer mix (8 µM each), and 5 µL 2X QuantiFast SYBR Green PCR Master Mix (Qiagen). I oligonucleotide sequences are listed in Table S1.

Among the *MTP* and *HMA* genes identified in eggplant with the above described in silico procedure, we analyzed the expression of genes previously reported to be directly involved in Cd transport or in Zn transport, a metal which is known to show strong chemical affinity with Cd [41]. We thus focused on gene expression of the Zn transporters *MTP1* to *MTP5* and *MTP12*, and on Zn/Cd/Co/Pb transporters *HMA1* to *HMA4* [42].

To quantify variation in the specific gene expression, we used the reference gene *APRT* as control [43]. Quantification of gene expression was carried out using the $2^{-\Delta\Delta Ct}$ method [44].

Student's t-test was performed to detect differences in gene expression between treated and untreated samples and between the two selected cultivars.

2.8. Statistical Analysis

Statistical analyses were performed on all the investigated parameters. For germination, hydroponic and qPCR analyses 180 seeds, 20 and 5 plants per cultivar, for each treatment, respectively, were used. Differences among treatments were assessed using the Kruskal–Wallis test with the Dunn–Kruskal–Wallis test for pairwise comparisons, and significance level was set to $p = 0.05$ (Bonferroni correction). All statistical analyses were performed using R 3.6.3.

3. Results

3.1. Germination Assays at Different Cd Concentrations

At low Cd concentrations (i.e., 5 and 10 µM), germination was not affected in both control and treated seeds. At higher Cd concentrations, we observed a gradual reduction in germination levels with only 52% of treated seeds germinating at the highest concentration treatment (100 µM) (Figure 1A). The susceptibility to Cd was different in the six investigated cultivars (Figure 1A, right panels). BN, PQ and VF showed a higher tolerance, with no significant differences even at the highest concentration treatments. In CV, RB and VL, germinability decreased with increasing Cd concentration and was statistically significant starting from treatments at 25 µM. VL was the cultivar showing the strongest effect of Cd on germination with no seed germinated at 100 µM. BN was, instead, the cultivar showing the highest germination rate at the highest Cd concentration (Figure 1A).

3.2. Hydroponic Culture of Plantlets at Different Cd Concentrations

Plantlets exposed to Cd treatments showed negative effects after a week of hydroponic culture. At concentrations higher than 10 µM, we observed chlorosis in leaves, whilst at concentrations higher than 50 µM, there was a visible reduction in leaf area; Cd in hydroponic culture also induced visible chlorosis, a reduction in leaves, modifications in roots length and architecture with a reduction in secondary roots (Figure 2).

After 15 days in the hydroponic culture, plants showed different susceptibility to different Cd concentrations (Figure 1B). Plants exposed to low concentrations of Cd (i.e., 5 µM) showed no differences from the control in terms of survival (Figure 1B). With the increase in Cd concentration, we observed a reduction in survival reaching a level of 2.8% at 100 µM. In our experiment, 10 µM was the concentration leading to statistically significant differences compared to the control.

In all the investigated cultivars, negative effects arose from 5 µM as at this concentration there was no difference with control. The general trend of a reduction in survival with increasing Cd concentrations was common to all the investigated cultivars. Survival frequency is significantly different from control starting from 10 µM (Figure 1B) in all the cultivars apart from RB which showed a significant increase in mortality only from 25 µM. The highest tested concentration of Cd (i.e., 100 µM) was the most toxic, with most plants dying after a short period of time; however, as previously reported for germination rate,

also in terms of survival frequency, the least susceptible cultivar was BN, which showed the highest tolerance to the highest Cd concentration with the 11.1% of plants surviving after 15 days.

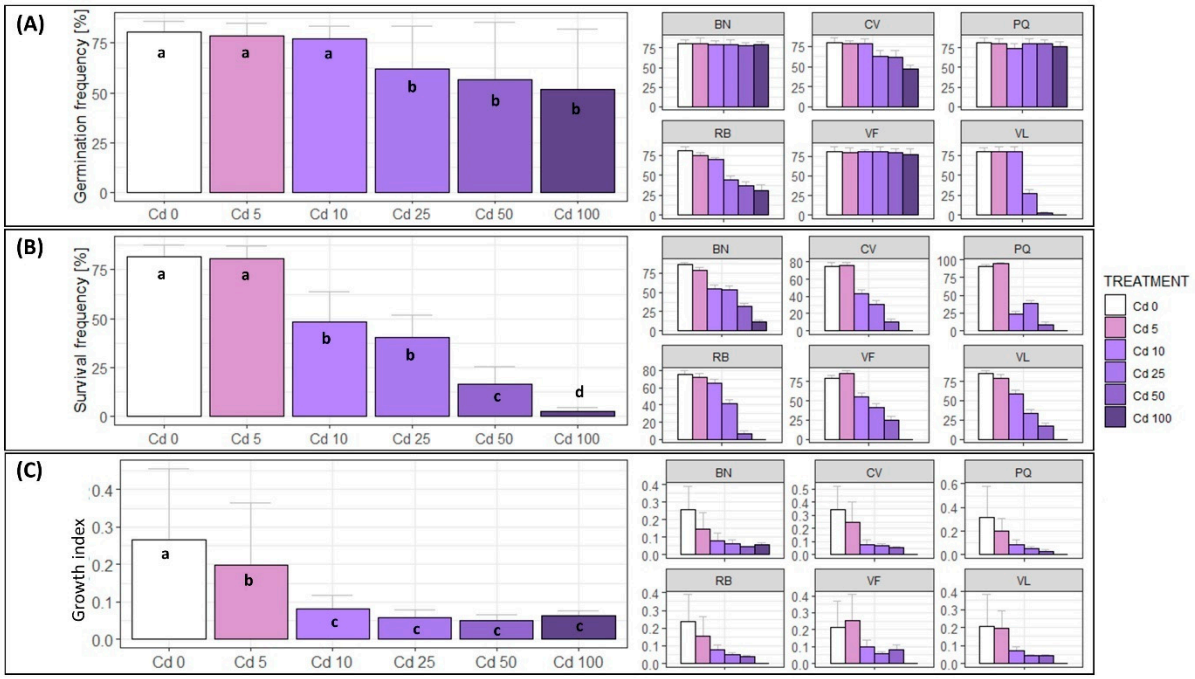

Figure 1. Influence of Cd on eggplant: (**A**) seed germination; (**B**) plantlet survival; (**C**) growth index. Data are shown together (left side) and independently (right side) for each investigated cultivar: BN = Bellezza Nera; CV = Cima Viola; PQ = Hybrid F1 Purple Queen; RB = Rotonda Bianca Sfumata di Rosa; VF = Violetta di Firenze; VL = Violetta Lunga. Error bars represent the standard deviation. Different letters indicate significant statistical differences ($p \leq 0.05$) among Cd treatments.

Figure 2. Effect of Cd on eggplant morphology in hydroponic culture. Each panel includes five plantlets treated with different Cd concentrations. (**A**) Bellezza Nera (BN); (**B**) Cima Viola (CV); (**C**) Hybrid F1 Purple Queen (PQ); (**D**) Rotonda Bianca Sfumata di Rosa (RB); (**E**) Violetta di Firenze (VF); (**F**) Violetta Lunga (VL).

The growth index was estimated only on plants survived after 15 days of treatment (Figure 1C). The growth index decreased with increasing Cd concentration and was significantly different from control starting from 5 µM (Figure 1C). Presence of Cd resulted in growth problems in all the investigated cultivars. VF and VL showed significant negative effects of Cd from a concentration of 10 µM. The remaining cultivars showed significant differences already when treated at 5 µM. Results on the tolerance index are reported in Table 1.

Table 1. Tolerance index and standard deviation (sd) for the six investigated eggplant cultivars.

Cultivar	Treatment	Tolerance Index [%]	sd
BN	Cd 5	90.63	4.32
	Cd 10	71.25	3.35
	Cd 25	60.9	5.54
	Cd 50	58.35	2.38
CV	Cd 5	88.23	6.98
	Cd 10	51.76	5.85
	Cd 25	50.89	3.99
	Cd 50	48.76	4.01
PQ	Cd 5	60.08	6.87
	Cd 10	35.71	5.72
	Cd 25	20.15	4.88
	Cd 50	4.89	1.32
RB	Cd 5	100	4.84
	Cd 10	55	3.83
	Cd 25	21	2.15
	Cd 50	18.74	3.44
VF	Cd 5	73.38	7.02
	Cd 10	48.38	3.66
	Cd 25	39.12	2.83
	Cd 50	35.48	2.46
VL	Cd 5	60.07	7.10
	Cd 10	28.12	6.77
	Cd 25	29.31	5.44
	Cd 50	27.96	4.32

3.3. Ionome Analysis, Bioaccumulation Coefficients and the Translocation Index

Eggplants grown with 5 µM Cd showed a dramatic increase in Cd content in the three plant organs and roots are clearly the primary accumulation site (Figure 3A). In this organ, Cd content was variable among the investigated cultivars reaching values ranging from 662 mg kg^{-1} dry weight in RB to 1466 mg kg^{-1} dry weight in BN. In stems and leaves, Cd concentration was lower than in roots (Figure 3A). Lowest Cd content was detected in RB for all the organs (Figure 3B).

Differences were detected in the bioaccumulation index of the six investigated cultivars (Table 2). BN, identified as the most tolerant cultivar for the above-mentioned parameters, also showed the highest ability of sequestrating Cd from the nutritive solution (bioaccumulation index = 2.61); RB, instead, was the one with the lowest ability (bioaccumulation index = 1.18). In eggplant, Cd is translocated from roots to aboveground organs with a low efficiency (Figure 3C). Translocation ability from root to shoots is not significantly different in the six investigated cultivars.

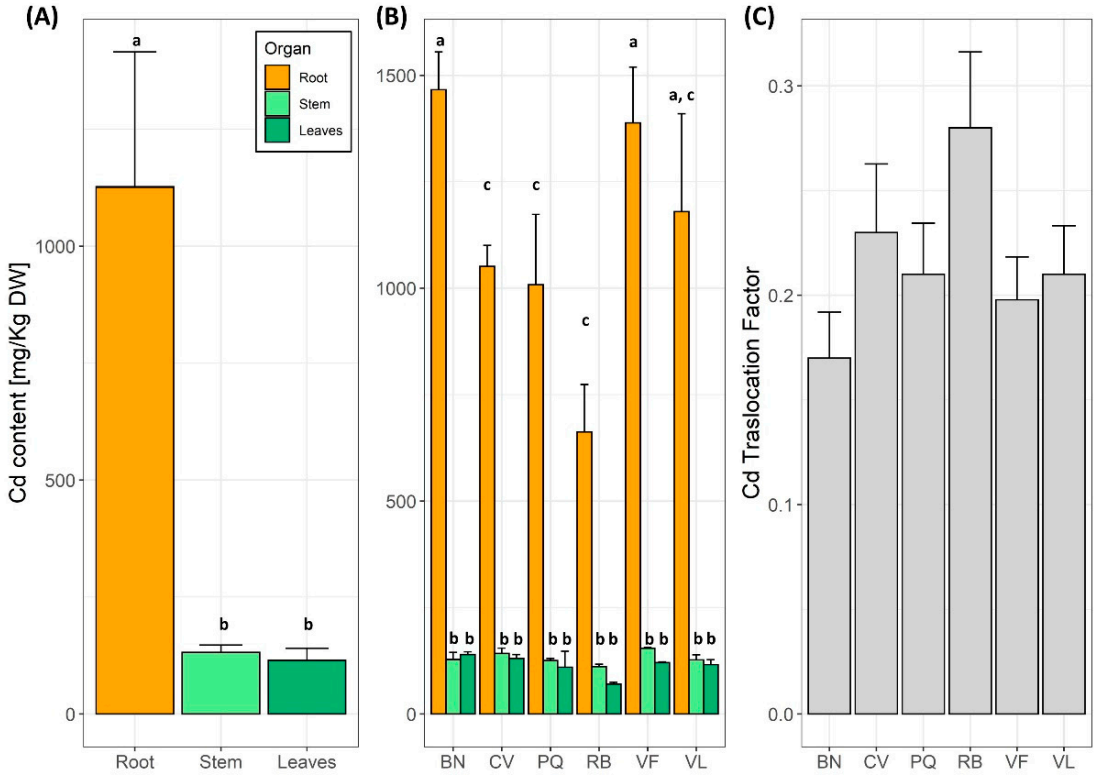

Figure 3. Cd content (**A**,**B**) and translocation factor (**C**) for eggplants grown with 5 µM Cd. Cd content in the three plant organs is showed for all the cultivars together (**A**) and for each investigated cultivar (**B**): BN = Bellezza Nera; CV = Cima Viola; PQ = Hybrid F1 Purple Queen; RB = Rotonda Bianca Sfumata di Rosa; VF = Violetta di Firenze; VL = Violetta Lunga. Error bars represent the standard deviation. Different letters indicate significant statistical differences ($p \leq 0.05$).

Table 2. Bioaccumulation index and standard deviation (sd) for the six investigated eggplant cultivars.

Cultivar	Bioaccumulation Index	sd
BN	2.61	0.16
CV	1.87	0.09
PQ	1.79	0.29
RB	1.18	0.20
VF	2.47	0.23
VL	2.10	0.41

The presence of Cd in nutritive solution strongly modified ionome of eggplants compared with control untreated plants (see Figure 4). Significant differences were observed for eleven element contents in roots (As, Ca, Co, Mg, Mn, Mo, Pb, Se, Sr, V, and Zn), six in stem (Ca, Mn, Na, Se, Sr, and Zn), and six in leaves (Ba, Cr, Cu, Na, Se, and Sr) out of the seventeen micronutrients measured. Data were variable among investigated cultivars (Table S2).

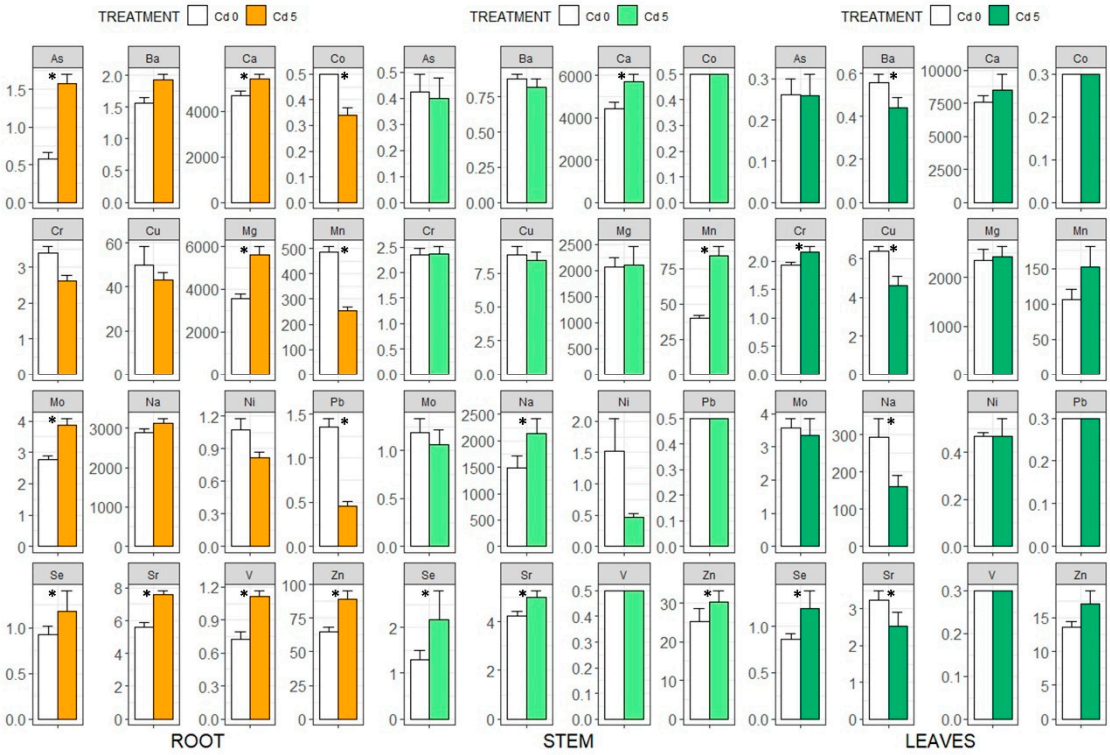

Figure 4. Effect of 5 µM Cd on micronutrient contents in each eggplant organ: root, stem and leaves. Error bars represent the standard deviation (* = $p \leq 0.05$).

3.4. Analysis of Photosynthetic Pigments

The negative effects described above are also mirrored in photosynthetic pigments content of plantlets exposed to Cd. Chlorophylls pools showed a reduction with increasing Cd concentration in the hydroponic culture solution. Chlorophyll *a* was the pigment which was most affected by the presence of Cd in the nutritive solution, its content was significantly lower than control at a concentration of 5 µM. Chlorophyll *b* content, instead, started to be significantly lower than in control plants from a concentration of 10 µM. Total carotenoid content showed an opposite trend, increasing with increasing Cd concentrations. The increase in carotenoid content was significant starting from a concentration of 25 µM, at this Cd concentration total carotenoid content in leaves reached 830.4 µg g^{-1} compared to 622.5 µg g^{-1} observed in control plants (Table 3).

Table 3. Effect of each Cd treatment on chlorophyll *a* and *b* (Chl *a*, Chl *b*) and carotenoid content for all the investigated cultivars. Means and standard deviations (sd) followed by different letters within each column indicate statistically significant differences based on the Dunn–Kruskal–Wallis test at $p \leq 0.05$.

Treatment	Chl *a* (µg g^{-1} Fw)	Sd	Chl *b* (µg g^{-1} Fw)	Sd	Chl tot (µg g^{-1} Fw)	Sd	Carotenoid (µg g^{-1} Fw)	Sd
Cd 0	660.18 ^{a}	182.64	241.20 ^{a}	60.37	901.38 ^{a}	242.58	622.54 ^{a}	184.32
Cd 5	548.94 ^{b}	213.51	201.75 ^{a}	85.00	750.69 ^{a}	292.80	643.88 ^{a}	201.88
Cd 10	282.80 ^{c}	61.18	175.12 ^{b}	65.26	457.93 ^{b}	125.53	721.58 ^{a}	229.03
Cd 25	183.22 ^{c}	17.63	147.47 ^{b}	52.60	330.69 ^{b}	70.04	830.39 ^{b}	279.56
Cd 50	89.08 ^{c}	48.20	166.74 ^{b}	177.37	255.82 ^{b}	187.56	1006.52 ^{b}	277.82

This general picture of a decrease in Chlorophyll *a* and *b* content and of an increase in carotenoid content due to Cd was similar in all the investigated cultivars (Table 3). In CV and VL, total chlorophyll content was significantly lower than in control plants starting

from the lowest tested Cd concentration (5 µM). In BN, PQ, RB and VF, instead, it started to be significantly different from controls at a concentration of 10 µM. Carotenoid content was significantly different from control at concentrations of 10 µM for CV, RB and VL and at concentrations of 25 µM for PQ and VF. BN showed an increase in carotenoids only starting from a concentration of 50 µM.

3.5. Quantitative Analysis of Gene Expression of HMA and MTP Genes

The *in silico* analysis identified 11 polypeptides belonging to the *MTP* gene family and 13 belonging to the *HMA* gene family in *Solanum melongena* (hereafter referred to as *SmeMTP*, and *SmeHMA*; Table S3). Phylogenetic analysis of these gene families in eggplant, in other Solanaceae and in *Arabidospsis* are reported in the Appendix A.

For *SmeMTP* genes, the length of the coding sequences (CDS) ranged between 639 bp (*SmeMTP12*) and 1404 bp (*SmeMTP11*), with 212 to 467 amino acids and a relative molecular weight (MW) ranging between 23,807.48 (*SmeMTP12*) and 53,184.51 KDa (*SmeMTP11*). The total average of hydropathicity (GRAVY) of the *SmeMTP* proteins ranged from −0.020 (*SmeMTP9-10B*) to 0.203 (*SmeMTP4*). *SmeMTP5* showed the highest isoelectric point (pI), i.e., 7.07, whereas all the other *SmeMTP*s showed a value lower than 7. All *SmeMTP* proteins were expected to be localized in the vacuole, nevertheless *SmeMTP9-10A* was localized also in the cell membrane. In addition, most *SmeMTP* proteins contained 4–6 typical transmembrane domains (TMDs), whereas *SmeMTP8C* and *SmeMTP5* had only 2, and none was found in *SmeMTP12*.

For *SmeHMA* genes, the length of the coding sequence (CDS) ranged from 438 bp (*SmeHMA1*) to 3792 bp (*SmeHMA2-3*), with 148 to 1236 amino acids, as well as a relative molecular weight (MW) ranging from 15,666.5 to 136,627.44 kDa. The total average of hydropathicity (GRAVY) ranged from −0.204 (*SmeHMA2-3*) to 0.769 (*SmeHMA1*). Moreover, *SmeHMA8* has the highest isoelectric point (pI), i.e., 9.76, whereas the other *SmeHMA*s showed a value lower than 9.5. The *SmeHMA* proteins were expected to be localized in the cell membrane, nevertheless *SmeHMA1* was found in the chloroplast, *SmeHMA2-3* in the cell membrane and in the nucleus, *SmeHMA5C* and *SmeHMA5E* were localized both in the cell membrane and in the chloroplast and *SmeHMA9* was localized in the vacuole (Table S3). *SmeHMA5D*, *SmeHMA5E*, *SmeHMA5F* and *SmeHMA8* did not contain TMDs, *SmeHMA1* was found to have only 1 TMD, *SmeHMA5B* and *SmeHMA5C* had 2 TMDs, *SmeHMA2-3*, *SmeHMA5A*, *SmeHMA6A*, *SmeHMA6B*, and *SmeHMA7* were found to have from 5 to 8 TMDs, and *SmeHMA9* was found to have 12 TMDs (Table S3).

As the effect of the 5 µM Cd treatment, we observed different gene expression trends in the two investigated cultivars PQ and RB (Figure 5). With regard to *SmeMTP* genes, *SmeMTP1/2/3* in PQ was over-expressed in stems, while, in the same organ, it was under-expressed in RB; *SmeMTP4* in PQ was over-expressed in root, while it was under-expressed in stem and over-expressed in leaves in RB; *SmeMTP5* in PQ was over-expressed in root and under-expressed in leaves; *SmeMTP12*, instead, was under-expressed in root of both cultivars and in PQ stem and over-expressed in leaves of both cultivars.

Differences were similarly found in *SmeHMA* genes: *SmeHMA1* showed an opposite expression profile in the two cultivars, it was under-expressed in PQ in root and leaves and no differences were detected in stems, while it was over-expressed in RB in root and leaves and under-expressed in stems. Finally, *SmeHMA2/3/4* was over-expressed in RB leaves.

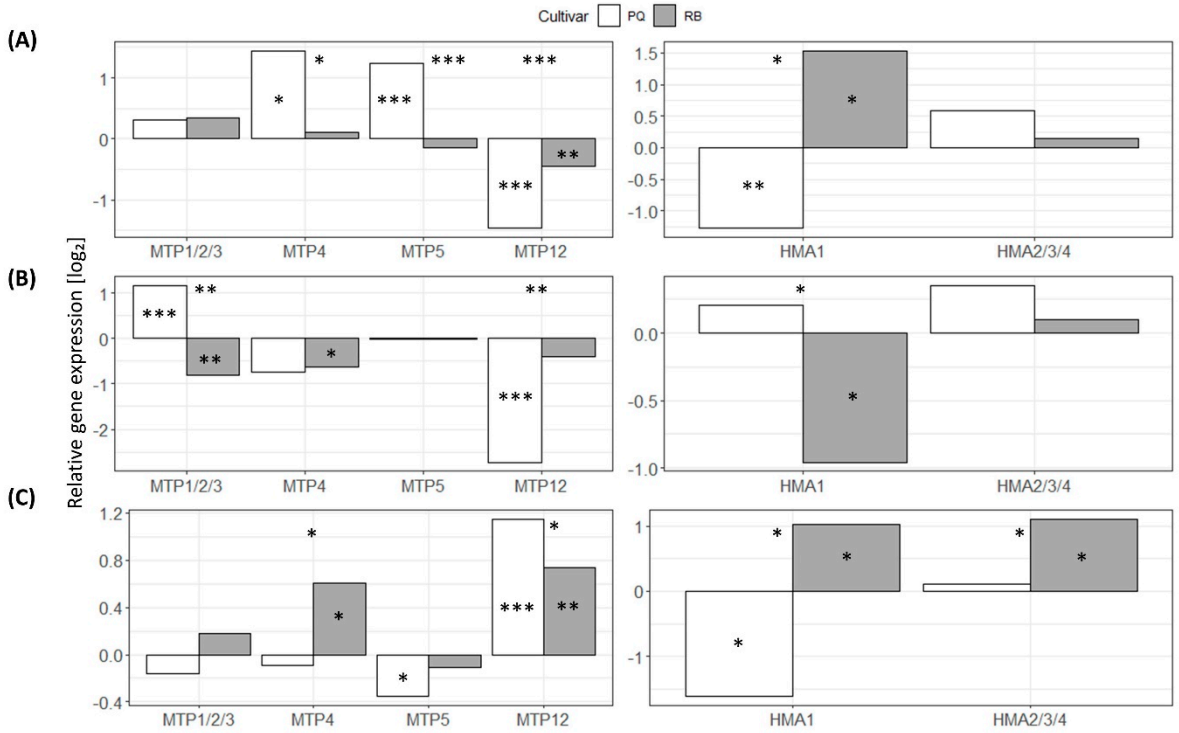

Figure 5. Relative expression of *MTP* and *HMA* genes in roots (**A**), stems (**B**) and leaves (**C**) of PQ (Hybrid F1 Purple Queen) and RB (Rotonda Bianca Sfumata di Rosa). Gene expression was evaluated after 15 days on plantlets growing in hydroponic culture with 5 µM Cd. The $\log_2$ of gene expression calculated using the $2^{-\Delta\Delta Ct}$ method is reported in the graph. Statistical comparison was performed using Student's t-test; statistical differences with control samples are represented inside the bar plots; statistical differences between the two cultivars are represented outside the bar plots (* = $p \leq 0.05$, ** = $p \leq 0.01$; *** =$p \leq 0.001$).

4. Discussion

The characterization of crop performances in contaminated soils is a primary aim of crop research. In eggplant, several cultivars have been characterized for the effects of Cd on plant performance in the primary diversification center [32,33], but less is known about cultivars from secondary diversification centers such as the Mediterranean basin. In this study, we comparatively analyzed seed germination, seedling growth, photosynthetic pigments content, among organs translocation patterns, ionic content balance and expression of genes involved in the Cd uptake in six Mediterranean eggplant cultivars treated with different concentrations of Cd. Overall, we found that Cd influences all the investigated parameters but with notable cultivar-level differences (Figures 1–4). Our study enables evaluating the potential of different Mediterranean eggplant cultivars for food safety.

4.1. Effects of Cd on Eggplant Germination

The ability of different trace metals to penetrate seeds during germination and to affect radicle protrusion is highly variable among flowering plants [45]. Among different trace metals, Cd was reported to inhibit the hydrolysis of reserve carbohydrates and their translocation, hence resulting in decreased germination and disruption of seedling growth mechanism [46]. Accordingly, in our study, we found that Cd has a detrimental effect on eggplant germination (Figure 1A). Germinability was not significantly different from control at low concentrations (5 and 10 µM) but was significantly lower in treatments at higher concentrations (25 and 50 e 100 µM; see Figure 1A).

The effects of Cd on germinability were different in the six investigated cultivars (Figure 1A), with three of them (BN, PQ and VF) suffering no effect from Cd on germination even at the highest treatment concentrations (Figure 1A). In VL, CV and RB,

germinability decreased instead with increasing Cd concentration, with the RB cultivar showing a significant decreasing even at 10 μM. VL was the cultivar showing strongest effects of Cd on germinability.

4.2. Effects of Cd on Eggplant Survival and Growth Rates

The effects of Cd on survival and growth in eggplant were previously investigated and were showed to induce some toxic effects [47–49]. The growth inhibitory effect of Cd is mainly attributable to its effect on photosynthesis efficiency [33]; Cd is able to inhibit the photosynthetic activity, altering the chlorophyll content as also showed by our analysis. Accordingly, our results clearly show a toxic effect of Cd both on survival and growth rate. After 15 days of exposition, plants showed different susceptibility to different Cd concentrations in terms of survival (Figure 1B).

Plants exposed to low Cd concentrations (i.e., 5 μM) showed survival rates similar to the control plants and there was a common trend towards a reduction in survival starting from 10 μM in the investigated cultivars. RB instead showed a significant increase in mortality only from 25 μM. RB was thus the more tolerant among the tested cultivars in terms of survival. The highest tested concentration was instead highly toxic for all the cultivars with all plants showing necrosis. It was only in BN that 11.1% of the plants were able to survive at 100 μM (Figure 1B).

Previous studies investigating the effects of Cd on different life history traits in eggplant reported toxic effects on growth and yield of this trace metal at high concentrations (i.e., 10^{-2} M), but also a promotor effect at lower concentrations (i.e., 10^{-8} M; [32,47,50]). In our study, we observed growth problems in all the investigated cultivars. For the VF and VL cultivars, significant negative effects of Cd started at 10 μM concentrations (Figure 1C). For all the other cultivars significant differences already arose when treated at 5 μM.

4.3. Effects of Cd on Eggplant Accumulation and Translocation

Interactions of plants with trace metals have been largely investigated for different purposes [51]. As expected, Cd accumulates preferentially in roots where Cd content increased by 1000 fold compared to control plants. These results are consistent with those reported in [33,52]. In particular, BN showed the highest Cd content in roots.

In stems and leaves, Cd concentration was 10-fold lower than in roots (Figure 3A). The lowest Cd content, concordantly in the three organs, was detected in RB, thus showing the lowest ability, among the six cultivars, to accumulate Cd (Figure 3B).

To assess the bioaccumulation ability of each cultivar we adopted the four-degree scale described in [53]. According to this scale, plants with a bioaccumulation index above 1 can be considered hyperaccumulators.

Our results show significant differences in the bioaccumulation index in the six investigated cultivars; however, all the cultivars have values above 1 (Table 2), hence showing a high ability of sequestrating Cd. BN was the cultivar with the highest bioaccumulation index, i.e., 2.61) while RB showed the lowest index (i.e., 1.18). Nevertheless, the observed high bioaccumulation ability of the investigated cultivars is mainly explained by elevated Cd concentration in roots, rather than in stems and leaves. To assess the capability of plants to grow in presence of a given concentration of Cd, we also calculated a tolerance index (T_i) and identified tolerant cultivars following [54] (i.e., T_i higher than 60%). As reported in Table 1, all the cultivars were tolerant at 5 μM of Cd; however, BN was the cultivar showing the highest tolerance, with a T_i higher than 60% even at 10 and 25 μM.

Tolerance to trace metals can be mediated by different processes such as exclusion, secretion of metal-chelating macromolecules, metal distribution in specific tissues and their compartmentation in organelles as vacuoles. Tolerant species often limit the entrance and translocation from roots to aboveground organs [52]. Here, we found that in eggplant, Cd is translocated from roots to epigean organs with a low efficiency: all the cultivars showed low translocation indices and no significant differences were detected among them (Figure 3C). These results are consistent with other studies, which demonstrate that approximately

60–90% of heavy metals accumulated by plants are adsorbed on the root cell walls [55]. A low Cd mobility could be explained by elevated Cd retention and sequestration levels of root cell walls and vacuoles, which can cause a reduction in Cd transport from roots to aboveground organs. However, contrasting results have been provided on eggplant translocation ability, thus suggesting a highly variable response among different cultivars (e.g., [52]). Recently, the authors of [32] have identified both low- and high-Cd eggplant cultivars, confirming this hypothesis. Interestingly, in this study, high-Cd and low-Cd cultivars showed a Cd content in fruits significantly lower than in leaves. Although we did not test Cd content in parts of the plant generally used for food consumption, these results suggest that Cd content in leaves might be used for a rapid screening of different cultivars.

4.4. Effects of Cd on Eggplant on Photosynthetic Pigments Content

The above-described problems in terms of growth rate and survival of eggplant in Cd treatments can be partly explained by the negative effects of Cd on photosynthesis [8,56–58]. A reduction in photosynthetic pigments in leaves is indeed among the first visible symptoms of Cd toxicity (e.g., [56,59]) This effect may be explained by the fact that Cd may act by reducing transcription of genes involved in photosynthesis as psbA, psaB and rbcL [58] or can influence cellular and sub-cellular structures modifying chloroplast ultrastructure [60]. Cd was also showed to spontaneously form a complex with chlorophyll, being incorporated at the central position of the chlorophyll molecule porphyrin ring, where it replaces magnesium (e.g., [61]).

In this study, we found that chlorophyll content decreased with increasing Cd concentration, but this pattern was higher for chlorophyll *a* than for chlorophyll *b*. A negative correlation between chlorophyll content and increasing Cd concentrations has already been reported in recent studies (e.g., [33]). Parallel to chlorophyll decay, we observed an increase in carotenoid content with increasing Cd concentrations. This general picture of a decreasing of chlorophyll *a* and *b* content and of an increase in carotenoid content due to Cd was similar in all the investigated cultivars (Table 3). Several previous studies indicate that Cd stimulates the production of Reactive Oxygen Species (ROS, e.g., [62]) that can induce peroxidation of photosynthetic structures. Carotenoids are key pigments for plant response to this stress as they are involved in photoprotection [63]. Therefore, the observed trend of an increase in carotenoid content (a trend already reported in sunflowers; [64]), can be explained as a response to peroxidative stress induced by Cd. Inter-cultivars differences were also observed for photosynthetic pigments related parameters (Figure 4).

In CV and VL, total chlorophyll content was significantly lower than in control plants starting from the lowest tested Cd concentration (5 µM). In BN, PQ, RB and VF, instead, it started to be significantly different from controls at a concentration of 10 µM.

Carotenoid content was significantly different from control at a concentration of 10 µM in CV, RB and VL; in PQ and VF, significant differences were observed at 25 µM; and BN, instead, showed an increase in total carotenoid only starting from a concentration of 50 µM.

4.5. Effects of Cd on Eggplant Ionic Balance

Coordination of uptake, translocation and accumulation is crucial for the maintenance of mineral element concentration in tissues within physiological limits [60]. Non-essential trace metals may be present in elevated concentrations in soils and may impact on this subtle coordination of uptake, translocation and accumulation of other elements [65]. Plant ionome is influenced by genotype, organ and environmental conditions [66–70]. Here, we found that Cd presence modified micronutrient content of different elements with respect to control plants (see Figure 4). These differences were higher in roots, where the concentration of eleven elements was altered; and lower in stem and leaves, where six elements were altered. Cd thus affects transport of the mineral elements in the different plant organs and their content is regulated independently.

4.6. Effects of Cd on Eggplant HMP and MTP Gene Expression

In this study, we also analyzed the expression of the *MTP* and *HMA* gene families, i.e., two classes of genes encoding for proteins and involved in Cd and other trace metals uptake [71]. Among these genes, we specifically focused on those reported to have a role in Cd or to chemically affine Zn uptake [41,42], i.e., *SmeMTP1/2/3*, *SmeMTP4*, *SmeMTP5* and *SmeMTP12*. We thus gained expression profiles, using the selected genes, in leaves, stems and roots of eggplant cultivars grown in 5 µM Cd for 15 days. For this analysis, we selected two cultivars, i.e., PQ and RB that show different physiological responses to Cd and different accumulation patterns in the three organs. *SmeMTP1/2/3* (an orthologous of the well characterized *MTP1* of *Arabidopsis*) was differently expressed in the two analyzed cultivars: PQ, which showed a higher Cd content, also showed a higher expression of this gene in stems, while *SmeMTP1/2/3* is under-expressed in stems in RB. *SmeMTP4* also showed different expression patterns in the two cultivars being over-expressed in roots of PQ, under-expressed in stems of RB and over-expressed in leaves the same cultivar. *SmeMTP5* showed deviation from the control treatment in PQ (over-expression in roots and under-expression in leaves) but showed no deviation from control in RB. Finally, *SmeMTP12* was concordantly under-expressed in roots and over-expressed in leaves of both cultivars and under-expressed in PQ stems.

In the other analyzed gene family, *HMA*, we focused on two genes, namely *SmeHMA1* and *SmeHMA2/3/4*. Genes from the *HMA* family have a crucial role in plant nutrition [72,73]. Results of our RT-qPCR analysis showed no effects of Cd on *HMA2/3/4* expression in roots and stems. The only deviation from this pattern was observed in leaves of RB where *HMA2/3/4* was over-expressed. Our results instead showed that Cd treatments influenced *HMA1* expression in all RB organs and in root and leaves of PQ. In PQ, *HMA1* gene expression is reduced in leaves and roots if compared to control; in RB, instead, it is reduced in stems and over-expressed in leaves and roots.

5. Conclusions

Our study highlights the importance of elucidating mechanisms involved in the interaction between a widely cultivated crop, *S. melongena*, and Cd, a heavy metal known for its severe toxic effects on plants and human health. Our results showed a variable Cd tolerance among the investigated cultivars, with Bellezza Nera (BN) being the least susceptible at high-Cd contents in terms of germination rate, survival frequency and the growth index. BN was the cultivar with the highest tolerance index in plants exposed to 5, 10 and 25 µM of Cd (i.e., 90.63, 71.25 and 60.9, respectively) and showed the highest bioaccumulation ability, with an index of 2.61. All the investigated cultivars have values above 1, highlighting a high ability in sequestrating Cd from the nutritive solution. This finding is likely affected by an elevated root cadmium uptake capacity. However, we found a low mobility of Cd from roots to epigean organs, even in cultivars with high bioaccumulation, confirming that the majority of absorbed Cd was restricted to the roots rather than transported into aboveground tissues in all the investigated cultivars. BN was the most promising as a pollution-safe cultivar, due to its low translocation index and its high Cd tolerance. However, further studies are needed in order to characterize Cd content in the edible parts of the plant to finally confirm our results. The safe use of Cd contaminated soils is an urgent issue in Mediterranean highly contaminated soils and in volcanic soils naturally rich in heavy metals common in Southern Italy. Therefore, in order to identify other pollution-safe cultivars for food safety, further investigations are needed. Finally, this study identified and characterized putative transporter genes belonging to the *MTP* and *HMA* gene families, which are known to be involved in Cd uptake, for the first time in *Solanum melongena*. Our results will provide useful information for understanding the complex molecular mechanism of response in eggplant under Cd stress, which could be exploited in the future for food safety purposes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12071059/s1, Table S1: List of oligonucleotides used for gene expression analysis of MTP and HMA genes in eggplant. Table S2: Effect of 5 µM Cd on micronutrients content in organs of six eggplant cultivars: BN = Bellezza Nera; CV = Cima Viola; PQ = Hybrid F1 Purple Queen; RB = Rotonda Bianca Sfumata di Rosa; VF = Violetta di Firenze; VL = Violetta Lunga (* = $p \leq 0.05$). Table S3: *SmeMTP* and *SmeHMA* genes identified with the in silico procedure.

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Appendix A. Phylogenetic Analysis on MTP and HMA Genes

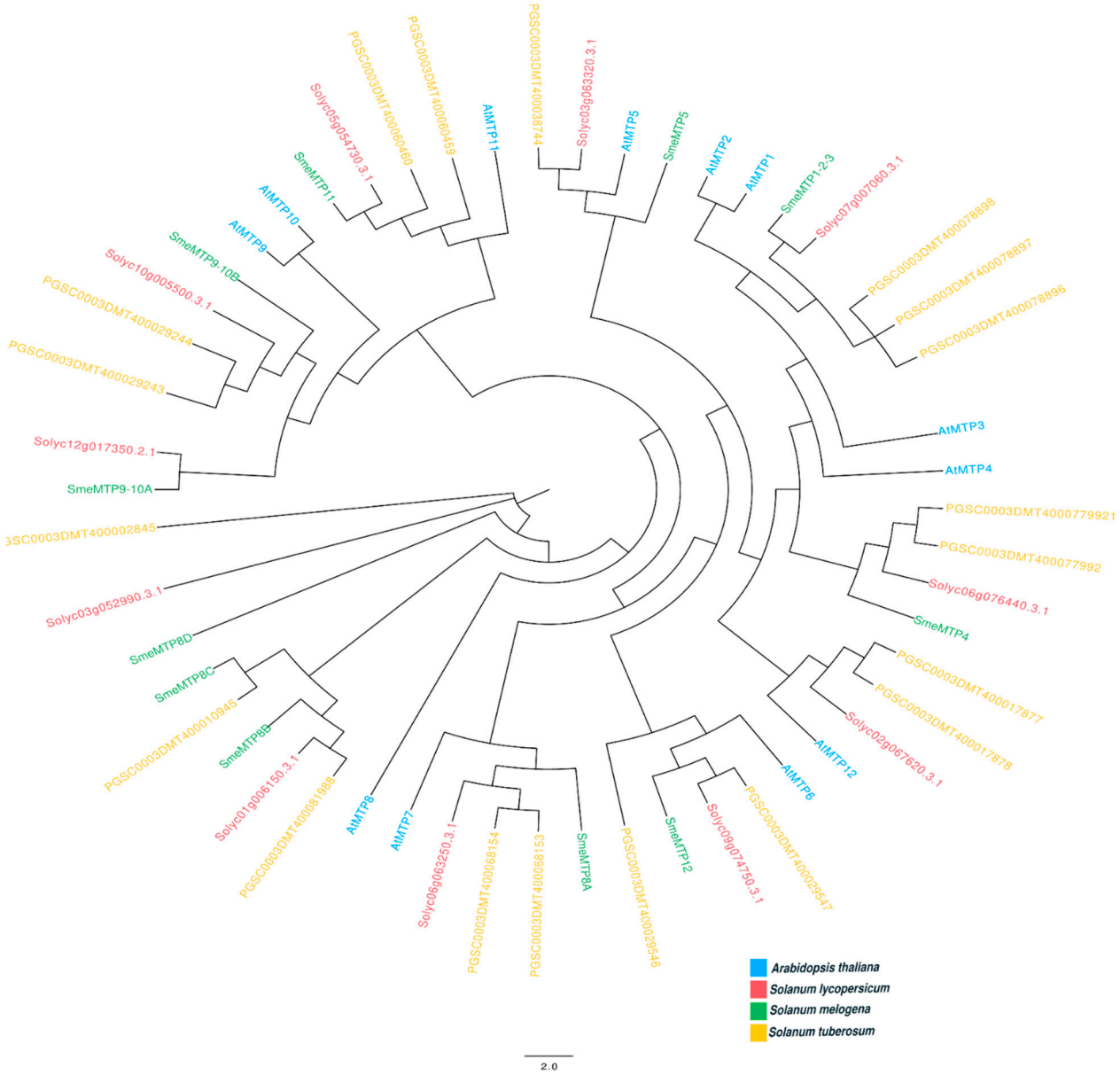
To understand the origin of these *MTP* and *HMA* gene families in eggplant, we analyzed their phylogenetic relationship in eggplant and other Solanaceae using *Arabidopsis thaliana* as outgroup. To do this, using ClustalW [74], we aligned the aminoacidic sequences of eggplant and *Solanum lycopersicum*, *S. tuberosum* and *A. thaliana* obtained from Sol Genomics Network database (SGN) and we built the phylogenetic trees using multiple amino acids sequence alignment with Neighbor Joining (NJ) method by phangorn R package.

Phylogenetic relationships among *SmeMTP* genes and *SmeHMA* genes in Potato, Tomato and *Arabidopsis thaliana* are showed in Fig. A1. Phylogenetic analysis carried out on the *SmeMTP* showed the presence of seven different groups (Figure A1). Phylogenetic analyses identified six main proteins in the *HMA* transporter family. Among genes involved in Cd transport *HMA2*, *HMA3* and *HMA4* have a common ancestor (See Figure A1) occurring in dicots and monocots.

Phylogenetic analysis carried out on the *MTP* gene family on different model species showed the presence of different groups. Five of these groups are at the basis of the tree, whilst two others originated from a duplication event. Group one, which includes *MTP1*, *MTP2*, *MTP3* and *MTP4* originated from a common ancestor and plays a role in transport in dicots [75]. Our in silico analysis showed the presence of a single sequence for these three genes (*MTP1/2/3*) in eggplant. These analyses also showed a gene duplication in gene *MTP8*, a pattern already reported in *Oryza* and *Sorghum* [75] and *Populus trichocarpa* [76].

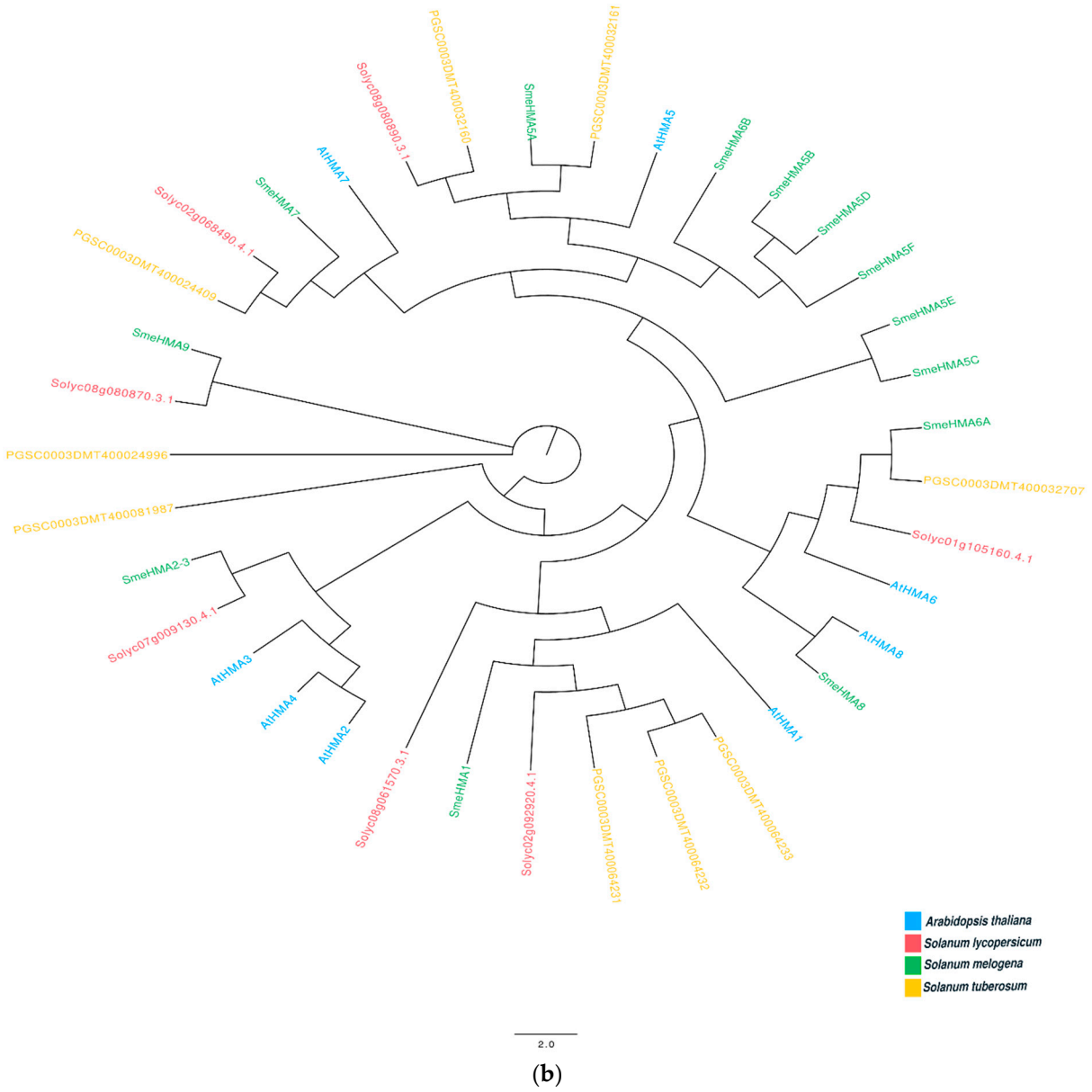
The transporter *HMA*, which includes integral membrane proteins that couple ATP hydrolysis to metal cation transport, is found in many organisms, and phylogenetic analyses identified six main proteins in this group.

To the second group belong the genes *HMA2*, *HMA3*, which suggests the presence of a common ancestor for these sequences occurring in both dicots and monocots [77].



(**a**)

Figure A1. *Cont.*



(**b**)

Figure A1. An unrooted, neighbor-joining (NJ) tree of the *MTP* family (**a**) and *HMA* family (**b**) in the selected plants. Branch lengths are proportional to phylogenetic distances.

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