

Article

Rapid Hormetic Responses of Photosystem II Photochemistry to Cadmium Exposure

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Abstract: Five-day exposure of clary sage (*Salvia sclarea*) to 100 μ M cadmium (Cd) in hydroponics was sufficient to increase Cd concentrations significantly in roots and aboveground parts and affect negatively whole plant levels of calcium (Ca) and magnesium (Mg), since Cd competes for Ca channels, while reduced Mg concentrations are associated with increased Cd tolerance. Total zinc (Zn), copper (Cu), and iron (Fe) uptake increased but their translocation to the aboveground parts decreased possible due to translocation barriers. Despite the substantial levels of Cd in leaves, without any observed defects on chloroplast ultrastructure, an enhanced photosystem II (PSII) efficiency was observed, with a higher fraction of absorbed light energy to be directed to photochemistry (Φ_{PSII}). The concomitant increase in the photoprotective mechanism of non-photochemical quenching of photosynthesis (NPQ) resulted in an important decrease in the dissipated non-regulated energy (Φ_{NO}), modifying the homeostasis of reactive oxygen species (ROS), through a decreased singlet oxygen (1O_2) formation. Thus, when clary sage was exposed to Cd for a short period, tolerance mechanisms were triggered, with PSII photochemistry to be regulated by NPQ in such a way that PSII efficiency to be enhanced. However, exposure to a combination of Cd and high light or for longer duration (8 days) to Cd alone, resulted in an inhibition of PSII functionality pointing out towards Cd toxicity. Thus, the rapid activation of PSII functionality at short time exposures and the inhibition at longer duration suggests a hormetic response and describes these effects in terms of “adaptive response” and “toxicity”, respectively.

Keywords: *Salvia sclarea*; chlorophyll fluorescence imaging; non-photochemical quenching; tolerance mechanism; photoprotective mechanism; photosynthesis; reactive oxygen species; toxicity; adaptive response, photochemical efficiency

1. Introduction

Cadmium (Cd) is occurring in soils at low concentrations but can arise to high concentrations as a result of numerous human activities, while being not biodegradable in the soil is considered as one of the most toxic elements, and also a non-essential element for plants [1-6]. Cadmium is taken up by roots and its translocation from the roots to the shoots and leaves with subsequent accumulation in the chloroplasts, will eventually disturb photosynthesis [7]. However, many plant species have established numerous special effective mechanisms for Cd detoxification and tolerance [4-6].

Cadmium tolerance is related with declines of the internal Cd accumulation [8] but some plant species are able to sustain growth or even improve their growth and functioning under Cd exposure, despite a high Cd accumulation in roots and shoots [9-11]. Foliar Cd content above 0.01 % dry

biomass ($100 \mu\text{g g}^{-1}$) is considered extraordinary and a limit value for Cd hyperaccumulation [7,12,13].

Plants can cope with stress by plethora of structural and functional mechanisms while low-level stress or short duration exposure stimulates plant performance [14-21]. This can be achieved through the involvement of a basal level of reactive oxygen species (ROS) [22-27], that are regulated by the non-photochemical quenching (NPQ) photoprotective mechanism of photosynthesis [21,24,28,29]. An elevated NPQ reduces electron transport rate (ETR) avoiding ROS formation [30,31]. ROS generation can be a direct consequence to photosystem II (PSII) damage or can inhibit the repair of PSII reaction centres [31-34].

Dose-response studies are proposing hormesis as a central dose-response phenomenon for a variety of stressors [8,35-40]. Hormesis is a widespread phenomenon usual in nature, independent of the kind of stressor, the physiological process or the organism it occurs [8,35-38]. It is described as the stimulatory effect of low doses or short exposure times, described by a biphasic dose response with a low dose stimulation and a high dose inhibition [41-43] representing an "over-compensation" response to a disruption in homeostasis [44].

Salvia sclarea (clary sage) is a flowering herb that is native in the Mediterranean basin area, possessing pharmacological activities, and being traditional used as a treatment for eye health and hair tonic. It is used for pharmaceutical purposes having antibacterial properties while in aromatherapy clary sage essential oil is used to alleviate stress acting as an anti-stressor and when inhaled it elicits feelings of relaxation and helps to reduce blood pressure [45]. *S. sclarea* is tolerant to heavy metals and has been characterized as Zn and Cd accumulator, while its essential oils are not contaminated with heavy metals [46].

Since any substantial effect on plant growth after exposure to Cd can be detected only if photosynthesis is disturbed [47], a hormetic response to Cd is frequently coupled with changes in the mechanisms of photosynthesis or the photosynthetic apparatus [8]. The toxic effects of Cd provoke oxidative stress in plants and are related with the production of ROS [4,38,48,49] that can be regulated by NPQ in such a way so that plants can cope with the stress [21,24,28,29]. Therefore, it was hypothesized that after exposure of clary sage to Cd, the photosynthetic machinery could display an hormetic response to Cd, if plant tolerance mechanisms were activated and photosystem II photochemistry could be regulated by the photoprotective mechanism of NPQ in a such way that PSII functionality to be enhanced without any chloroplast ultrastructure perturbations.

2. Results

2.1. Cadmium Accumulation and Elemental Concentrations

Upon exposure of plants to $100 \mu\text{M}$ Cd in hydroponics, Cd concentrations of aboveground tissues (shoot-leaves) and roots increased ($p < 0.05$) by 31-fold (Figure 1a) and 2900-fold (Figure 1b) respectively, with Cd ions to be retained almost exclusively in the roots and reaching $23941 \pm 715 \mu\text{g g}^{-1}$ vs $53.3 \pm 1.6 \mu\text{g g}^{-1}$ in the aboveground tissues. Cadmium exposure enhanced total Zn, Cu, and Fe uptake by 2.1-fold (Figure 2a), 1.7-fold (Figure 2b) and 1.5-fold (Figure 2c) respectively, but decreased their translocation to the leaves by 45%, 16% and 60%, respectively. Zinc, Cu, and Fe content in roots after Cd exposure reached 542 ± 16 , 112.2 ± 3.5 and $1696 \pm 51 \mu\text{g g}^{-1}$ from 195.4 ± 5.8 , 60.6 ± 1.8 and $945 \pm 28 \mu\text{g g}^{-1}$ respectively, while in aboveground tissues from 72.9 ± 2.2 , 12.24 ± 0.36 and $224.8 \pm 6.7 \mu\text{g g}^{-1}$ decreased to 32.58 ± 1.6 , 10.29 ± 0.28 and $88.4 \pm 2.6 \mu\text{g g}^{-1}$, respectively. Total Ca uptake was significantly reduced (58%), with root Ca accumulation to remain almost unaffected but Ca translocation to shoot-leaves to be significantly reduced (84%) (Figure 3a). Thus, after Cd exposure Ca content in roots was $10122 \pm 304 \mu\text{g g}^{-1}$ from 10005 ± 300 before, but in the aboveground tissues decreased to $3820 \pm 115 \mu\text{g g}^{-1}$ from that of $23484 \pm 704 \mu\text{g g}^{-1}$ before Cd treatment. Total Mn (Figure 2d) and Mg (Figure 3b) uptake decreased ($p < 0.05$) by 5% and 49%

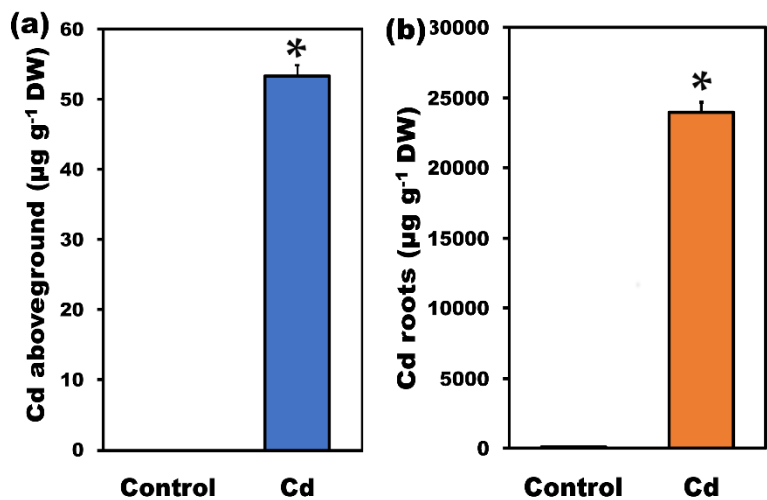


Figure 1. Changes of Cd accumulation in (a) aboveground (shoots-leaves) tissues and (b) roots, in $\mu\text{g g}^{-1}$ dry weight, after 5 days Cd treatment of *Salvia sclarea* plants. Error bars are standard deviations (n=5). Means between the two treatments that are statistically different ($p < 0.05$) are marked by an asterisk (*).

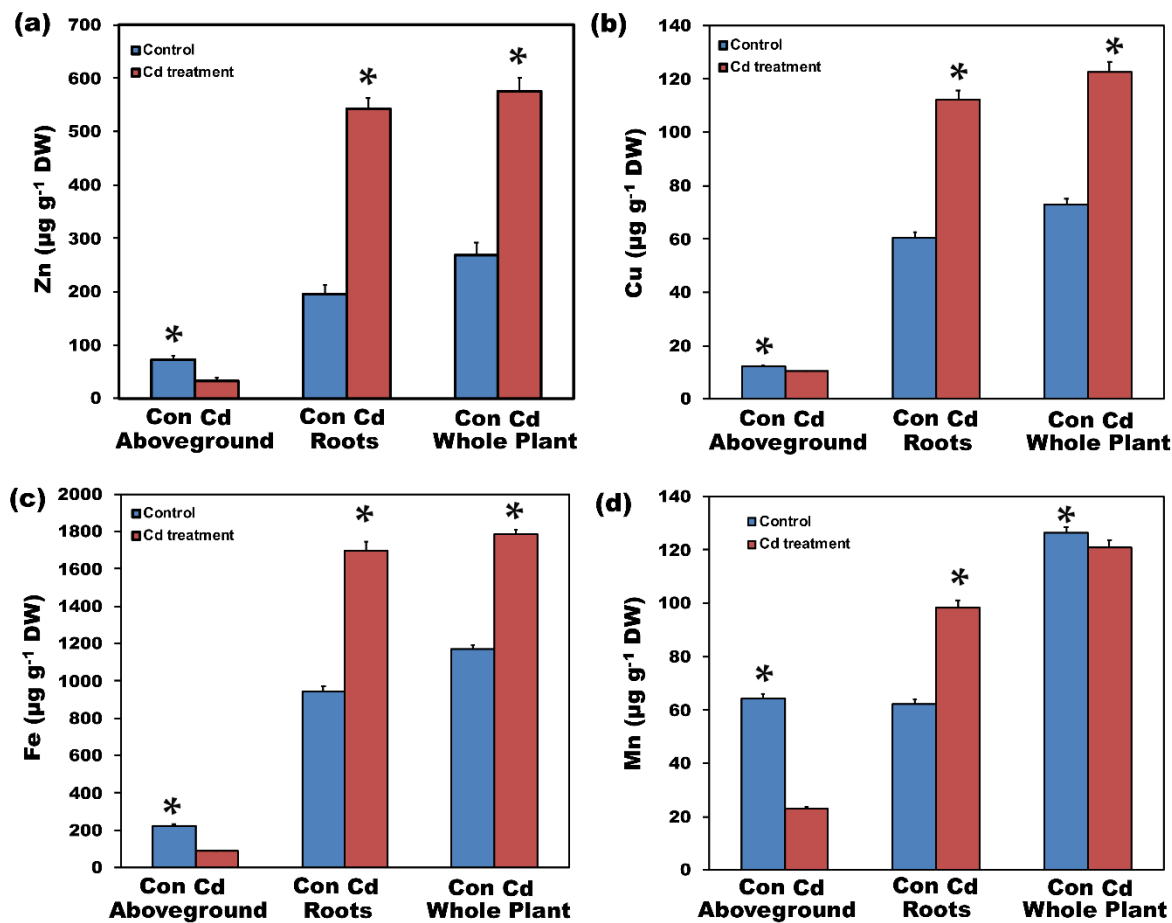


Figure 2. Zinc (a), Cu (b), Fe (c), and Mn (d) content, in $\mu\text{g g}^{-1}$ dry weight, of control (con) and 5 days Cd treated *Salvia sclarea* aboveground (shoots-leaves) tissues, roots and whole plants. Error bars are standard deviations (n=5). Means between the two treatments that are statistically different ($p < 0.05$) are marked by an asterisk (*).

respectively, but while Mg accumulation decreased in both roots (43%) and shoots-leaves (53%) (Figure 3b), Mn accumulation increased in roots (1.6-fold) and its translocation to the shoots decreased (65%) (Figure 2d). Magnesium, the next most negatively affected element after Ca, decreased in roots from $4184 \pm 126 \mu\text{g g}^{-1}$ before Cd treatment to $2391 \pm 72 \mu\text{g g}^{-1}$, while in aboveground tissues from 5851 ± 175 to $2778 \pm 83 \mu\text{g g}^{-1}$. Manganese increased in roots from $62.01 \pm 1.8 \mu\text{g g}^{-1}$ before Cd treatment to $98.1 \pm 2.9 \mu\text{g g}^{-1}$, while in aboveground tissues decreased from 64.2 ± 1.9 to $22.88 \pm 0.69 \mu\text{g g}^{-1}$.

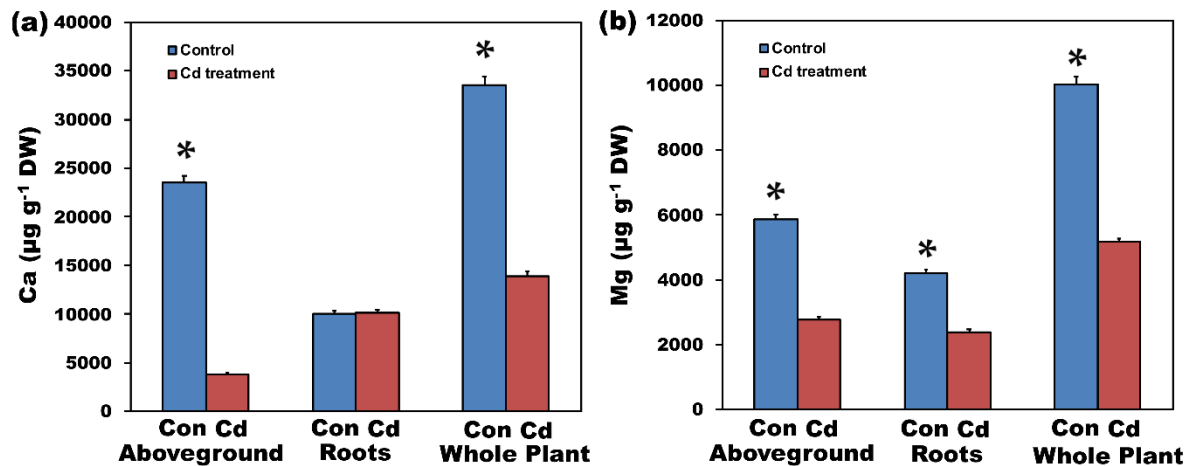


Figure 3. Calcium (a) and Mg (b) content, in $\mu\text{g g}^{-1}$ dry weight, of control (con) and 5 days Cd treated *Salvia sclarea* aboveground (shoots-leaves) tissues, roots and whole plants. Error bars are standard deviations ($n=5$). Means between the two treatments that are statistically different ($p < 0.05$) are marked by an asterisk (*).

2.2. The Efficiency of Photosystem II after Cadmium Exposure

In order to understand how PSII functionality is affected by exposure of plants to Cd, we measured the maximum efficiency of PSII photochemistry (F_v/F_m) (Figure 4a) and the efficiency of the water-splitting complex on the donor side of PSII (F_v/F_o) (Figure 4b). Both parameters increased ($p < 0.05$) in *S. sclarea* plants exposed to Cd stress for 2 and 5 days compared to their respective controls, indicating an enhanced PSII functionality under Cd stress.

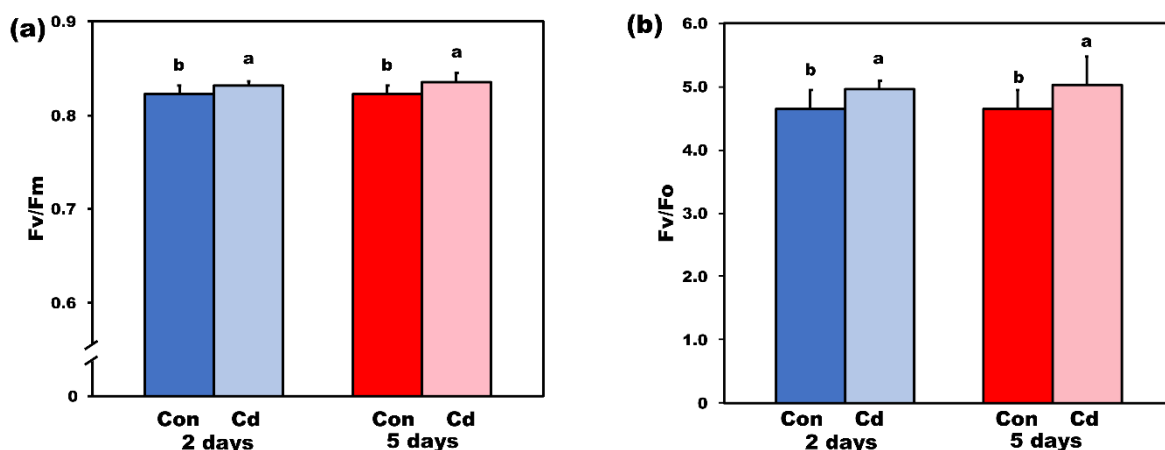


Figure 4. The maximum efficiency of PSII photochemistry (F_v/F_m) (a) and the efficiency of the water-splitting complex on the donor side of PSII (F_v/F_o) (b), of control (con) and 2- and 5-days Cd treated *Salvia sclarea* plants. Error bars are standard deviations ($n=6$). Columns with different letters are statistically different ($p < 0.05$).

2.3. Changes in the Quantum Yields and the Fraction of Open Photosystem II Reaction Centers after Cadmium Exposure under Low Light

The quantum efficiency of PSII photochemistry (Φ_{PSII}) measured at low light (LL, 220 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) (Figure 5a) increased ($p < 0.05$) in *S. sclarea* plants exposed to Cd stress for 2 and 5 days compared to their respective controls, indicating a higher fraction of absorbed light energy to be directed to photochemistry under Cd stress. The concomitant increase in the quantum yield of regulated heat dissipation in PSII (Φ_{NPQ}) (Figure 5b) under 2- and 5-days Cd stress resulted in a significant decrease in the quantum yield of non-regulated energy dissipated in PSII (Φ_{NO}) (Figure 5c), indicating a better use of the absorbed light energy in *S. sclarea* Cd stressed plants. The fraction of open PSII reaction centers (q_p), at LL (Figure 5d), increased ($p < 0.05$) in *S. sclarea* plants exposed to Cd stress for 2 days, while at 5 days exposure remain the same, compared to controls.

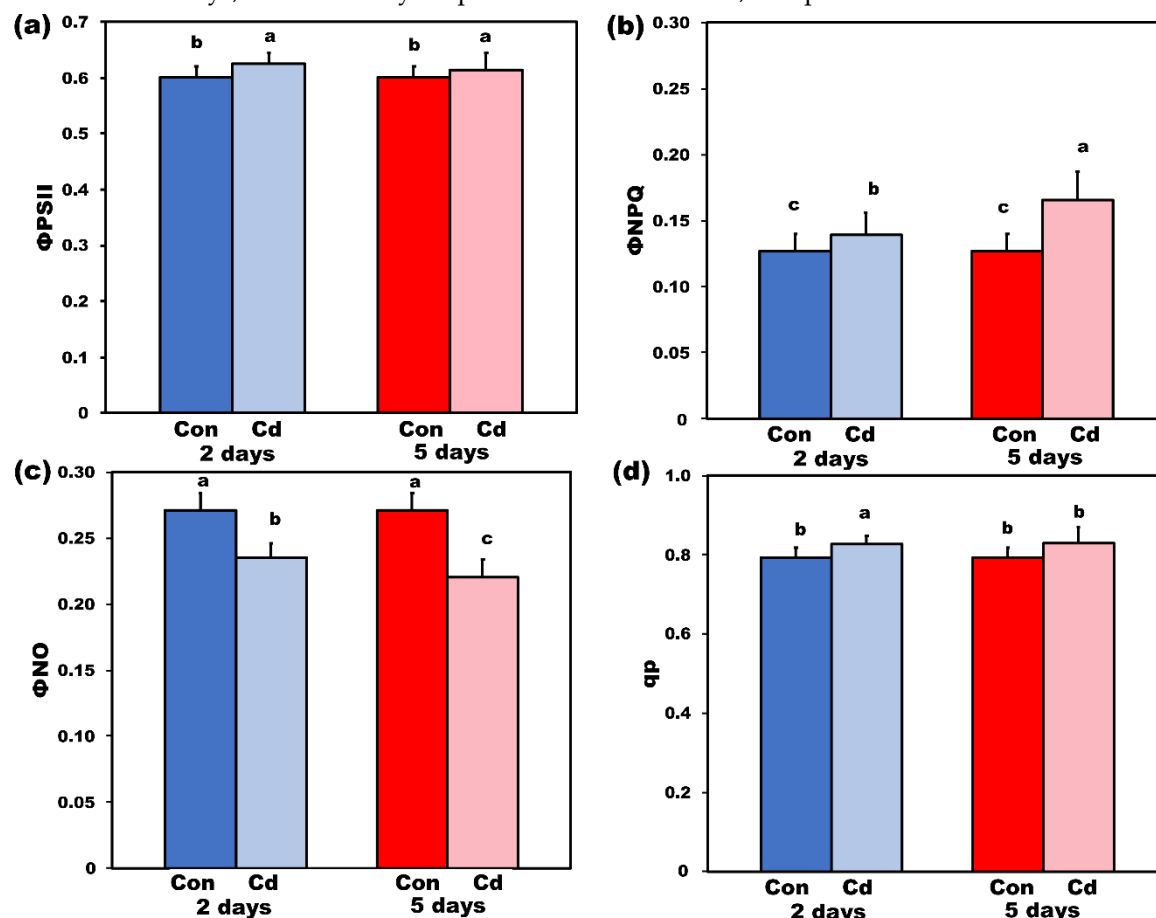


Figure 5. Changes in Φ_{PSII} (a), Φ_{NPQ} (b), Φ_{NO} (c), and q_p (d), measured at 220 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; of control (con) and 2- and 5-days Cd treated *Salvia sclarea* plants. Error bars are standard deviations (n=6). Columns with different letters are statistically different ($p < 0.05$).

2.4. Changes in Non-photochemical Fluorescence Quenching and Electron Transport Rate after Cadmium Exposure under Low Light

Non-photochemical quenching (NPQ) increased ($p < 0.05$) in plants exposed to Cd stress for 2 and 5 days compared to their respective controls (Figure 6a). Electron transport rate measured at 220 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Figure 6b) increased ($p < 0.05$) in *S. sclarea* plants exposed to Cd stress for 2 and 5 days compared to their respective controls, following the pattern of Φ_{PSII} (Figure 5a).

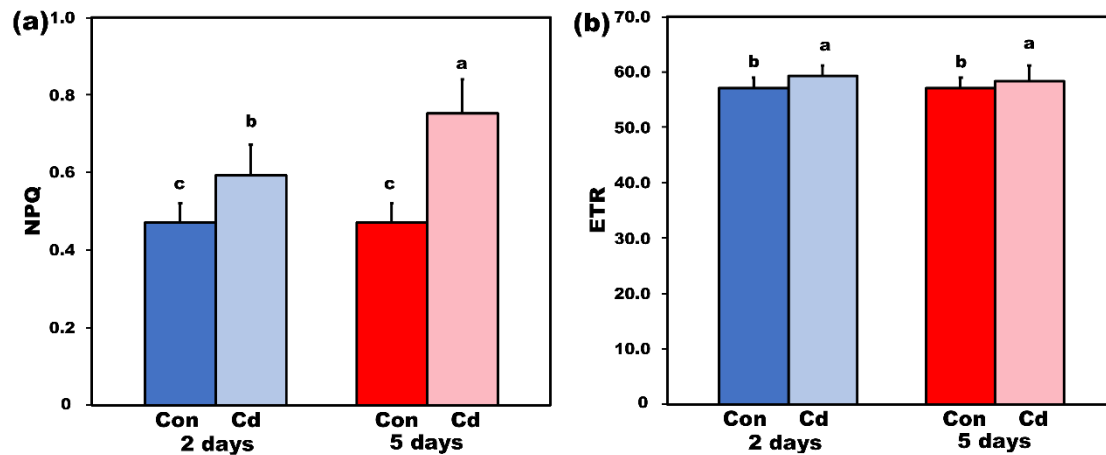


Figure 6. Changes in NPQ (a) and ETR (b), measured at 220 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; of control (con) and 2- and 5-days Cd treated *Salvia sclarea* plants. Error bars are standard deviations (n=6). Columns with different letters are statistically different ($p < 0.05$).

2.5. Changes in Excess Excitation Energy under Low and High Light after Cadmium Exposure

The excess excitation energy (EXC) at PSII in *S. sclarea*, at 220 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (LL) after 2 days Cd exposure, decreased ($p < 0.05$) compared to control while, after 5 days Cd exposure was at the same level to control (Figure 7a). However, under high light (HL, 900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) after 5 days Cd exposure, EXC increased ($p < 0.05$) compared to control (Figure 7b), indicating that the synergistic effect of Cd stress and HL resulted in a lower efficiency of light energy use by PSII.

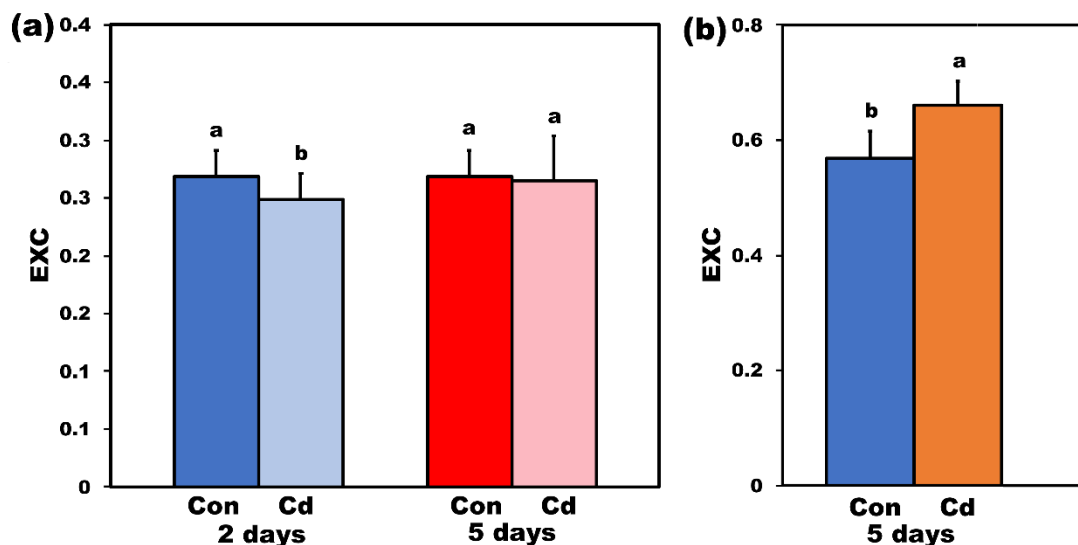


Figure 7. Changes in the excess excitation energy (EXC) measured at 220 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of control (con) and 2- and 5-days Cd treated *Salvia sclarea* plants (a) and the excess excitation energy (EXC) measured at 900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of control (con) and 5-days Cd treated *Salvia sclarea* plants. Error bars are standard deviations (n=6). Columns with different letters are statistically different ($p < 0.05$).

2.6. Changes in the Quantum Yields under High Light after Cadmium Exposure

The allocation of absorbed light energy to PSII photochemistry (Φ_{PSII}) measured at HL in *S. sclarea* plants exposed to Cd for 5 days decreased ($p < 0.05$) compared to controls, while the regulated heat dissipation (Φ_{NPQ}) increased ($p < 0.05$) in such a degree that the non-regulated energy dissipation (Φ_{NO}) in *S. sclarea* plants exposed to Cd for 5 days did not differ compared to controls (Figure 8).

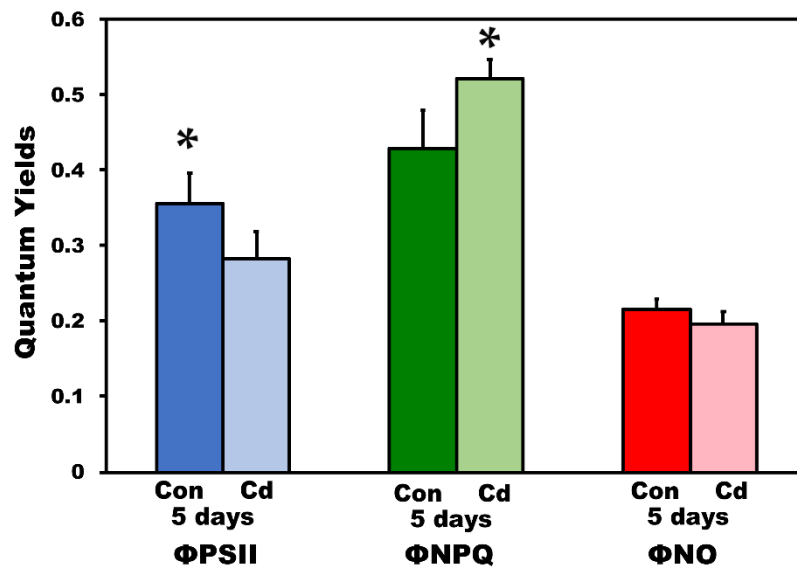


Figure 8. The quantum yields of PSII photochemistry (Φ_{PSII}), the regulated heat dissipation (Φ_{NPQ}), and the non-regulated energy dissipation (Φ_{NO}), measured at $900 \mu\text{mol photons m}^{-2} \text{s}^{-1}$; of control (con) and 5-days Cd treated *Salvia sclarea* plants. Error bars are standard deviations ($n=6$). Means between the two treatments that are statistically different ($p < 0.05$) are marked by an asterisk (*).

2.7. Changes in Non-photochemical Fluorescence Quenching, Electron Transport Rate and the Fraction of Open Photosystem II Reaction Centers under High Light after Cadmium Exposure

Non-photochemical quenching (NPQ), measured at HL, increased ($p < 0.05$) in *S. sclarea* plants exposed for 5 days to Cd compared to control plants (Figure 9a), while PSII electron transport rate decreased ($p < 0.05$) compared to controls (Figure 9b) following the pattern of Φ_{PSII} (Figure 8). The fraction of open PSII reaction centers (q_P) decreased ($p < 0.05$) in *S. sclarea* plants exposed for 5 days to Cd compared to control plants (Figure 9c).

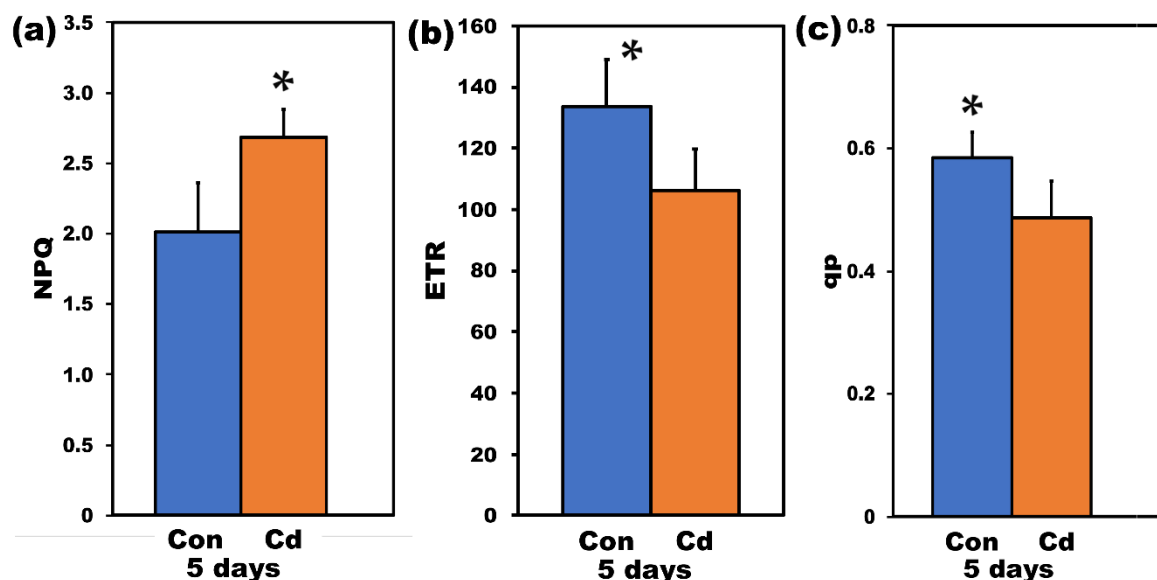


Figure 9. The non-photochemical fluorescence quenching (NPQ) (a), the relative PSII electron transport rate (ETR) (b), and the relative reduction state of Q_A , reflecting the fraction of open PSII reaction centers (q_P) (c), measured at $900 \mu\text{mol photons m}^{-2} \text{s}^{-1}$; of control (con) and 5-days Cd treated *Salvia sclarea* plants. Error bars are standard deviations ($n=6$). Means between the two treatments that are statistically different ($p < 0.05$) are marked by an asterisk (*).

2.8. Chlorophyll *a* Fluorescence Images under Low and High Light

Chlorophyll *a* fluorescence images of the fluorescence parameters Φ_{PSII} and Φ_{NO} , measured at LL and HL, of control and 5-days Cd treated *S. sclarea* plants, revealed a spatial heterogeneity over the whole leaf area (Figures 10), especially under HL. The heterogeneity was higher under Cd exposure with Φ_{PSII} values at the center of the leaf and near the main leaf vein to have lower values compared to marginal, while the spatial heterogeneity was even higher under HL and Cd exposure (Figures 10). The effective quantum yield of PSII photochemistry (Φ_{PSII}) after 5 days Cd exposure, was higher under LL compared to control, but it was lower under HL compared to control (Figures 10). The non-regulated energy loss in PSII (Φ_{NO}), under both LL and HL, was lower in 5-days Cd treated *S. sclarea* plants compared to controls (Figures 10).

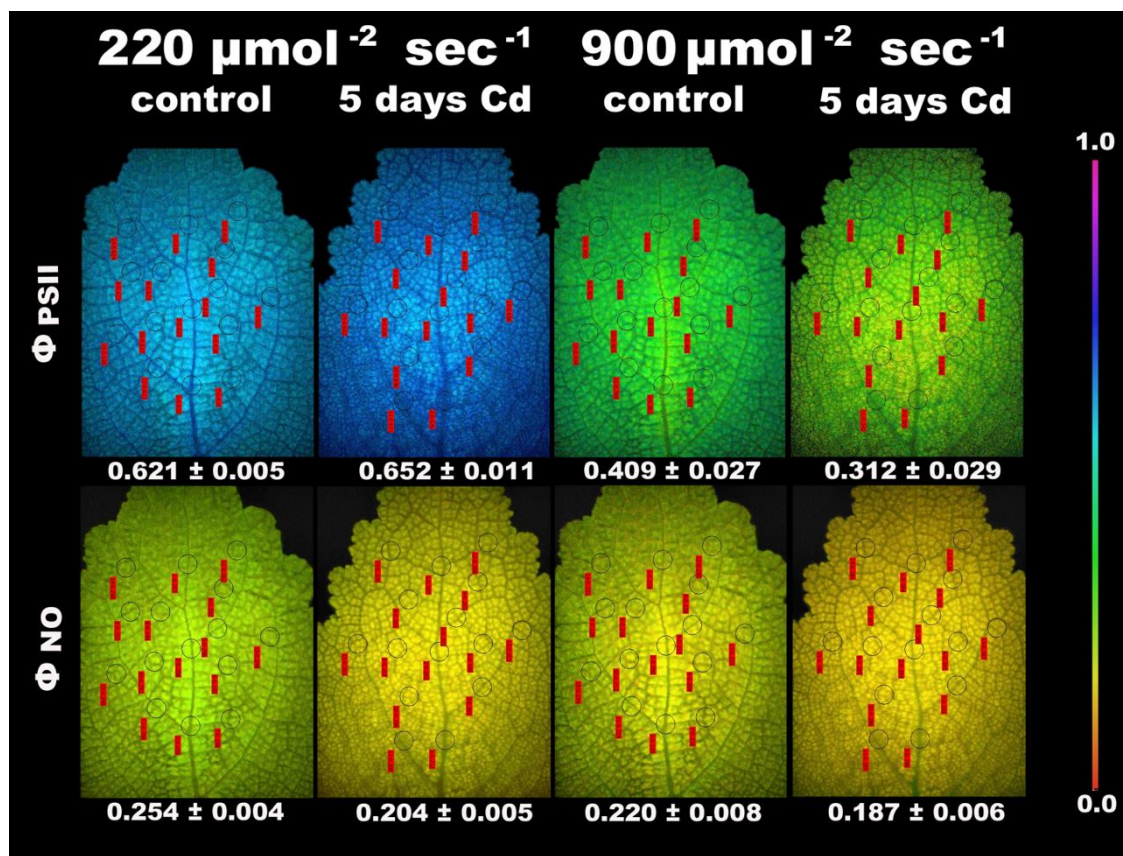


Figure 10. Chlorophyll fluorescence images of Φ_{PSII} and Φ_{NO} (measured at 220 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and 900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) of control and 5-days Cd treated *Salvia sclarea* plants. The color code depicted at the right-side ranges from values 0.0 to 1.0. The fifteen circles in each image denote the areas of interest (AOI) that are complemented by red labels with the values of the fluorescence parameter, while whole leaf value is presented.

At longer duration exposure (8 days) to Cd under LL, an inhibition of PSII functionality is observed (Figure S2). More specifically, at 8 days exposure of *S. sclarea* plants to Cd, the lower F_v/F_m values were found near the midvein while the lower Φ_{PSII} values at the leaf area behind the leaf edge (Figure S2).

2.9. Chloroplast Ultrastructure after Cadmium Exposure

Leaves from untreated plants exhibited rather electronically dense mesophyll chloroplasts (Figure 11a) that showed a typical internal membrane structure with well-organized grana and stroma thylakoids (Figure 11a). Both 2-day (Figure 11b) and 5-day (Figure 11c) Cd treated plastids did not show any noticeable structural disruption having a similar appearance to the control, with

the 5-day Cd treated plastids to appear more electronically dense (Figure 11c). In both treated and untreated plastids starch grains were noticeable.

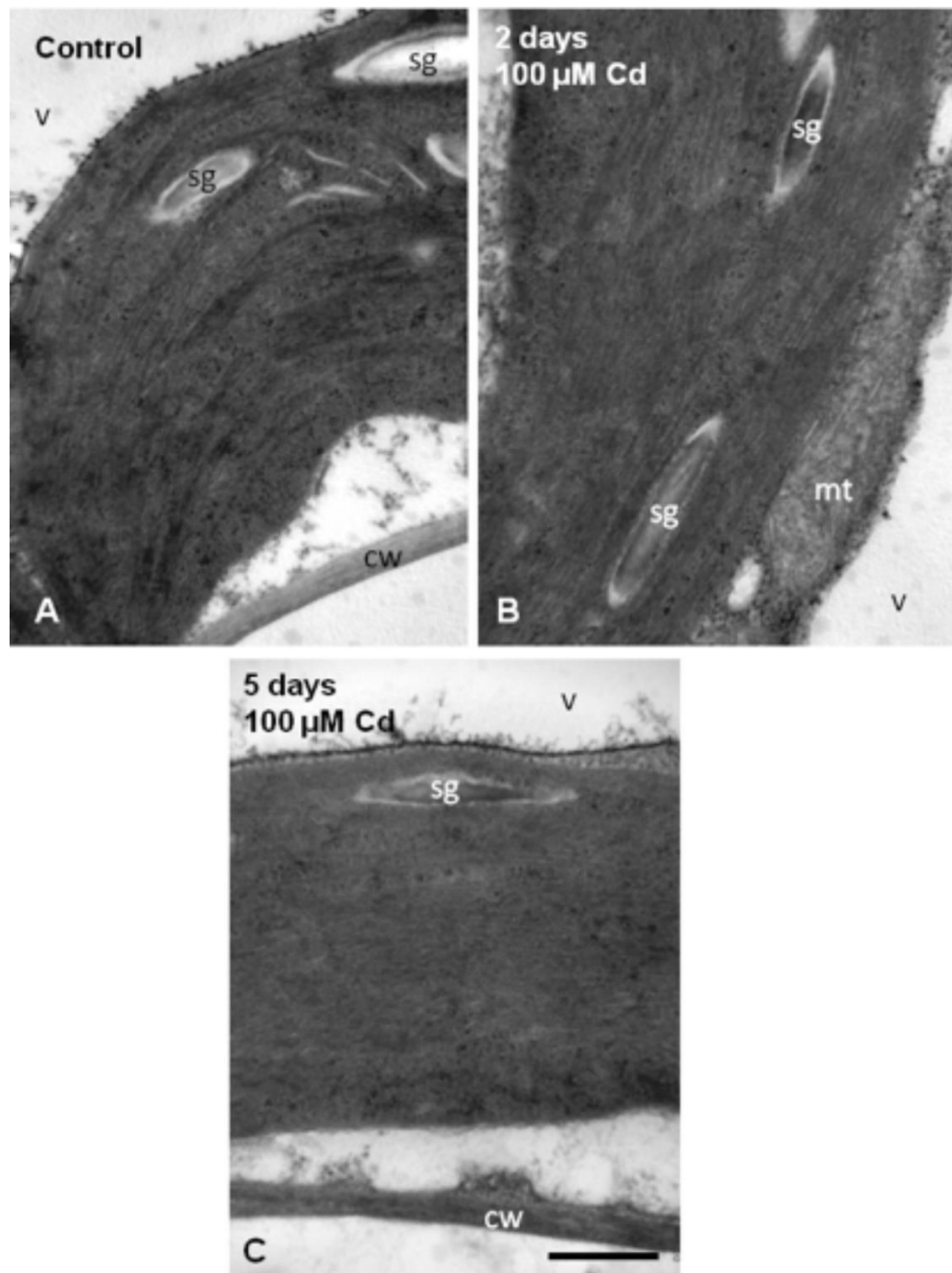


Figure 11. Transmission Electron Microscopy (TEM) images of control (untreated) chloroplasts (a) and 2-day (b) or 5-day (c) Cd-treated *S. clarea* leaves. Chloroplasts appear electronically dense and upon Cd treatment (b, c) no noticeable disruption has been detected. cw: cell wall; mt: mitochondria; sg: starch grain; v: vacuole. Scale bar: 500 nm.

3. Discussion

Exposure of *S. sclarea* plants to 100 µM Cd for 5 days in hydroponics resulted in a high Cd uptake with a 2400-fold increase at the whole plant level but with Cd ions to be retained almost exclusively in the roots (Figure 1b) and only 53.3 µg g⁻¹ to be translocated to the aboveground tissues (Figure 1a). Low Cd accumulation in leaves may represent a tolerance mechanism that protects the photosynthetic equipment against additional oxidative stress [50-52]. In the tolerant plant species,

the excess heavy metals in roots play a significant role by sequestering and detoxifying the extreme amount of heavy metal in order to protect the delicate aboveground photosynthetic tissues [53,54]. Since leaf Cd contents greater than 5–10 $\mu\text{g g}^{-1}$ have been characterized toxic to most plants [55], it seems that *S. sclarea* could have kept Cd concentration in the photosynthetic tissues in non-toxic forms. This, could be done by depositing it in the vacuoles of leaf epidermal cells [56] and/or by complexation with cellular ligands [7,57–59]. Hyperaccumulators can accumulate Cd to levels above 100 $\mu\text{g g}^{-1}$ of shoot dry weight, without showing any toxicity symptoms [7,12,60]. Our results agree with those of He et al. [61] that roots of Cd tolerant plants (non-hyperaccumulators) retain considerably higher Cd concentrations than the aboveground parts, and only minor Cd is translocated to the aerial parts. In comparison to the above-ground tissues, *S. sclarea* roots showed a higher bioaccumulation ability of Cd with translocation to shoots-leaves to be restricted.

Cadmium uptake is affected by Ca levels because Cd competes for Ca channels [7,62–64] and the low Ca content of the hydroponic solution may enhance Cd uptake [63,65] resulting in enhanced Cd and decreased Ca in many plant species [7,64], as we also observed in *S. sclarea* experiments, with Ca being the most affected element (58% total uptake decrease, Figure 3a). Likewise, in *Oryza sativa* seedlings exposed to Cd, uptake of Ca was decreased, and Ca content in both roots and aboveground parts was significantly reduced [66]. Magnesium was the next most negatively affected element after Ca in our experiments, but low Mg status has been associated with increased Cd tolerance [67–72], indicating that plants regulate nutrient concentrations to mitigate Cd toxicity [72].

Antagonistic effects of Cd with Fe [52,64,73] and Zn [74–76] have been frequently reported. However, in *S. sclarea* exposed to 100 μM Cd for 5 days, total Zn, Cu, and Fe uptake increased but their translocation to the aboveground parts decreased possible due to translocation barriers. It seems that Cd uptake in *S. sclarea* is not taking place through Fe or Zn pathway, while conditions that lead to increased Cd uptake in plants may also favor increased Fe uptake [7]. Cd treatment has been frequently mentioned that it increases Fe retention in roots but obstructs its translocation to shoots, thus reducing Fe concentrations in aboveground parts [61,77,78]. In rice Cd has been shown to be taken up predominantly via the Mn pathway [79,80] but this was not the case in *S. sclarea*, since Mn was the less negatively affected element.

Cadmium contamination of soil has become a serious environmental alarm as it is estimated that around 30,000 t of Cd are released annually into the environment with a consequence to the food chain and a threat to human health [81,82]. The use of plants for heavy metals elimination from pollutant soils and water is a technique known as phytoremediation [83]. In phytoremediation, plants that absorb heavy metals from soils and translocate them to the harvestable shoots are used for phytoextraction, while those that stabilize metal contaminants through accumulation in the root zones are used for phytostabilization [82,84]. Plant species with high bioconcentration factor but relatively low translocation factor (< 1) may be considered as potential phytostabilizers [85]. *Salvia sclarea* exhibited high accumulation capacity for Cd and by limiting its translocation from roots to shoots may be considered a potential phytostabilizer that can be used in heavy metal contaminated environments. Other plant species that have been proposed as Cd phytostabilizers are *Iris lactea* [82] and *Sesuvium portulacastrum* [86,87].

Despite the significant levels of Cd in leaves, a higher fraction of absorbed light energy was directed to photochemistry (Φ_{PSII}) under 2- and 5-days Cd stress, with a concomitant increase in Φ_{NPQ} that resulted in a significant decrease in Φ_{NO} . The non-regulated energy loss in PSII (Φ_{NO}) encompasses internal conversions and intersystem crossing, that results to singlet oxygen ($^1\text{O}_2$) creation via the triplet state of chlorophyll ($^3\text{chl}^*$) [31,88–91]. To optimize photosynthesis and growth under stressful conditions plants have evolved a variety of mechanisms against photodamage and photoinhibition [92,93]. Non-photochemical quenching is the key photoprotective process that dissipates excess light energy as heat and protects photosynthesis [88,94–98]. Thus, the increased non-photochemical quenching of photosynthesis (NPQ) decreased ROS through a $^1\text{O}_2$ formation. Consequently, in *S. sclarea* plants exposed to 100 μM Cd, ROS homeostasis could be regulated by NPQ in such a way so that plants can cope with Cd stress [21,24,28,29].

The potential PSII efficiency of *S. sclarea* plants exposed to 100 μM Cd estimated by the maximum efficiency of PSII photochemistry (F_v/F_m) (Figure 4a) and the efficiency of the water-splitting complex on the donor side of PSII (F_v/F_o) (Figure 4b) indicated an enhanced PSII functionality under Cd stress. In accordance, *S. sclarea* plants exposed to 100 μM Cd show an increased capacity to keep quinone (QA) oxidized, thus, to have a higher fraction of open PSII reaction centers (q_p) compared to controls. In other words, *S. sclarea* plants exposed to 100 μM Cd show a low PSII excitation pressure associated with toxicity tolerance mechanisms [99,100]. High excitation pressure defines excess energy and consequently a disproportion between energy resource and requirement [101]. This discrepancy leads to an increase in the energy transmitted from chlorophyll to oxygen, resulting in $^1\text{O}_2$ generation [102]. Control *S. sclarea* plants that show increased excess excitation energy (EXC) at PSII (Figure 7a), show also increased $^1\text{O}_2$ creation via $^3\text{chl}^*$ (Figure 5c), compared to plants exposed to Cd for 2 days. A basal level of ROS is needed for optimal growth [22,27], with a low level of ROS to be beneficial in accomplishing the maximum fitness of the biological plasticity, and a high level of ROS out of the boundaries to be harmful to plants [22,36]. Photosystem II responses of *S. sclarea* plants to short time Cd exposure can be described as a hormetic response (Figure 12) representing an “over-compensation” response to a disruption in homeostasis [44].

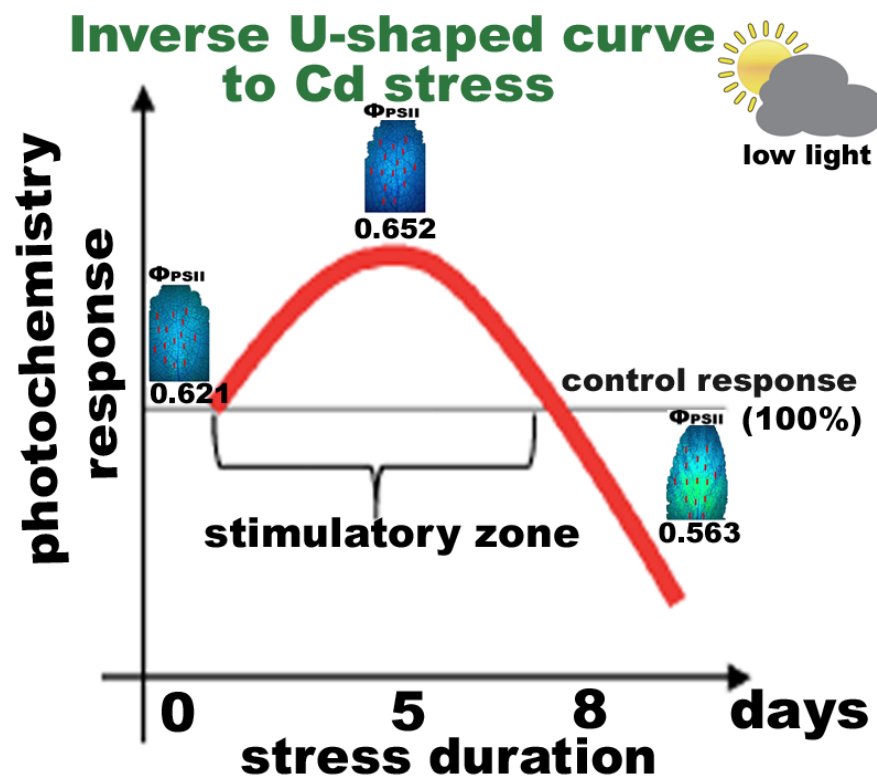


Figure 12. Overview of the hormetic response of photosystem II photochemistry to Cd exposure. Hormesis [103] is defined as the stimulatory effect of short exposure times of toxic constituents, e.g., Cd on a biological factor (photosystem II photochemistry), of a particular organism (*S. sclarea*). The hormetic effect is defined by an inverse U-shaped biphasic curve [8,36] in which short exposure time has a stimulatory effect; however, at longer exposure time, a toxic effect is evident.

Although excess Cd accumulation is detrimental to plants, different strategies of Cd tolerance and accumulation are adopted by plants [104]. Tang et al. [105] described a stimulation of plant growth, increase of photosynthesis and an up-regulation of the related genes in *Sedum alfredii* exposed to 5 μM Cd. Similar results with stimulation of growth when *Noccea caerulescens* was exposed to 100 μM Cd were reported by Lombi et al. [106]. A stimulatory effect of Cd on the photosynthetic

apparatus of *Arabidopsis halleri* was also described recently [35]. Małkowski et al. [38] reported a stimulation of the photosynthetic rate by Cd only at low concentrations, whereas at higher Cd concentrations there was a significant decrease compared to controls. However, other studies have shown a dose dependent negative impact of Cd that increases with the generation of ROS and oxidative damage and the inhibition of photosynthetic rate to follow [107-116new 1, new 2]. On the other hand, protection to stress through the production of ROS [117,118] has been shown that it can be regulated by NPQ in such a way so that plants can cope with stress [24,28,29].

A negative impact of Cd on photosynthesis has been assigned to decreases in chlorophylls; ascribed to Cd-induced damage in chloroplasts' ultrastructure [119,120]. Cadmium has been reported to alter chloroplast ultrastructure, reduce photosynthesis [64,116,121], and inactivate enzymes involved in CO₂ fixation [62]. Ultrastructural changes that are observed in Cd exposed leaves of sensitive plants (thylakoid dismantling, increase of lipid droplets, etc) [122-126], are similar to those occurring at leaf ageing [127]. In Cd tolerant species the only ultrastructural alteration observed in leaves was a reduction of starch grains in chloroplasts [52,128,129] that may be due to disorders in the photoassimilate transport or to nutrient deficiency [52,130]. In *S. sclarea* Cd-treated plants an increase in deposited electron-dense material was observed, as reported also by Mizushima et al. [52], but no other noticeable alteration, further consolidating the chlorophyll fluorescence imaging results. Thus, a hormetic response of PSII photochemistry to short term Cd exposure was observed, indicating an "over-compensation" response to Cd disruption in homeostasis, justifying the statement of Carvalho et al. [8] that Cd can be regarded from a toxic element, a beneficial one. Hormesis research data and data on priming (preconditioning), an expression of hormesis [17,36,131-133], indicate that stimulatory response detection of the low-dose or short-time exposure are highly dependent on the study strategy, including dose range and number with duration exposure and endpoint selected [14,15,17,36,54].

Exposure of *S. sclarea* plants to a combination of Cd and high light (900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) resulted in an inhibition of PSII functionality (Figures 8,10), while the increased NPQ (Figure 9a) was inefficient to keep the same number of open reaction centers PSII (q_p , Figure 9c) compared to control plants. Dissipation of excess light energy as heat (NPQ) under environmental pressure conditions is effective only if it is regulated so as to maintain the same fraction of open reaction centers as in unstressed conditions [96,97,134-137], as was observed under low light exposure of *S. sclarea* plants to Cd (Figure 5d). Thus, the combination of Cd and high light is pointing out to Cd toxicity. The same conclusion is reached at longer duration exposure (8 days) to Cd at LL, with an inhibition of PSII functionality to be observed (Figure S2) [138].

4. Materials and Methods

4.1. Plant Material and Growth Conditions

Seeds of *Salvia sclarea* L. used for the experiments were collected from the Rose Valley (Bulgaria). After germination on soil in a growth room for about a month, the seedlings were transferred to pots containing continuously aerated modified Hoagland nutrient solution (described in detail before) [6]. The nutrient solution was adjusted to pH 6.0 and changed every 3 days. The growth room conditions were $24 \pm 1 / 20 \pm 1$ °C day/night temperature, 14/10 h day/night photoperiod with photon flux density $200 \pm 20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$.

4.2. Cadmium Treatment

Two-month-old *S. sclarea* plants in the hydroponic culture experiments were subjected to 0 or 100 μM Cd (as $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$) for a period up to five days. The pots containing only Hoagland nutrient solution served as control, while all solutions were renewed every two days.

4.3. Determination of Elemental Concentration by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

After 5 days treatment with 0 (control) or 100 μM Cd, *Salvia* plants were harvested, separated in roots and aboveground (shoots-leaves) tissues, washed three times in deionized water and then dried at 65 °C to constant biomass, milled and finally sieved. Dried sieved samples of 0.3g were transferred in 10 mL quartz vessels with 65% (v/v) nitric acid (Suprapur, Merck, Germany) and 30% (v/v) hydrogen peroxide (Suprapur, Merck, Germany) in 3:1 ratio. Digestion was carried out in the microwave assisted digestion system Ethos One (Milestone Sorisole, BG, Italy). The process run out in 3 stages: ramp time – 20 min to reach 200 °C and 1500W; hold time – 30 min at 200 °C and 1500W; cooling – 30 min. Next step was the quantitative transfer of digested samples into polypropylene tubes and dilution with demineralized water (Direct-Q 3 UV, Merck, Germany). All prepared samples were diluted immediately prior to inductively coupled plasma mass spectrometer (ICP-MS) analysis. Samples were analyzed in an ICP-MS model ELAN DRC II (PerkinElmer Sciex, Toronto, Canada) [139]. ICP-MS operational conditions, instrumental settings calibration solutions, data validation and validation parameters are given in Appendix A. Elemental analysis was performed for Cd, Cu, Ca, Mg, Mn, Fe and Zn.

4.4. Chlorophyll Fluorescence Imaging Analysis

Chlorophyll fluorescence measurements were conducted on dark adapted (20 min) leaves of *S. sclarea* plants, treated for 2- and 5-days with 0 (control) or 100 μM Cd, using an *Imaging PAM M-Series* system (Heinz Walz Instruments, Effeltrich, Germany) as described in detail previously [140]. Two light intensities were used for measurements of photosynthetic efficiency of *S. sclarea* leaves, a LL, similar to the growth light (220 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$), and a HL (900 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). In each leaf representative areas of interest (AOIs) were selected so as to have measurements of the whole leaf area. The definitions of the five main chlorophyll fluorescence parameters (F_o , F_m , F_o' , F_m' and F_s) measured by the *Imaging PAM M-Series* system are presented in Table S1, while a typical modulated fluorescence trace showing how the main five parameters are formed is presented in Figure S1. The chlorophyll fluorescence parameters calculated from the five main parameters with their definitions are described in Table 1. Representative results are also shown as color-coded images of Φ_{PSII} and Φ_{NO} , after 5 min illumination with 220 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (LL) and 900 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (HL) intensity.

4.5. Leaf Ultrastructure Observations by Transmission Electron Microscopy

To study leaf ultrastructure alterations after 2- and 5-days of Cd treatment, leaves from both Cd-treated and untreated plants were excised and segmented with a razor blade into small pieces of 0.5 x 1 mm. Leaf segments were fixed with 2% paraformaldehyde plus 4% glutaraldehyde, in 0.05 M sodium cacodylate buffer, pH 7.0 solution [140]. After a 5h fixation at room temperature the samples were washed with a 0.05 M sodium cacodylate buffer and post-fixed for another 3 h in a similarly buffered 2% osmium tetroxide solution (Agar Scientific, Essex, UK). Afterwards samples were dehydrated in an acetone series, treated with propylene oxide, and embedded in Durcupan ACM resin (Fluka Chemie AG, Buchs, Switzerland). Ultrathin sections (80-90 nm) were cut in a ULTRATOME III TYPE 8801A ultramicrotome (LKB, Stockholm, Sweden) equipped with a glass knife., collected on nickel grids. Ultrathin sections were stained with 2% uranyl acetate and 1% lead citrate and examined in a JEOL JEM 1011 transmission electron microscope equipped with a Gatan ES500W digital camera. Digital electron micrographs were obtained with the DigitalMicrograph 3.11.2 software according to the manufacturer's instructions.

4.6. Statistical analyses

Mean values were calculated from three independent treatments (biological replicates). Statistically significant differences among the means were determined using one-way analysis of variance or two-way ANOVA. Means (\pm SD) were considered statistically different at a level of $p < 0.05$.

Table 1. Definitions of the chlorophyll fluorescence parameters calculated from the five main chlorophyll fluorescence parameters listed in Table S1.

Parameter	Definition	Calculation
Fv/Fm	Maximum efficiency of PSII photochemistry	Calculated as $(Fm - Fo)/Fm$
Fv/Fo	Efficiency of the water-splitting complex on the donor side of PSII	Calculated as $(Fm - Fo)/Fo$
Fv'/Fm'	The efficiency of open PSII reaction centers	Calculated as $(Fm' - Fo)/Fm'$
Φ_{PSII}	The effective quantum yield of PSII photochemistry	Calculated as $(Fm' - Fs)/Fm'$
q_p	The photochemical quenching, that is the redox state of the plastoquinone pool, is a measure of the number of open PSII reaction centers	Calculated as $(Fm' - Fs)/(Fm' - Fo)$
NPQ	The non-photochemical quenching that reflects heat dissipation of excitation energy	Calculated as $(Fm - Fm')/Fm'$
ETR	The relative PSII electron transport rate	Calculated as $\Phi_{PSII} \times PAR \times c \times abs$, where PAR is the photosynthetically active radiation c is 0.5, and abs is the total light absorption of the leaf taken as 0.84
Φ_{NPQ}	The quantum yield of regulated non-photochemical energy loss in PSII, that is heat dissipation for photoprotection	Calculated as $Fs/Fm' - Fs/Fm$
Φ_{NO}	The quantum yield of non-regulated energy loss in PSII	Calculated as Fs/Fm
EXC	Excess excitation energy	Calculated as $(Fv/Fm - \Phi_{PSII}) / (Fv/Fm)$

5. Conclusions

Although surplus Cd accumulation is detrimental to all plants different strategies of Cd tolerance and accumulation are adopted by different plants [104]. When clary sage was exposed to Cd for a short time, tolerance mechanisms were triggered, with PSII photochemistry to be regulated by the photoprotective mechanism of NPQ in such a way that PSII functionality to be enhanced (Figures 4-6) without any defects to chloroplasts, as observed by transmission electron microscopy (Figure 11). However, exposure to a combination of Cd and high light (Figures 8,10) or longer duration exposure to Cd alone (8 days, Figure S2) [138] resulted in an inhibition of PSII functionality pointing out to Cd toxicity. Thus, an activation of PSII function at short time exposures and an inhibition at longer duration suggests a hormetic response (Figure 12), and describes these effects in terms of “adaptive response” and “toxicity”, respectively.

Supplementary Materials: The following is available online at www.mdpi.com/xxx/s1, Table S1: The definitions of the five main chlorophyll fluorescence parameters (F_0 , F_m , F_0' , F_m' and F_s) measured by the *Imaging PAM M-Series* system. Figure S1: A typical modulated fluorescence trace showing how F_0 , F_m , F_0' , F_m' and F_s are formed to measure photochemical and non-photochemical parameters. Figure S2: Representative chlorophyll fluorescence images of F_v/F_m and Φ_{PSII} of *Salvia sclarea* leaves from control and 8-days Cd treated plants.

Author Contributions: Conceptualization, A.D. and M.M.; methodology, I-D.S.A., I.S. and M.M.; software, I.S., A.H. and M.M.; validation, A.H. and M.M.; formal analysis, I-D.S.A., I.S., A.H. and M.M.; investigation, I-D.S.A., I.S. and A.H.; resources, I-D.S.A., A.H., A.D., E.A. and M.M.; data curation, I-D.S.A., I.S., A.H., A.D. and M.M.; writing—original draft preparation, I-D.S.A., A.H. and M.M.; writing—review and editing, I-D.S.A., A.H., I.S., A.D., E.A. and M.M.; visualization, A.H. and M.M.; supervision, A.D. and M.M.; project administration, A.D., E.A. and M.M.; funding acquisition, I-D.S.A., A.H., A.D., E.A. and M.M.

Funding: This work was supported by the Agreement for scientific cooperation between the Bulgarian Academy of Sciences and the Aristotle University of Thessaloniki, Greece. AH was supported by the Ministry of Science and Higher Education in Poland for statutory activities in Adam Mickiewicz University, Poznań.

Acknowledgments: Seeds of *Salvia sclarea* used for the experiments were kindly provided by Bio Cultures Ltd.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Appendix A

ICP-MS operational conditions

Operational conditions were optimized daily, using a solution of Mg, In, and U at a concentration of 1 mg L⁻¹ and Ba at a concentration of 10 mg L⁻¹ (Smart Tune Solution e Elan DRC II/plus, Atomic Spectroscopy Standard, Perkin Elmer Pure). Whilst tuning the ICP-MS, compromise conditions for maximum signal intensity of the analyte (²⁴Mg⁺, ¹¹⁵In⁺, ²³⁸U⁺) and minimum ratio of oxide (¹⁴⁰Ce¹⁶O⁺/¹⁴⁰Ce < 3%) and doubly charged ions (¹²⁸Ba²⁺/¹²⁸Ba⁺ < 3%) were found.

Calibration solutions

Calibration solutions were prepared by appropriate dilution of 10 mg L⁻¹ multi-elemental stock solution in 5% HNO₃ (Multi-Element Calibration Standard 3, PerkinElmer, MA, USA). The calibration curves were constructed in the concentration ranges: 0.1–100 µg L⁻¹ for Cd, Cu, Mn, Zn and 50–1500 µg L⁻¹ for Ca, Fe and Mg.

ICP-MS instrumental settings

The ICP-MS instrumental settings were as follows: (0.89–0.91) L min⁻¹ sample gas flow, 16 L min⁻¹ plasma gas flow, 1.2 L min⁻¹ auxiliary gas flow, 1250W RF generator power, dual detector mode, autolens mode. Dynamic reaction cell (DRC) mode with ammonia as reaction gas was used in order to remove the polyatomic interferences. The non-spectral interferences were reduced by diluting the sample and using 10 µg L⁻¹ of Ge and Rh as internal standard.

Data validation

For data validation of the applied analytical procedure, two CRMs were used: Trace elements in spinach leaves NIST SRM 1570a (NIST, USA) and TM-27.4 (National Water Research Institute, Canada).

Validation parameters

Validation parameters such as linearity, precision, LOD and trueness were evaluated. The calibration curves for the determined elements were linear in the range of calibration standards. Coefficient of correlation (R) values were estimated daily and were greater than 0.999 for all analytes. Residual plots showed a random distribution of residuals around the vertical axis. Precision values were calculated as coefficient of variation (CV) (%) ranged from 1.7% to 3.7% for all elements. Trueness was evaluated by applying the certified reference materials and expressed as recovery (%).

Recovery values ranged from 95% to 106% respectively. LOD values were estimated as $3.3 S/b$, where S means the standard deviation of the result obtained for the blank sample and b is the sensitivity ($n=5$). The LODs were as follows: Cd $0.02 \mu\text{g g}^{-1}$, Cu $0.05 \mu\text{g g}^{-1}$, Ca $30 \mu\text{g g}^{-1}$, Mg $0.8 \mu\text{g g}^{-1}$, Mn $0.03 \mu\text{g g}^{-1}$, Fe $40 \mu\text{g g}^{-1}$ and Zn $0.01 \mu\text{g g}^{-1}$. Measurement traceability was established by applying the certified reference materials.

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