Cadmium toxicity in *Salvia sclarea* L.: An integrative response of element uptake, oxidative stress markers, leaf structure and photosynthesis

Anelia G. Dobrikova a,*, Emilia L. Apostolova a, Anetta Han b, Ekaterina Yotsova a, Preslava Borisova a, Ilektra Sperdouli c, Ioannis-Dimosthenis S. Adamakis d, Michael Moustakas e

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**A B S T R A C T**

The herbal plant *Salvia sclarea* L. (clary sage) is classified to cadmium (Cd) accumulators and considered as a potential plant for phytoremediation of heavy metal polluted soil. However, the effect of Cd only treatment on the function of the photosynthetic apparatus of *S. sclarea*, as well as the mechanisms involved in Cd tolerance have not yet been studied in detail. This study was conducted to examine the integrative responses of *S. sclarea* plants exposed to a high Cd supply (100 \( \mu M \)) for 3 and 8 days by investigating element nutrient uptake, oxidative stress markers, pigment composition, photosynthetic performance and leaf structure. Measurements of the functional activities of photosystem I (PSI, by P700 photooxidation), photosystem II (PSII, by chlorophyll fluorescence parameters), the oxygen-evolving complex (oxygen evolution by Joliot- and Clark-type electrodes), as well as the leaf pigment and phenolic contents, were used to evaluate the protective mechanisms of the photosynthetic apparatus under Cd stress. Data suggested that the molecular mechanisms included in the photosynthetic tolerance to Cd toxicity involve strongly increased phenolic and anthocyanin contents, as well as an increased non-photochemical quenching and accelerated cyclic electron transport around PSI up to 61%, which protect the function of the photosynthetic apparatus under stress. Furthermore, the tolerance of *S. sclarea* to Cd stress is also associated with increased accumulation of Fe in leaves by 25%. All the above, clearly suggest that *S. sclarea* plants employ several different mechanisms to protect the function of the photosynthetic apparatus against Cd stress, which are discussed here.

**1. Introduction**

Cadmium (Cd) soil contamination from both natural and anthropogenic sources represents a growing problem for the environmental quality and, consequently, for food safety and human health (Ismael et al., 2019). Cd is considered to be one of the most phytotoxic metals and does not seem to have any biological function in plants, while its potential plant for phytoremediation of heavy metal polluted soil. However, the effect of Cd only treatment on the function of the photosynthetic apparatus of *S. sclarea*, as well as the mechanisms involved in Cd tolerance have not yet been studied in detail. This study was conducted to examine the integrative responses of *S. sclarea* plants exposed to a high Cd supply (100 \( \mu M \)) for 3 and 8 days by investigating element nutrient uptake, oxidative stress markers, pigment composition, photosynthetic performance and leaf structure. Measurements of the functional activities of photosystem I (PSI, by P700 photooxidation), photosystem II (PSII, by chlorophyll fluorescence parameters), the oxygen-evolving complex (oxygen evolution by Joliot- and Clark-type electrodes), as well as the leaf pigment and phenolic contents, were used to evaluate the protective mechanisms of the photosynthetic apparatus under Cd stress. Data suggested that the molecular mechanisms included in the photosynthetic tolerance to Cd toxicity involve strongly increased phenolic and anthocyanin contents, as well as an increased non-photochemical quenching and accelerated cyclic electron transport around PSI up to 61%, which protect the function of the photosynthetic apparatus under stress. Furthermore, the tolerance of *S. sclarea* to Cd stress is also associated with increased accumulation of Fe in leaves by 25%. All the above, clearly suggest that *S. sclarea* plants employ several different mechanisms to protect the function of the photosynthetic apparatus against Cd stress, which are discussed here.

Malecka et al., 2019). It is also well established that high doses of Cd exposure increase the formation of reactive oxygen species (ROS) including hydrogen peroxide (H\(_2\)O\(_2\)) and cause subsequent oxidative stress in plant tissues (Schützendübel et al., 2001; Romero-Puertas et al., 2004; Cho and Seo, 2005; Kapoor et al., 2019). The visible Cd toxicity symptoms include reduced growth, leaf chlorosis and necrosis, stomata closure, water uptake imbalance, and damage of the photosynthetic apparatus (Shi and Cai, 2008; Ali et al., 2013; Xue et al., 2013; Arivazhagan and Sharavanian, 2015; Bayçu et al., 2018; Yotsova et al., 2018; Szopinski et al., 2019; Xin et al., 2019).

Photosynthesis is considered the primary physiological process affected by Cd in all its phases (the “light” and “dark” reactions) either directly or indirectly (Greger and Ögren, 1991; Küpper et al., 2007;
Mobin and Khan, 2007; Parmar et al., 2013). Moreover, the photosynthetic membranes are suggested to be very sensitive to Cd, as their damage is caused at different levels of their organization: ultrastructure of chloroplasts, pigment, protein and lipid composition (Vassilev et al., 2004; Hakmaoui et al., 2007; Basa et al., 2014; Dobrikova and Apostolova, 2019). It has also been shown that Cd firstly affects chlorophyll content, then inhibits the photochemical activity of PSII and oxygen-evolving complex (OEC), and later the PSI activity (Wodala et al., 2012; Basa et al., 2014; Dobrikova et al., 2017; Yotova et al., 2018). Recently, the chlorophyll fluorescence analysis, examining the photosynthetic performance in vivo, has been used extensively as a biomarker for environmental stress, as well as for studying the defence mechanisms of the photosynthetic apparatus under heavy metal stress and especially Cd toxicity (Sitko et al., 2017; Murchie and Lawson, 2013; Kalaji et al., 2016; Bayçu et al., 2017, 2018; Chu et al., 2018; Moustakas et al., 2019a).

The metal hyperaccumulating plants have an ability to accumulate a relatively high level of Cd in their plant tissues (Baker et al., 2000; Reeves and Baker, 2000) as they have developed a number of Cd-detoxification mechanisms that lead to acclimation and tolerance (Sytrar et al., 2013; Mesnoua et al., 2016; Bayçu et al., 2018; Rehman et al., 2017, 2019 and refs. therein). During the last years, increased interest has been focused on some economically important or industrial plant species with high Cd-accumulating capacity as promising tools for phytoremediation of Cd-contaminated soils (Chen et al., 2011; Rehman et al., 2017, 2019; Sorrentino et al., 2018). Moreover, increasing attention has also been paid to the herbal plants, as an alternative for ecologically safe and cost-effective phytoremediation, since these aromatic plants are mainly used for secondary products (Pandey et al., 2019).

The herbal plant Salvia sclarea L. (clary sage) is classified to the Zn, Pb, Cr and Cd accumulators, and considered to have potential for phytoremediation of heavy-metal polluted areas as a substitute for some other edible plants (Chand et al., 2015). It has also been demonstrated that S. sclarea grown in contaminated industrial fields accumulates heavy metals through the root system and then translocates them to the aboveground parts, as the accumulation of heavy metals in the leaves does not affect the quantity and quality of essential oils (Angelova et al., 2016). Among the aboveground parts, the accumulation of Cd is greatest in the leaves where photosynthesis takes place, which is essential for the survival of plants. However, to the best of our knowledge, the impact of Cd on the function of the photosynthetic apparatus and defence mechanisms of S. sclarea in detail almost missing. Our previous study (Moustakas et al., 2019b) has demonstrated for the first time that the exposure of S. sclarea plants to 100 μM Cd for 5 days caused spatial leaf heterogeneity of PSII photochemistry (ΦPSII), which showed a high resistance to Cd stress at these conditions.

Based on all above, in this study we sought to clarify in more detail the defence mechanisms of S. sclarea against Cd toxicity in order to evaluate its potential for phytoremediation or phytoextraction of Cd contaminated soils. For this purpose, the impact of a high Cd supply (100 μM) on S. sclarea for 3 and 8 days was investigated by following changes in the oxidative stress markers, the content of leaf photosynthetic pigments, anthocyanins and total phenolics, as well as the leaf anatomy. The functional activity of the photosynthetic apparatus, evaluated by PAM chlorophyll fluorescence, P700 photooxidation and oxygen-evolving activity was also assessed with respect to the time of treatment. The responses of S. sclarea to Cd treatment were also evaluated by the uptake and distribution of nutrient elements in plant tissues (roots and leaves) after the 8th day when the Cd accumulation in leaves reached (and exceeded) the criteria for hyperaccumulating plants. The obtained data will reveal the possible mechanisms of adaptation of the photosynthetic apparatus to Cd toxicity. Knowledge of the defence mechanisms and plant responses to Cd stress will be of high importance for a deeper understanding of plant tolerance mechanisms, as well as would be useful for genetic engineering in hyperaccumulator plants and for optimizing management practices in the phytoremediation/phytoextraction.

2. Materials and methods

2.1. Plant growth conditions and Cd treatment

Seeds of clary sage (Salvia sclarea L., belongs to the family Lamiacae) were provided by Bio Cultures Ltd. (Karlov, Bulgaria). After the initial seed germination followed by about 40 days soil cultivation (with 40% perlite), the equally developed seedlings (6–7 cm high), were transferred to 1-L containers (3–4 seedlings per container) filled with a continuously aerated half-strange Hoagland nutrient solution (pH 6.0) with some modifications: 1.5 mM KNO₃, 1.5 mM Ca(NO₃)₂, 0.5 mM NH₄NO₃, 0.25 mM KH₂PO₄, 0.5 mM MgSO₄, 50 μM NaFe(III)EDTA, 23 μM H₂BO₃, 5 μM ZnSO₄, 4.5 μM MnCl₂, 0.2 μM Na₂MoO₄ and 0.2 μM CuSO₄, as described previously (Moustakas et al., 2019b). Plants were grown in a greenhouse under controlled conditions: photon flux density of about 220 μmol m⁻² s⁻¹ and 14/10 h light/dark photoperiod at 25/20 °C. About 2-month-old uniform Salvia plants were selected and treated with 0 or 100 μM Cd (as 3CdSO₄·8H₂O) for 8 days. For each treatment, 4 containers were prepared. Measurements were performed at the 3rd and the 8th day after Cd exposure. The nutrient solutions were renewed every three days. The bioconcentration factor (BF = Cd content in roots (μg g⁻¹ DW)/Cd content in the solution (μg mL⁻¹)) was estimated according to Chen et al. (2011) and Zoufan et al. (2020). Different plants were harvested after the 3rd and 8th day of Cd exposure. Plant biomass parameters such as dry weight (DW) of roots and shoots were determined as DW per plant. The relative water content (RWC) of leaves was calculated as: RWC(%) = [(FW – DW)/(SW – DW)] × 100, where FW is the fresh weight and SW is the water-saturated weight.

2.2. Elemental concentration determination by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Preliminary measurements of the Cd accumulation in plant tissues showed that the Cd content in leaves up to the 5th day of Cd exposure was less than 55 μg g⁻¹ dry weight (Adamakis, 2021b). Therefore, we performed the analysis of elemental uptake only at the 8th day of Cd exposure when Cd hyperaccumulation concentration reached the threshold of 0.01% in the leaves.

Fresh plant material (roots and leaves) was rinsed with distilled water and dried at 60 °C to constant biomass. Dried biomass was then pulverized in a mortar and kept in sealed polyethylene bags under dry conditions. The dried samples were mineralized in a micro-wave digestion oven (Ethos One, Milestone, Italy). A quantity of 0.3 g of dried plant materials were accurately weighed in a quartz closed flask. Approximately 2 mL of nitric acid 65% (v/v) (Merck Suprapur) and 1 mL of hydrogen peroxide 33% (v/v) (Sigma Aldrich, USA) were added to each flask. The digests were adjusted to 40 mL with ultrapure water. In parallel, the procedural blanks, including the same reagents as the samples, were prepared and digested in the same way as the samples in each digestion run. Distilled, ultrapure water (Direct-Q 3 UV Water Purification System, Merck, Germany) was used throughout the experiments (see Konkolewska et al., 2020).

An Elan DRC II ICP-MS (PerkinElmer SCIEX, Ontario, Canada) was used to determine 111Cd, 66Zn, 57Fe, 55Mn, 63Cu, 24Mg and 44Ca concentrations. An ICP-MS spectrometer equipped with a Meinhard concentric nebulizer, cyclonic spray chamber, Pt cones, and quadrupole
mass analyzer was used for this study. The operating conditions for ICP-MS were optimized on a daily basis and were as follows: RF power was 1250 W; the plasma gas flow rate was 16 L min⁻¹; the nebulizer gas flow rate was 0.89–0.91 L min⁻¹ and the auxiliary gas flow rate was 1.2 L min⁻¹. Smart Tune Solution – ELAN DRC/PLUS/II was used to check the daily performance of ICP-DRC-MS. The dynamic reaction cell (DRC) mode was used in order to eliminate spectral interferences with high-purity ammonia (Linde Gas, Poland) for 44Ca and 52Mn analyzed. To eliminate non-spectral interferences, a 10 µg L⁻¹ solution of 45Sc, 74Ge, and 103Rh was used as an internal standard. A multi-element stock solution (Multi-element Calibration Standard 3, Atomic Spectroscopy Standard, PerkinElmer Pure) containing the analyzed elements at a concentration of 10 mg L⁻¹ was used to prepare a series of standard solutions for calibration. The validity of the analytical method was assessed by analyzing the certified reference material NIST SRM Spinach Leaves 1575a (Kołodziej et al., 2020).

During the validation process parameters such as linearity, limit of detection (LOD), precision and trueness were evaluated. Calibration curves were constructed based on six points over a concentration range of: 50–1500 µg L⁻¹ for 25Mg, 44Ca and 57Fe, 0.1–100 µg L⁻¹ for 60Zn, 55Mn, 65Cu, 111Cd. The correlation coefficient R exceeded a value of 0.999. The trueness of the analytical results was assessed as recovery (R, %). For all elements the recovery values were equal to 95% and 106% respectively. The obtained precision values, expressed as the coefficient of variation (CV, %) for all elements were as follows: 1.8% for Cd, 2.8% for Ca, 2.5% for Mg, 1.7% for Mn, 3.5% for Zn and 3.7% for Fe and 2.7% for Cu. The limits of detection (LOD) for the determined elements were counted according to LOD = 3.3σ/b, where b means standard deviation of the result obtained for the blank samples and b is the sensitivity. The LODs for the ICP-MS method were found to be 0.02 µg g⁻¹ (Cd), 0.05 µg g⁻¹ (Cu), 30 µg g⁻¹ (Ca), 0.8 µg g⁻¹ (Mg), 0.03 µg g⁻¹ (Mn), 40 µg g⁻¹ (Fe) and 0.01 µg g⁻¹ (Zn).

2.3. Oxidative stress markers

2.3.1. Malondialdehyde and hydrogen peroxide content

Fresh mature leaves from randomly selected plants were collected and stored in a deep freezer (−80 °C) until measurements. The evaluation of the hydrogen peroxide (H₂O₂) content and the level of lipid peroxidation, by the content of malondialdehyde (MDA) was measured as described in Yotsova et al. (2018). Frozen leaf tissues (100 mg) were homogenized at 4 °C in 1% (w/v) trichloroacetic acid (TCA) and then centrifuged at 12,000 × g for 20 min. For estimation of H₂O₂ content, the reaction mixture consisted of: 0.5 mL supernatant, 0.5 mL of 100 mM Na–K–phosphate buffer (pH 7.6) and 1 mL of 1 M KI, was vortexed and left in the dark for 1 h, then the absorbance was measured spectrophotometrically at 390 nm. The amount of H₂O₂ was calculated using a molar extinction coefficient (0.28 µM⁻¹ cm⁻¹). For estimation of MDA content, the reaction mixture consisted of: 0.5 mL supernatant, 0.5 mL of 100 mM Na–K–phosphate buffer (pH 7.6) and 1 mL reagent (20% TCA containing 0.5% thioibarbituric acid), was vortexed and heated at 95 °C for 30 min and then cooled in an ice bath to stop the reaction. After centrifugation at 12,000 × g for 5 min, the supernatant was measured spectrophotometrically. The amount of MDA was calculated by subtracting the absorbance at 600 nm from the absorbance at 532 nm using a molar extinction coefficient (0.155 µM⁻¹ cm⁻¹). Measurements were performed at 3rd and 8th day after the Cd exposure. The mean values were averaged from three independent treatments with at least 3 repetitions for each treatment.

2.3.2. Electrolyte leakage

For the determination of electrolyte leakage, some fully expanded leaves from different selected plants were cut into pieces (about 4 cm²) and immersed in 40 mL tubes with distilled water at room temperature in the dark. After 24 h of incubation electrical conductivity of the solutions (EC1) was measured with a conductometer (Hydromat LM302, Germany). After that, the samples were boiled for 30 min, then cooled to room temperature and their final electrical conductivity was measured again (EC2). The electrolyte leakage (EL) was estimated from the equation: EL (%) = (EC1/EC2) × 100.

2.4. Photosynthetic pigment analysis

The photosynthetic pigments were extracted from frozen leaf tissues (50 mg) by grinding with 10 mL ice-cold 80% (v/v) acetone and centrifuged at 5000 × g for 5 min at 4 °C. The supernatant was measured spectrophotometrically (Spectord 210 Plus, Ed. 2010, Analytik Jena AG, Germany) at 470, 646.8 and 663.2 nm. The amounts of chlorophyll a (Chl a), chlorophyll b (Chl b) and total carotenoids (Car) were determined according to the equations of Lichtenthaler (1987):

\[ \text{Chl a (µg mL⁻¹)} = 12.25 \text{A}_{436.2} - 2.79 \text{A}_{469.8}; \]
\[ \text{Chl b (µg mL⁻¹)} = 21.50 \text{A}_{466.8} - 5.10 \text{A}_{663.2}; \]
\[ \text{Car (µg mL⁻¹)} = (1000 \text{A}_{649} - 1.82 \text{Chl a} - 85.02 \text{Chl b})/198. \]

The mean values were averaged from three independent treatments with at least 3 repetitions for each treatment.

2.5. Anthocyanins and total phenolic content

Fresh mature leaves from randomly selected plants were collected and stored in a deep freezer (−80 °C) until phytochemical evaluations. The frozen leaf samples (100 mg) were ground and extracted with 10 mL acidified methanol (1% HCl) in darkness and left at 4 °C in the dark for 24 h (see Zoufan et al. (2020)). Total phenolic content (TPC) was determined by the Folin-Ciocalteu’s colorimetric method as described in Sripakdee et al. (2015). Briefly, 0.5 mL of the diluted leaf extract (1 mg mL⁻¹) was added to 2.5 mL of Folin-Ciocalteu’s reagent and after that 2 mL of sodium carbonate solution (7.5%) were added and vortexed regularly for 2 h. The optical density of the mixture was measured spectrophotometrically at 765 nm using a Spectord 210 Plus (Edition 2010, Analytik Jena AG, Germany) and TPC was calculated using standard curves of gallic acid and expressed as mg of gallic acid equivalent per g FW of leaf tissues. The anthocyanins content was estimated by measuring of the leaf extracts spectrophotometrically at 535 and 657 nm by method of Mancinelli et al. (1991) and was expressed as mg of cyanidin-3-glucoside equivalent per g FW using an extinction coefficient (33000 M⁻¹ cm⁻¹) (Zoufan et al., 2020). Quantification data were the mean of 3 replicates for each treatment.

2.6. Light microscopy

Pieces of S. sclarea leaves from plants exposed to 100 µM Cd for 0 (control) or 3- and 8-days plants, were fixed in a 3% paraformaldehyde + 3% glutaraldehyde solution buffered with 0.05 M sodium cacodylate pH 7.0 at room temperature for 6 h. Subsequently, the leaf segments were post-fixed in 2% osmium tetroxide similarly buffered for 3 h, afterwards dehydrated in an ace tone series, treated with propylene oxide, and finally embedded in Durcupan ACM resin (Moustaka et al., 2018b). An ultramicromtore (LK8 8801 A, Stockholm, Sweden) equipped with a glass knife was used to obtained semi-thin sections (0.5–2 µm) which were stained with 0.5% (w/v) toluidine blue O and observed with a Zeiss Axiosplan light microscope equipped with a digital AxioCam MRc 5 camera (Adamakis et al., 2014).

2.7. Chlorophyll fluorescence measurements

Chlorophyll a fluorescence was measured in dark-adapted leaves of S. sclarea plants, grown with 0 (control) or 100 µM Cd for 3 and 8 days, using an Imaging-PAM Fluorometer M-Series MINI-Version (Walz,
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4. Results

3.1. Cadmium accumulation and nutrient element content

Exposure of \textit{S. sclarea} to Cd for 8 days resulted in increased Cd accumulation in both roots and leaves (Fig. 1A and B), as the calculated BF value for roots was BF = 1.113. This was accompanied by an increased Zn and Fe uptake that resulted in significantly increased accumulation of these elements in roots over 13 and 2 times, respectively (Fig. 1C), while Zn translocation to leaves decreased significantly by about 63% (p < 0.01) (Fig. 1D). In contrast, the translocation of Fe to the leaves was increased by about 25% (p < 0.05) after the Cd treatment (Fig. 1D). Furthermore, under Cd exposure it is also observed an increased accumulation of Ca and Mn in roots (p < 0.05) (Fig. 2A and B), as well as strongly increased accumulation of Cu in roots over 10 times (Fig. 2D), while their concentrations in leaves was decreased significantly (p < 0.05) compared to control plants (Fig. 2A, B and D). Finally, Cd exposure decreased Mg uptake (p < 0.05) that resulted in decreased accumulation in roots (by 46%), as well as decreased accumulation in leaves by 30% (Fig. 2C).

3.2. Alterations in the plant biomass and oxidative stress markers

Under Cd exposure for 8 days, clary sage plants showed reduced root and shoot dry weight per plant by about 36% and 15%, respectively, while the 3-day Cd exposure caused no significantly different changes compared to the control (Fig. 3A and Supplemental Table 1S). The responses of plant biomass parameters and morphological alterations after the Cd treatment are presented in more detail in Supplemental Table 1S and Fig. 1S. The leaf RWC gradually decreased with increasing time of Cd treatments reaching a reduction up to 27% (p < 0.01) on the 8th day (Fig. 3A, Table 1S).

The oxidative stress and lipid peroxidation, causing disruption of membrane integrity in \textit{Salvia} leaves under Cd exposure were estimated by \textit{H$_2$O$_2$} content and MDA content, respectively. Data presented on Fig. 3B showed that the leaf content of \textit{H$_2$O$_2$} and MDA gradually increased with time of exposure by up to 48% and 42% (p < 0.01), respectively, at the 8th day after Cd treatment. The electrolyte leakage (EL) from leaf cell membranes was investigated to assess the extent of membrane damage in \textit{S. sclarea} leaves in response to Cd exposure. Results showed that the values of EL were increased gradually by 12% and 43% after Cd exposure for 3 and 8 days (Fig. 3B) in comparison to the control, which is in line with the observed increase in lipid peroxidation of membranes (MDA).

3.3. Pigment and total phenolic content

After Cd treatment, the amount of photosynthetic pigments (Chl \textit{a}, Chl \textit{b} and Car) decreased (p < 0.05) in comparison to the control (Table 1). Data showed that the content of Chl \textit{a} significantly decreased by about 25% at the 3rd day and 36% at the 8th day after the Cd treatment. Slightly less decrease was observed for Chl \textit{b} after 3 and 8 days of the treatment (by 19% and 32%, respectively). The reduction of the Chl \textit{a} content was more considerable than that of the Car content (by 13%) after Cd exposure for 3 days and reached control values at the 8th day. The Cd-induced changes in the pigment composition resulted in a significant decrease of the Chl \textit{a/b} ratio (p < 0.05) in comparison to the control due to the stronger reduction in Chl \textit{a} content (Table 1).

On the other hand, the leaf content of total phenolics and anthocyanins increased with time of exposure up to 1.3-folds and 1.5-folds, respectively, under Cd exposure for 8 days when compared to the control.
3.4. Changes in the chlorophyll fluorescence parameters

The maximum efficiency of PSII photochemistry (Fv/Fm) and the efficiency of OEC on the donor side of PSII (Fv/Fo, Moustakas et al., 2020) did not change after 3 days exposure to Cd, but both of them decreased (p < 0.05) compared to control after 8-day exposure (Fig. 4A).

Fig. 1. Effects of 100 µM Cd exposure for 8 days on the content of Cd (A, B), Zn and Fe (C, D) in roots and leaves of S. sclarea plants. Mean values (± SD; n = 3) were subjected to one-way ANOVA and different letters indicate significant differences for the same element at p < 0.05 for Fe and p < 0.01 for Zn after Fisher’s least significant difference post-hoc test.

Fig. 2. Effects of 100 µM Cd exposure for 8 days on the content of Ca (A), Mn (B), Mg (C) and Cu (D) in roots and leaves of S. sclarea plants. Mean values (± SD; n = 3) were subjected to two-way ANOVA and different letters indicate significant differences for the same element at p < 0.05 after Fisher’s least significant difference post-hoc test.
The effective quantum yield of PSII photochemistry (ΦPSII) and the relative PSII electron transport rate (ETR) increased both after 3-day exposure treatment, but decreased (p < 0.05) after 8-day Cd exposure at low-light (LL) intensity measurements (Fig. 4C and D, respectively). At high-light (HL) intensity, ΦPSII and ETR did not change after 3-day exposure to Cd, but both decreased (p < 0.05) compared to the control after 8-day exposure (Fig. 4C and D, respectively).

The non-photochemical quenching that reflects heat dissipation of excitation energy (NPQ) increased (p < 0.05) by about 13% (p < 0.05) after 8-day Cd exposure compared to the control plants. In addition, the subsequent dark reduction kinetics of P700+ (Fig. 5B) and half-times of P700+ dark reduction (Fig. 5F) at high light, qI, did not change after 3-day exposure to Cd, but decreased (p < 0.05) compared to the control after 8-day exposure (Fig. 5F).

3.5. Photosynthetic oxygen evolution

Compared to control, the Cd treatment led to a decrease in the flash-induced oxygen yields (Y) (Fig. 5A). In this case, the measurements were made without an exogenous acceptor i.e., electrons are accepted from the plastoquinone (PQ) in the thylakoid membranes. In addition, the number of PSII centres in the initial reduced state (S0 = 100 - S1) increased by 22% (p < 0.01) and the amount of misses (a) by 9% (p < 0.05) indicating an alteration on the donor side of PSII and modification of the Mn4Ca cluster of OEC. PSII-mediated electron transport (H2O→BQ) was measured in vitro to assess the Cd-induced inhibition of PSII electron transport using an exogenous acceptor BQ, which accepts electrons from the secondary quinone electron acceptor (Qa). The results presented on Fig. 5A showed that the oxygen evolution in the presence of BQ was less affected (by about 10%, p < 0.05) by the Cd treatment than the flash-induced oxygen yields (by 29%, p < 0.01) when the electrons are accepted from PQ in the thylakoid membranes.

3.6. P700 photooxidation

To characterize the effect of Cd stress on the PSI photochemistry of *Salvia* leaves, we measured steady-state P700 oxidation-reduction kinetics by FR light-induced absorbance changes at 830 nm (ΔA/A830). The P700+ reduction in the dark after turning off the FR light was characterized by half-time of the exponential decay (t1/2) (Dobrikova et al., 2017). The dependence of the relative absorbance changes of P700+ (ΔA/A830) and half-times of P700+ dark reduction (t1/2) on the time of exposure to 100 μM Cd are presented on Fig. 5B. As can be seen, the PSI photochemistry (measured as ΔA/A830) was slightly decreased by about 13% (p < 0.05) on the 8th day and not statistically changed on the 3rd day after Cd exposure compared to the control plants. In addition, the subsequent dark reduction kinetics of P700+ (t1/2) in Cd-treated plants were found to be faster by 30% and 61% (p < 0.01) than those of the control plants after 3-day and 8-day Cd exposure, respectively (Fig. 5B).

3.7. Leaf structure features

Upon increasing exposure time, leaf structural changes included the reduction of the epidermal cell size (Fig. 6) while at the same time the cell vacuoles were filled with osmiophilic granules (arrows in Fig. 6B and C). Moreover, especially after 8 days of Cd exposure, the spongy parenchyma seemed to bare less intercellular spaces compared to control leaves (Fig. 6), while mesophyll cells appeared more densely stained.
4. Discussion

Heavy metal accumulation is an active process and the efficient uptake depends on the plant’s tolerance mechanisms to cope with the accumulated toxic levels of metals and/or on the mechanisms for their neutralization (DalCorso et al., 2008). In soils containing Cd concentrations above 35 μM, the only plants that can survive are the Cd-hyperaccumulating species (Sanit`a di Toppi and Gabbrielli, 1999). The so-called hyperaccumulator plants can accumulate high levels of Cd in their aboveground tissues without showing symptoms of

Fig. 4. Changes in (A) the maximum efficiency of PSII photochemistry (Fv/Fm) and (B) the efficiency of the OEC on the donor side of PSII (Fv/Fo) in Salvia sclarea plants grown at 0 (control) or 100 μM Cd for 3 and 8 days, and changes in (C) the quantum efficiency of photosystem II (PSII) photochemistry (ΦPSII), (D) the relative PSII electron transport rate (ETR), (E) the non-photochemical fluorescence quenching (NPQ), that reflects heat dissipation of excitation energy and (F) the coefficient of photochemical quenching (qP), reflecting the fraction of open PSII reaction centers, under low light (LL, 230 μmol photons m⁻² s⁻¹) and high light (HL, 900 μmol photons m⁻² s⁻¹) in S. sclarea plants grown at 0 (control) or 100 μM Cd for 3 and 8 days. Means (± SD; n = 3) were subjected to two-way ANOVA with treatment (0 or 100 μM Cd) and sampling-time (3 and 8 days) as the source of variations. Means with the same letter are not statistically different at p < 0.05 after Fisher’s least significant difference post-hoc test.
phytotoxicity (Dar et al., 2015; Rehman et al., 2017). Accordingly, in the treatment, and on (B) P700 photooxidation (P700
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Effects of the Cd exposure on (A) PSII activity and flash-induced oxygen yields (Y) of thylakoid membranes isolated from S. sclarea leaves at the 8th day after the treatment, and on (B) P700 photooxidation (P700− measured as ΔA/A<sub>0</sub>) and half-times of P700− dark reduction (t_{1/2}) in Salvia leaves. The values are presented as % of the control, as 100% corresponds to: PSII activity ~ 80.2 ± 0.9 μmol O₂ mg Chl<sup>−1</sup> h<sup>−1</sup>, ΔA/A<sub>0</sub> = 11.15 ± 0.39 (x 10<sup>−3</sup>) and t_{1/2} = 2.35 ± 0.21 s. S₈ (%) - the populations of PSII centres in the most reduced S₈ state in the dark (S₈ (%) = 100- S₄); α (%) – the misses. Mean values (± SD; n = 6) were considered as statistically significant with *p < 0.05 and **p < 0.01 using two-sample Student’s t-test.

It is known that the most evident symptom of Cd toxicity is chlorosis (DalCorso et al., 2008), which is thought to be caused by Cd-induced changes in the Fe/Zn ratio, as well as the negative effects on chlorophyll metabolism. In addition, it has been found that the Cd-induced inhibition of the root Fe(III) reductase causes a Fe(II) deficiency in sugar beet and cucumber (Alcantara et al., 1994). Conversely, our results demonstrated significantly increased Fe uptake by roots, and its translocation to leaves under Cd exposure compared to the control Salvia plants, causing an increased Fe leaf content by 25% (Fig. 1D). The increased accumulation of Fe in Salvia plants is most likely a protective response to a possible risk of chlorosis (Fe deficiency) in the leaves (van de Mortel et al., 2006). Additionally, it is considered that Fe can protect PSII from damage caused by the Fe deficiency in the leaves (Saito et al., 2010), as well as the Fe supplementation can also protect the photosynthetic electron transport, thus improving Cd tolerance (Sebastian and Prasad, 2015).

Our results showed that the plant biomass decreased after Cd exposure for 8 days compared to the control more pronounced for root than for shoot dry weight (Fig. 3A). One of the reasons for these observations can be the bigger Cd accumulation in roots and the induction of oxidative stress in the plant tissues.

Elevated ROS generation under Cd exposure usually results in oxidative injuries that promote lipid peroxidation of membranes causing disruption of their integrity, i.e. MDA increased (Schützendübel et al., 2001; Romero-Puertas et al., 2004). Additionally, the electrolyte leakage (EL) from leaf cell membranes has also reported as one of the most important criteria for loss of cell membrane integrity (membrane permeability), as well as for identification of heavy metal tolerance and cell viability (Gonzalez-Mendoza et al., 2009). In the current study amounts of MDA and H₂O₂, as well as EL values did not show a strong increasing pattern during Cd exposure as these markers increased about 1.43 folds at 8th day in comparison to the control (Fig. 3B). However, it is not clear if the overproduction of ROS (including H₂O₂) during Cd exposure is the cause for redox cellular imbalance or it is a stress mechanism that is activated in the cells in order to cope with the toxic effects of Cd ions (Romero-Puertas et al., 2004). Moreover, it has been implied that a lower H₂O₂ accumulation confers Cd-tolerance in Arabidopsis thaliana (Cho and Seo, 2005), since it can play a role as a signal molecule in the induction of protective mechanisms against different...
stresses (Jiménez et al., 2019). A previous study with sage plants grown on heavy metal contaminated soil demonstrated that the H$_2$O$_2$ scavenging is rather non-enzymatic than enzymatic process as registered weak activities of the most antioxidant enzymes (Stancheva et al., 2010). Our data also revealed increased accumulation of the non-enzymatic antioxidants such as total phenolics and anthocyanins, as well as carotenoids in leaves under Cd exposure (Table 1), which have a significant role in ROS scavenging during Cd treatment in accordance with previous observations in the tolerant species as Brassica juncea and Malva herb (Kapoor et al., 2019; Zoufan et al., 2020). The protective effect of carotenoids is attributed to their contribution in quenching chlorophyll singlets and triplets thus reducing oxidative stress in chloroplasts (see in Zoufan et al. (2020)). Furthermore, the accumulation of various polyphenolic compounds under abiotic stress (including heavy metals) is due to the up-regulation of the biosynthesis of phenylpropanoid enzymes (the phenylpropanoid biosynthetic pathway) that have also the potential to scavenge harmful ROS (reviewed in Sharma et al. (2019)). The recent study has also proposed that an increase in PAL (phenylalanine ammonia-lyase) activity is accompanied by an increase in the synthesis of phenolic compounds in the Cd treated plants (Zoufan et al., 2020). Additionally, polyphenolics can also act as antioxidants for detoxification of H$_2$O$_2$ by donating electrons to guaiacol-type peroxides (Sytar et al., 2013). Therefore, they can complete the activity of enzymatic antioxidants to reduce Cd-induced oxidative stress in plants confirming their role in the heavy metal stress responses and in increasing plant tolerance (Chen et al., 2019; Farouk and Al-Amri, 2019). Moreover, Vidal et al. (2020) have proposed that plants which produce high amounts of phenolic compounds under the heavy metal stress could be good candidates for phytoremediation and/or phytostabilization. In addition, the anthocyanins have been also reported to have high antioxidant activity, acting as scavengers of H$_2$O$_2$ and peroxide anions in the vacuoles (Moustaka et al., 2018b, 2020; Sytar et al., 2013). Therefore, their localization probably has an adaptive role as a barrier against Cd stress, due either to their antioxidant capacity, which increases upon heavy metals stress, or to the accumulation of anthocyanins which coincides with the highest content of Cd in plant tissues (Sytar et al., 2013; Kapoor et al., 2019). The accumulation of anthocyanin-chelated metal (n+-) complexes has also been shown to be a protective mechanism leading to the higher plant tolerance (Baldisserotto et al., 2010; Landi, 2015; Landi et al., 2015). One of the most convincing hypotheses is that the formation of anthocyanin-metal (n+-) complex in plant tissue may alleviate the heavy metal toxicity by storing metals in the peripheral cell layers and/or cell vacuoles when they will be less harmful than in the chloroplasts (Landi, 2015). Salvia sclarea leaf blades are dorsosentral and amphistomatic. The upper epidermis cells are larger than lower epidermis cells, while the mesophyll comprises of a 2–3-layered palisade and 1–2-layered spongy parenchyma (Özdemir and Şenel, 1999). Upon Cd application the basic anatomy remained unaltered, however, the vacuoles of the upper epidermal cells were filled with osmiophilic granules and reduced in size, while the mesophyll intercellular space also decreased (Fig. 6). Usually leaf epidermal and mesophyll cells are sites of increased Cd accumulation as it has been observed in the Cd hyperaccumulators Thlaspi caerulescens, T. praecox (Vogel-Mikus et al., 2008a, 2008b), Arabidopsis halleri (Fukuda et al., 2008) and Sedum alfredii (Tian et al., 2011). If the Cd accumulation occurs mainly in the epidermis and the mesophyll in the leaves of S. sclarea treated plants (a issue that remains to be further elucidated), this could explain the observed defects. At the cellular level, it has been also found that Cd damages the photosynthetic membranes, as the main target sites are the chlorophyll molecules, OEC and both photosystems (DalCorso et al., 2008; Dobrikova and Apostolova, 2019 and refs. therein). One reason for this could be a substitution of the Mg$^{2+}$, with Cd$^{2+}$ ions in the chemical structure of chlorophyll (Küpper et al., 1998). In addition, our results showed a reduced chlorophyll content in the Cd-treated leaves by 36% (Table 1), associated with the reduction of Mg leaf content by about 30% (Fig. 2C). Previously, it has also been proposed that the decreased chlorophyll content is associated with lower Mg uptake under Cd exposure of Capsicum annuum or soybean plants (Abdel Latief, 2013; Andresen et al., 2020) or the inhibition of enzymes involved in chlorophyll synthesis (Arena et al., 2017; Zoufan et al., 2018; Kapoor et al., 2019). Photosynthesis in higher plants is extremely sensitive to metals inhibiting the chlorophyll biosynthesis (Mobin and Khan, 2007; Fanoq et al., 2020). The first target of Cd action is considered to be PSII and OEC (Zobnin et al., 2018; Moustaka et al., 2018a; Moustakas et al., 2019a). The PSI function is harmed when electron flow from PSII to PSI exceeds the ability of PSI electron carriers to manage the electrons (Tikkkanen et al., 2014; Moustaka et al., 2018a). It is assumed that in the PSI complex, the Q$_{b}$-sited sites are both the donor (Mn$_A$ cluster) and the acceptor side (Q$_{a}$, and Q$_{o}$) (Szopinski et al., 2019). To conduct more detailed investigations of the Cd-induced changes in the Mn4Ca cluster of OEC, we used a Joliot-type oxygen electrode. These measurements examine the active PSI centres in grana domains i.e., PSII centres (Apostolova et al., 2006; Dobrikova et al., 2017). Data showed a decrease in the amount of PSI centres in the most reduced S$_0$ state in the dark (Fig. 5A) indicating alterations or modifications in the Mn4Ca cluster as also shown previously for Cd-treated wheat plants (Yotsova et al., 2019).
indirect evidence that Cd should be transferred to the chloroplast where Cd ions bind competitively to the Ca\(^{2+}\) cofactor in the Mn4Ca cluster, causing an inhibition of the oxygen evolution. Furthermore, data revealed stronger inhibition of the oxygen flash yields (\(Y\)) in comparison with the oxygen evolution measured with the exogenous electron acceptor BQ (Fig. 5A). Since the oxygen yields (\(Y\)) correlated with the functionally active PSI\(_h\) centres, located in the grana domains (Apostolova et al., 2006), the Cd-induced decrease of the oxygen yields (by about 29\%) could be due to inactivation of PSI\(_h\) centres and/or the conversion of PSI\(_h\) to PSI\(_i\) centres in the stroma, since the PSI\(_i\) centres in grana regions are more sensitive to stress. Nevertheless, these results suggest a high resistance of Salvia plants regarding oxygen evolution, taking into account the observed previously tolerant wheat Della mutant subjected to similar Cd stress (Dobrikova et al., 2017).

The slightly decreased \(\Phi_{PSII}\) after 8 days exposure to Cd (measured at low light, Fig. 4C), according to the model of PSI\(_h\) function (Genty et al., 1989), could be the result of either decreased fraction of open PSI\(_h\) reaction centres (\(q_p\)) or of the decreased efficiency of these centres (\(F_v/F_m^\prime\)). The coefficient of photochemical quenching (\(q_p\)) is a measure of the redox state of the plastoquinone pool PQ, while \(F_v/F_m^\prime\) is a measure of the energy of supply that reaches PSI reaction centres (Moustaka et al., 2016; Moustakas et al., 2020). Since the fraction of open PSI\(_h\) reaction centres increased after 8-day exposure to Cd at low light (Fig. 4F), it is evident that the decreased \(\Phi_{PSII}\) after 8-day-exposure to Cd (Fig. 4C) was due to a decreased efficiency of open PSI\(_h\) centres. Similar observations of decreased \(\Phi_{PSII}\) and decreased efficiency of open PSI\(_h\) reaction centres (\(F_v/F_m^\prime\)) after Cd treatment was observed in the hyperaccumulator \(Thlaspi caerulescens\) (Küpper et al., 2007). The mechanism of NPQ can reduce the energy that is transferred to the reaction centres, thus reducing \(\Phi_{PSII}\) (Simón et al., 2013). The NPQ parameter estimates the surplus light energy from the light-harvesting complex II that is dissipated as thermal energy from PSI\(_h\) via the zeaxanthin quencher (Müller et al., 2001; Demmg-Adams et al., 2012; Murchie and Ruban, 2020) and thus, decreases the efficiency of photochemical reactions of the photosynthesis (down-regulation of PSI\(_h\)) (Takahashi and Badger, 2011; Moustaka et al., 2015; Ruban, 2016). Consequently, the decreased \(\Phi_{PSII}\) after 8-day-exposure to Cd (measured at low light) was due to an increased NPQ (\(F_v/F_m^\prime\)) that reduced the efficiency of open PSI\(_h\) centres (\(F_v/F_m^\prime\)). However, this increased NPQ decreases ETR (Fig. 4D), preventing ROS formation (Roach et al., 2020). ROS can contribute directly to PSI damage or inhibit the repair of PSI reaction centres (Kale et al., 2017; Moustakas et al., 2020; Murata et al., 2007; Nishiyama et al., 2001). It is now accepted, that ROS generation can activate the plant’s defence mechanisms in order to cope with the oxidative stress damage, and that ROS are essential molecules involved in plethora of physiological functions serving as an antioxidant defence signal during stress in plants (Mittler et al., 2011; Foyer and Noctor, 2013; Sperdouli et al., 2019; Adamakis et al., 2020b). The increase of the photoprotective mechanism of NPQ after 8 days of Cd exposure (Fig. 4E) was effective at retaining the same fraction of open reaction centres with control leaves under low light, but it was not sufficient enough under high-light exposure (Fig. 4F). Non-photochemical quenching under stress conditions can be regarded as efficient only if it is adjusted in such a way to retain the same fraction of open reaction centres as in control conditions (Lambre et al., 2012; Adamakis et al., 2021a; Moustakas et al., 2019b; Moustaka et al., 2016).

The analysis of the P700 photooxidation (\(AA/AA_0\)) revealed that the function of PSI was slightly inhibited in \(S.\) \(sclarea\) leaves during Cd exposure for 8 days (Fig. 5B). Moreover, the data also showed that the half-times (\(t_{1/2}\)) of P700\(^+\) dark reduction gradually decreased with increasing the time of Cd exposure. This indicates that under Cd stress, clary sage plants accelerate CEF around PSI, in accordance with previous studies for other plant species (Wodala et al., 2012; Dobrikova et al., 2017). Furthermore, the PSI-dependent CEF has been proposed to play a defence role preventing the photosynthetic apparatus from the oxidative damage under stress conditions, since it can prevent of the PSI acceptor side from over-reduction by generating \(\Delta\)pH through the thylakoid membranes (Huang et al., 2017). The CEF also regulates the processes of light-harvesting via the enhancement of NPQ (Fig. 4E), thus contributing to the protection of PSII. Additionally, the CEF-dependent generation of \(\Delta\)pH was found to enhance the lumen acidification, which in turn activates NPQ processes and regulates P700 redox state (Huang et al., 2017). Therefore, the accelerated CEF around PSI observed in \(S.\) sclarea could be a possible defence mechanism to alleviate the oxidative damage under Cd stress.

All the above clearly suggest that \(S.\) sclarea plants can employ several different protective strategies against Cd toxicity to balance the functional characteristics of the photosynthetic apparatus. Further investigations should be performed to obtain more detailed information about metabolic pathways and antioxidant enzymes that also can be involved in the enhanced Cd tolerance in \(S.\) sclarea.

5. Conclusion

The current study demonstrates some tolerance mechanisms of the photosynthetic apparatus, as well as the element nutrient uptake and distribution in \(S.\) sclarea under Cd only exposure. The protective mechanisms of \(S.\) sclarea plants against Cd toxicity include: 1) an increased Fe accumulation in the leaves as a response to minimize Fe deficiency (chlorosis), as well as to maintain the photosynthetic electron transport; 2) an increased accumulation of phenolic compounds and anthocyanins as non-enzymatic antioxidants contributing to a higher degree of metal tolerance and 3) a strongly increased NPQ and accelerated CEF around PSI, thus protecting the photochemical functionality of the photosynthetic apparatus and alleviating oxidative damage under heavy metal stress.

CRediT authorship contribution statement


Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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