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Research Article

PHOTOSYNTHETIC ELECTRON TRANSPORT AND ANTIOXIDANT DEFENSE CAPACITY OF SUNFLOWER PLANTS UNDER COMBINED HEAVY METAL STRESS

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Summary: Sunflower plants (*Helianthus annuus* L.) cv. 1114 were used as test plants to investigate the combined toxicity effect of heavy metals present in soils collected from three different locations of a polluted area near a non-ferrous metal smelter. Soils were designated as highly contaminated, moderately contaminated and non-contaminated (control) based on the content analysis of soil samples. The photosynthetic performance was assessed by the changes in the functional activity of PS1 (photosystem 1) and PS2 (photosystem 2). Evidence for accelerated electron flow beyond the first qiunone acceptors in PS2 (Q_A) but not related with accelerated reduction of the first acceptor of light in PS1 (P700) is presented. The retarded rate of the slower (IDP) increase of the rapid phase of Kautsky effect (OIDP) as well as the lower level of Q_B-non-reducing PS2 reaction centers in HCS plants suggested an accelerated rate of electron flow in the PS2 acceptor side. The response of the antioxidant defense system included enhancement of superoxide dismutase (SOD) activity, total antioxidant activity and content of flavonoids. Changes in thylakoid electron transport chain reactions accompanied with altered oxidative stress-related metabolic activity could be considered as a protective mechanism operating in conditions of combined heavy metal toxicity.

Keywords: Heavy metal stress; photosynthetic electron transport; chlorophyll fluorescence; contaminated soil; antioxidant defense system; P700 redox state.

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INTRODUCTION

Heavy metal (HM) toxicity is one of the major abiotic stresses leading to hazardous effects in plants. Heavy metals in soils may be either naturally occurring elements due to mineral weathering, volcano eruptions and erosion or a consequence of the increased environmental pollution from industrial, agricultural, energetical and municipal sources (Chen et al. 2006; Antoniadis et al. 2017). The term HM includes only elements with specific gravity above five such as copper (Cu), iron (Fe), manganese (Mn), zinc (Zn), nickel (Ni), cobalt (Co), cadmium (Cd), and arsenic (As) which can become detrimental at excess concentrations to living organisms, including plants (Tchounwou et al. 2012). Heavy metals are also considered as trace elements because of their presence in trace concentrations (ppb range to less than 10 ppm) in soils.

Depending on their oxidation states, redox active (Fe, Cu, Cr, Co) and redox inactive (Cd, Zn, Ni, Al, etc.) heavy metals can be highly reactive, resulting in toxicity to plant cells in many ways (Antoniadis et al. 2017). Heavy metal toxicity is accompanied by disorders in plant metabolism and cell structure (Navari-Izzo and Rascio 2011; Doncheva et al. 2009, 2013). The effect of their toxic influence on plants is a strong and fast inhibition of plant growth processes as well as a decrease of the photosynthetic activity often in correlation with accelerated senescence (Maksymiec 2007; Masarovičová et al. 2011; Navari-Izzo and Rascio 2011). Heavy metal toxicity affects CO_2 fixation (Romanowska et al. 2002), electron transport in the light reactions (Rashid et al. 1994; Stefanov et al. 2014) and enzyme activities in the dark reactions of photosynthesis (Van Assche and Clijsters 1990; Solymosi and Bertrand 2011, 2012). Changes in the regulation of the photosynthetic electron transport in tomato and sunflower leaves in conditions of inhibited ferredoxin:NADP oxidoreductase (FNR) were found (Stefanov et al. 2012, 2014). Damages in PS2 after heavy metal stress in tomato plants were also observed (Stefanov et al. 2012). The rapid electron drain from PS2 measured by Chl fluorescence was suggested to be a result of an activated electron flow from the plastoquinone pool to O_2, a reaction catalyzed by the plastid terminal oxidase enzyme (PTOX) (chlororespiration) (Stefanov et al. 2014). In this case, chlororespiration could be considered as a protective mechanism against the inhibitory effect of heavy metals.

After metal uptake by the roots, plants make use of different defense strategies to maintain metal homeostasis and limit metal-induced cellular damages (Nagajyoti et al. 2010; Cuypers at al. 2013). A common consequence of heavy metal toxicity in plants is the generation of oxidative stress by stimulating the formation of free radicals and unstable reactive oxygen species (ROS) in plant cells, such as superoxide free radicals ($O_2^{\cdot -}$), hydroxyl free radicals ($OH^{\cdot -}$) and hydrogen peroxide (H_2O_2) which are more toxic and reactive than molecular O_2. ROS directly react with cellular organelles and macromolecules thus leading to multiple toxic effects such as ion leakage, oxidation of proteins and lipids, redox imbalance, damage and

conformational modifications of root and leaf cell structures and membranes, ultimately resulting in the activation of programmed cell death (PCD) pathways (Nagajyoti et al. 2010; Emamverdian et al. 2015). On the other hand, ROS can act as important secondary messengers for inducing plant defense system under stress conditions. Due to the dual function of the active oxygen species, cells possess mechanisms to strictly control their levels. These mechanisms involve antioxidant enzymes and low molecular weight non-enzymatic antioxidants which play major roles in scavenging ROS thereby protecting plants from oxidative damage (Gopal and Khurana 2011; Sytar et al. 2013).

Environmental pollution by heavy metals may lead to soil contamination, the most considerable being mainly due to mine production of Cu, Cd and Pb (Pinto et al. 2004). The mining and smelting of metal ores are one of the important sources of environmental pollution by metals and metalloids (Nriagu 1996). High levels of metal(loids) in topsoils are affected by non-ferrous metal smelters (Ettler 2016). Emissions from a long-term operation in a non-ferrous metal smelter situated in the Kuklen area near the town of Plovdiv (Bulgaria) have contaminated the arable soils which were sampled and analyzed for total acid extractable contents of Pb, Cd, Cu and Zn. In the present study, sunflower plants were grown in contaminated soils collected from three different locations of the polluted area near the non-ferrous metal smelter. This experimental approach allowed us to study the the response of plants under close to natural conditions of heavy metal pollution caused usually by the combined action of several heavy metals. We used sunflower (*Helianthus annuus*) plants cv. 1114 as test plants to assess the combined heavy metal toxicity as these plants were found to be more susceptible to heavy metal treatment in a previous comparative analysis of the responses of two sunflower genotypes to Pb excess (Doncheva et al. 2013; Stefanov et al. 2014). The assessment of heavy metal toxicity was based on changes in photosynthetic electron transport and capacity of the antioxidant defense system.

MATERIALS AND METHODS

Soil sampling

Soil samples were collected from three locations: Point 1 – "Green belt" (24°49′332″E 42°3′588″N), Point 2 – "Old lavender'" (24°49′607″E 42°3′125″N), Point 3 – "Airport" (24°49′534″E 42°3′298″N). The choice of sampling points was based on the distance from the non-ferrous metal smelter situated in the Kuklen area near the town of Plovdiv (Bulgaria). Sample units were collected from a sampling point in each of the segments by taking 9 cores per ha. Only the top soil layer (0-20 cm) was sampled at 3 to 5 spots per field to obtain an average sample.

Sample preservation, preparation and soil analysis

The samples were air dried in the laboratory and sieved with a 2 mm grid sieve. Soil analysis was carried out to determine pH in water with a glass electrode. The content of Pb, Cd, Cu and Zn in the soil samples was determined after microwave digestion with Milestone

MLS 1200 Mega system. The extraction solution was aqua regia and the used program included two steps of power (250 and 450 W) for 10 min. Determination of heavy metal content was carried out on an atomic absorption spectrometer (Analist 200, Perkin Elmer). Three replicates per each treatment were analyzed. Soils from the above-mentioned three locations were collected and used in the experiment.

Experimental scheme

Sunflower plants (*Helianthus annuus* L.) cv. 1114 were grown in pots with the above-described types of soil (FAO - Eutric Fluvisols) collected from the three different locations in the polluted area (Table 1). The experiment was carried out in a growth chamber at a photon flux density of 250 μmol m^{-2} s^{-1}, 22±2°C, a 14h/10h (day/night) photoperiod and 60% relative humidity. Plants were grown in contaminated soil (1 kg/pot) for 30 days. Analyses were performed on the 3rd differentiated leaf and intact root system.

Electrolyte leakage

Membrane integrity damage was monitored by measuring the increase in electrolyte leakage (EL) from different organs. The EL test was conducted according to Yamori et al. (2005). One gram of tissue was cut into 2 cm segments placed in test tubes containing 15 ml of bidistilled H_2O for 36 h at 25°C. The conductivity of the supernatant (initial EL - C_1) was measured with an electro-conductivity meter. The tubes were placed in a boiling water bath for 60 min, and the electrical conductivity was obtained after attaining equilibrium at 25°C (final EL - C_2). EL was calculated using the following equation: EL (%) = $C_1/C_2 \times 100$.

Chlorophyll fluorescence measurements

In vivo modulated chlorophyll (Chl) fluorescence was measured by a pulse amplitude modulation fluorometer (Heinz Walz, Effeltrich, Germany, model PAM 101-103) as was described in details by Schreiber et al. (1986). Measurements were carried out on leaf discs (1cm^2) detached from plants dark adapted for 15 min before analysis (details about fluorescence measurements were presented in Stefanov and Terashima, 2008). Data analysis (Tyystjärvi and Karunen 1990) was carried out using FIP software (Q_A-Data, Turku, Finland). The following parameters were used to calculate the maximal quantum yield of PS2, $F_v/F_m = (F_m-F_0)/F_m$ (Kitajima and Butler 1975), effective quantum yield, $\Phi_{PSII} = (F_m'- F)/F_m'$ (Genty et al. 1989), and non-photochemical quenching, NPQ = (Fm-Fm′)/Fm′ (Bilger and Björkman 1990).

Rapid fluorescence transient (OIDP) was measured in a separate experiment as was earlier described by Yordanov et al. (2008). Each transient represents the average of about 8 measurements.

P700 redox state measurements

The redox state of P700 was investigated *in vivo* with a dual wavelength (810/860 nm) unit (Heinz Walz ED 700DW-E) attached to a PAM101E main control unit in the reflectance mode (Schreiber et al. 1988). The dual wavelength system detects purer P700-related signals (Klughammer and Schreiber 1998). P700 was oxidized by irradiation with far-red light (13.4 Wm^{-2}), provided by a photodiode (FR-102, Walz, Effeltrich, Germany) that was controlled

by the PAM 102 unit. The A810/860 signal was normalized. The output signal is presented in volts.

Determination of chlorophyll content

Chlorophyll was extracted in N, N-dimethyl-formamide (5%, w/v) as described by Moran and Porath (1980). Chlorophyll content was measured using a spectrophotometer (Shimadzu UV-200, Japan) and calculations were based on the extinction coefficients proposed by Inskeep and Bloom (1985).

Activity of superoxide dismutase (SOD; E.C. 1.15.1.1)

SOD activity was measured spectrophotometrically at 560 nm based on the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) as described by Beauchamp and Fridovich (1971). One unit of SOD activity is defined as the quantity of the enzyme required to cause 50% inhibition of the initial NBT reduction under the assay conditions.

Protein content was evaluated according to the method of Lowry et al. (1951).

Analysis of non-enzymatic antioxidants

Finely grinded material (1.0 g) was extracted with 80% ethanol. The homogenate was centrifuged at 12,000 × *g* for 30 min at 0-4°C and the supernatant was used for analyses of antioxidant activity as well as flavonoid content.

Total antioxidant activity was investigated using the ferric reducing antioxidant power (FRAP) assay according to Benzie and Strain (1999). The FRAP reagent was freshly prepared by mixing 25 ml acetate buffer (300 mM, pH 3.6), 2.5 ml TPTZ (2,4,6-tripyridyl-s-triazine) solution (10 mM TPTZ in 0.04 M HCl) and 2.5 ml $FeCl_3$ (20 mM). An aliquot (0.05 ml) of the extract was added to 1.5 ml of the FRAP reagent and 0.15 ml dH_2O and after 15 min the absorbance was measured at 593 nm using FRAP solution as a blank. This procedure involves the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) in a blue colored complex in the presence of bioactive compounds (antioxidants).

Total flavonoid content was determined by the method of Lamaison and Carnet (1990) with modifications. An aliquot (1 ml) of the extract solution was mixed with 1 ml 2% $AlCl_3.6H_2O$ (w/v) solution. The absorbance of the mixture was measured after 10 min at 430 nm against 80% ethanol as a blank. Rutin was used as a standard. Results are expressed as rutin equivalents (mg rutin g^{-1} FW).

Statistical analysis

Data are expressed as means ± SE. Different letters indicate significant differences assessed by the Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis. The statistical software package StatGraphics Plus version 5.1 for Windows was used.

RESULTS

Heavy metal content in soils

The most contaminated soil was found in location 1 ("Green belt") situated nearby the non-ferrous metal smelter, further designated as highly contaminated soil (HCS) (Table 1). The concentrations of Pb, Cd, Cu and Zn in this sample strongly exceeded the maximal content levels. Soils collected from location 2

Table 1. Heavy metal content in soil samples (mg kg^{-1} DM) collected form three different locations of a polluted area near a non-ferrous metal smelter situated in Plovdiv region (Bulgaria). Maximal content levels: Pb>600 mg kg^{-1} DM; Cd>15 mg kg^{-1} DM; Zn>3000 mg kg^{-1} DM; Cu>50 mg kg^{-1} (according to Ordinance № 3/1 August 2008 on the values for permitted content of harmful substances in soils issued by the Ministry of Environment and Water, the Ministry of Health and the Ministry of Agriculture and Food of Republic of Bulgaria). Heavy metals with elevated concentrations are marked in bold.

Soil sample	Cu	Zn	Pb	Cd	pH
Point 1-HCS	**550±28^{c}**	**6500±103^{c}**	**6400±68^{b}**	**110±6.0^{b}**	7.5
Point 2-MCS	**60±10^{b}**	950±44^{b}	158±11^{a}	3.5±0.5^{a}	7.8
Point 3-C	32±5^{a}	150±9^{a}	75±8^{a}	1.5±0.3^{a}	7.2
LSD ≤ 0.05	94.84	725.01	2450.50	65.13	

Locations: Point 1 ("Green belt", HCS), point 2 ("Old Lavender", MCS), point 3 ("Airport", non-contaminated soil, control). HCS – highly contaminated soil; MCS – moderately contaminated soil. Different letters indicate significant differences assessed by the Fisher LSD test (P≤0.05) after performing ANOVA multifactor analysis.

("Old Lavender") situated at a larger distance from the non-ferrous metal smelter contained much lower amounts of heavy metals with the exception of Cu which was present above the maximal content level. This type of soil is referred to as moderately contaminated soil (MCS). Location 3 ("Airport") was situated far from the non-ferrous metal smelter and soil samples were non-contaminated (control).

Electrolyte leakage (EL)

Similar significant increases in EL were observed in leaves and roots of sunflower plants grown in soils with different degree of contamination with Pb, Cd, Cu and Zn (Fig. 1a, b). The highest values of EL were measured in plants grown in HCS both in leaves and roots (the increase was by 35% and 26%, respectively compared with the control) while in MCS plants the increase in EL was by 20 and 10%, respectively.

Chlorophyll content

Total chlorophyll content decreased in both HCS and MCS plants by 60% and 25%, respectively compared with the control (Fig. 2). The observed reduction was due mainly to decreased levels of Chl *b* (by 76% and 54% in HCS and MCS plants, respectively).

Functional activity of PS1 and PS2

Changes in PS2 acceptor side. A sudden illumination of a dark-adapted leaf with low actinic light intensities induced a rapid OIDP phase of Kautsky transient (Fig. 3). The fluorescence rise OI reflects the reduction in Q_B-non-reducing PS2 reaction centers (Chylla and Whitmarsh 1990; Cao and Govindjee 1990). Further IP fluorescence transient can be used to estimate the rate of Q_A reduction in the active PS2 reaction centers (Lu and Zhang 1998). A retarded rate of IP increase in sunflower plants grown in HCS was observed (Fig. 3). The trend of changes in the MCS curve was similar to the control. The proportion of the inactive PS2 centers

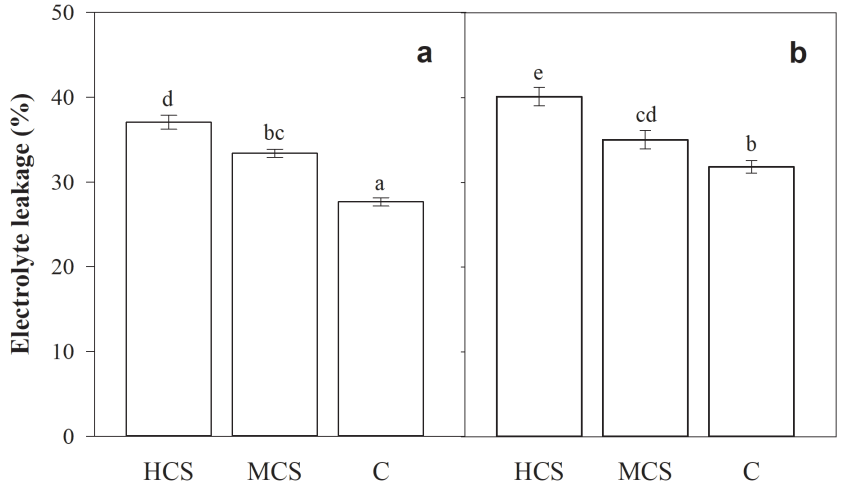

Figure 1. Electrolyte leakage from leaves (a) and roots (b) of sunflower plants grown in contaminated soils for 30 days in a growth chamber under controlled conditions. Soils were collected from three different locations of a polluted area near a non-ferrous metal smelter situated in Plovdiv region (Bulgaria). Analyses were performed on the 3rd differentiated leaf and intact root system. HCS – highly contaminated soil; MCS – moderately contaminated soil; C – control. Data are means ± SE (n=6). Different letters indicate significant differences assessed by the Fisher LSD test (P≤0.05) after performing ANOVA multifactor analysis.

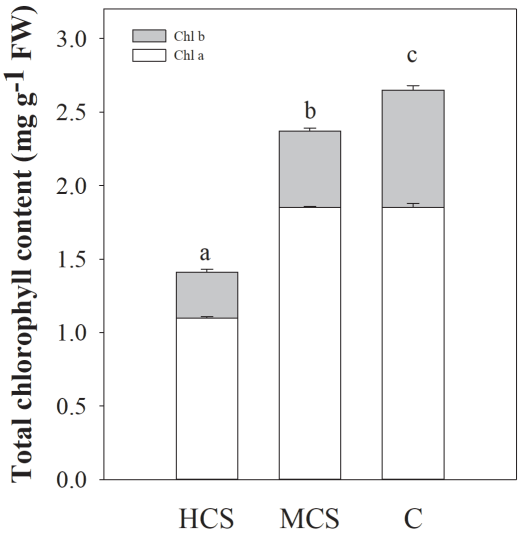

Figure 2. Chlorophyll content in sunflower plants grown in contaminated soils for 30 days in a growth chamber under controlled conditions. Soils were collected from three different locations of a polluted area near a non-ferrous metal smelter situated in Plovdiv region (Bulgaria). Analyses were performed on the 3rd differentiated leaf. HCS – highly contaminated soil; MCS – moderately contaminated soil; C-control. Data are means ± SE (n=6). Different letters indicate significant differences assessed by the Fisher LSD test (P≤0.05) after performing ANOVA multifactor analysis.

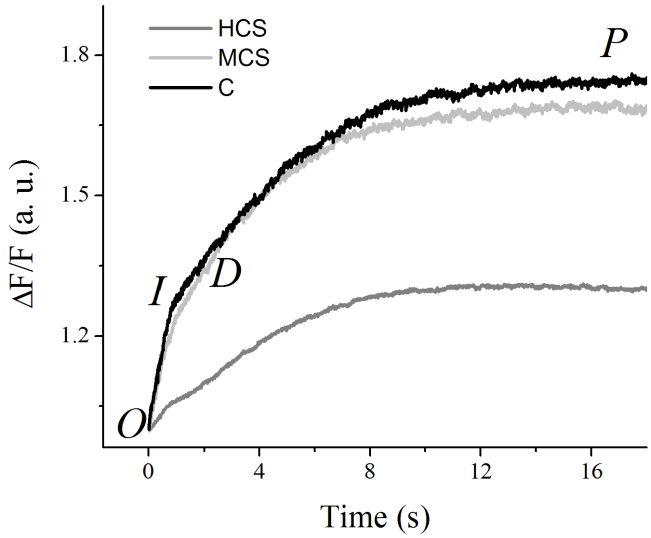

Figure 3. Rapid (OIDP) fluorescence transient during excitation by low actinic light intensities in sunflower plants grown in contaminated soils for 30 days in a growth chamber under controlled conditions Soils were collected from three different locations of a polluted area near a non-ferrous metal smelter situated in Plovdiv region (Bulgaria). Analyses were performed on the 3rd differentiated leaf. HCS – highly contaminated soil; MCS – moderately contaminated soil; C – control. Data are means of 3 to 5 independent measurements.

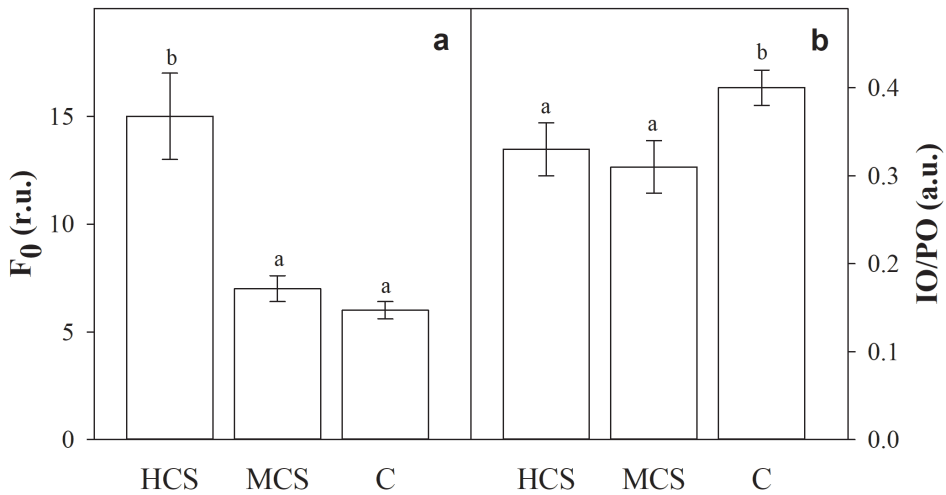

Figure 4. F_0 changes (a) and accumulation of Q_B non-reducing PS2 centers (b) in sunflower plants grown in contaminated soils for 30 days in a growth chamber under controlled conditions. Soils were collected from three different locations of a polluted area near a non-ferrous metal smelter situated in Plovdiv region (Bulgaria). Analyses were performed on the 3rd differentiated leaf. HCS – highly contaminated soil; MCS – moderately contaminated soil; C – control. Data are means of 3 to 5 independent measurements. Different letters indicate significant differences assessed by the Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

(Q_B-non-reducing PS2 reaction centers), is given by the OI/OP fluorescence ratio (Fig. 4b). While MCS plants expressed the lowest level of Q_B-non-reducing PS2 reaction centers (Fig. 4b), HCS plants showed a decreased rate of Q_A reduction in PS2 centers (Fig. 3). The slower rate of Q_A reduction in HCS plants (Fig. 3) was accompanied by the accumulation of Q_B non-reducing centers (Fig. 4b). The lowest values for the level of Q_B-non-reducing PS2 reaction centers (Fig. 4b) suggest a more active electron flow to PS1. An enhanced initial F_0 fluorescence level (Fig. 4a) together with a strongly modified OIDP curve in plants grown in HCS (Fig. 3) was also observed.

PS2 photochemistry and photoprotection induced by NPQ. A significant decrease (by 20 %) in the apparent PS2 quantum yield in HCS plants was found (Fig. 5a). It was not accompanied by delayed electron flow beyond Q_A as was estimated by the lack of changes in the photochemical fluorescence quenching coefficient, qP (Fig. 5b). A possible reason for these damages in PS2 could be the destroyed photoprotection mechanisms expressed as a decrease in NPQ (Fig. 5c). The lower NPQ (by 30%) in MCS plants that was accompanied by slightly increased qP suggests higher rates of the linear photosynthetic electron transport that could not be down-regulated by enhanced non-radiative dissipation of excessive excitation energy (Fig. 5b, c).

NPQ induction. To gain further insight into the differences in NPQ between control and heavy metal stressed plants, we investigated the changes in the fluorescence induction after a dark-to-light transition (Fig. 6). The actinic light (AL)-induced changes in NPQ showed

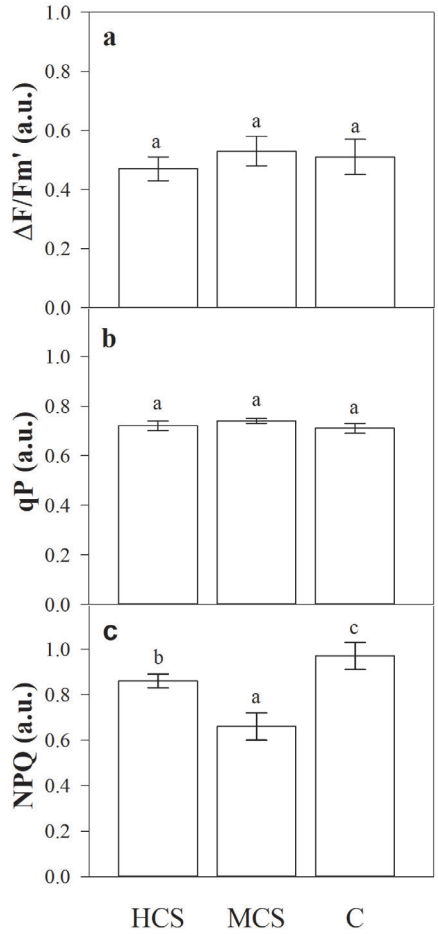

Figure 5. PS2 photochemistry (ΔF/Fm′ and qP) and non-photochemical fluorescence quenching (NPQ) as a measure for photoprotection in steady state fluorescence level in sunflower plants grown in contaminated soils for 30 days in a growth chamber under controlled conditions. Analyses were performed on the 3rd differentiated leaf. Actual PS2 quantum yield, ΔF/Fm′ (a); photochemical fluorescence quenching, qP parameters (b) and non-photochemical fluorescence quenching changes, NPQ (c). HCS – highly contaminated soil; MCS – moderately contaminated soil; C – control. Data are means of 3 to 5 independent measurements. Different letters indicate significant differences assessed by the Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

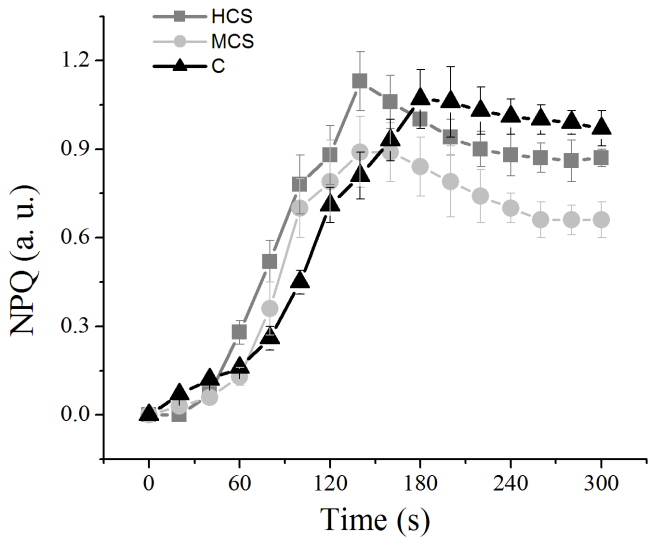

Figure 6. Induction changes in non-photochemical quenching in sunflower plants grown in contaminated soils for 30 days in a growth chamber under controlled conditions. Soils were collected from three different locations of a polluted area near a non-ferrous metal smelter situated in Plovdiv region (Bulgaria). Analyses were performed on the 3rd differentiated leaf. HCS – highly contaminated soil; MCS – moderately contaminated soil; C – control. Data are means of 3 to 5 independent measurements.

two phases. The rapid phase of NPQ induction occurred within 180 s, while a second phase appeared as a slow decrease to steady-state values. Probably, the rapid formation of a large proton gradient was responsible for the rise in the first phase (Stefanov and Terashima 2008), while the slower phase of the decrease would depend on the zeaxanthin-related reactions in the pigment bed (Govindjee 2002). Control plants displayed a typical two-phase curve, but the second phase showed a decreasing trend. A decrease in NPQ during the second phase of the NPQ induction transient in both MCS and HCS plants was observed (Fig. 6). The decrease in NPQ after reaching the maximum to the steady-state values could be due to (1) a decreased proton gradient in the later phases of photosynthetic induction because of activation of ATPase or (2) a decrease in the ascorbate concentration necessary for activation of the xanthophyll cycle which probably corresponds to the "middle phase" of the decrease in NPQ as shown in the study of Ivanov and Edwards (2000). A well-observable NPQ maximum was reached in HCS plants followed by the above-mentioned decrease in NPQ values (Fig. 6). The second phase of the NPQ decrease was expressed more strongly in MCS plants. Both types of heavy metal stressed plants showed an accelerated rate of the NPQ increase during the first phase of NPQ transient.

Far-red induced P700 oxidation. PS1 activity was assessed by P700 oxidation and was probed by measuring the far-red induced changes in leaf absorbance at 830 nm (A810/860 signal). An accelerated FR light induced rate of P700 oxidation was observed in plants grown in MCS (Fig.7).

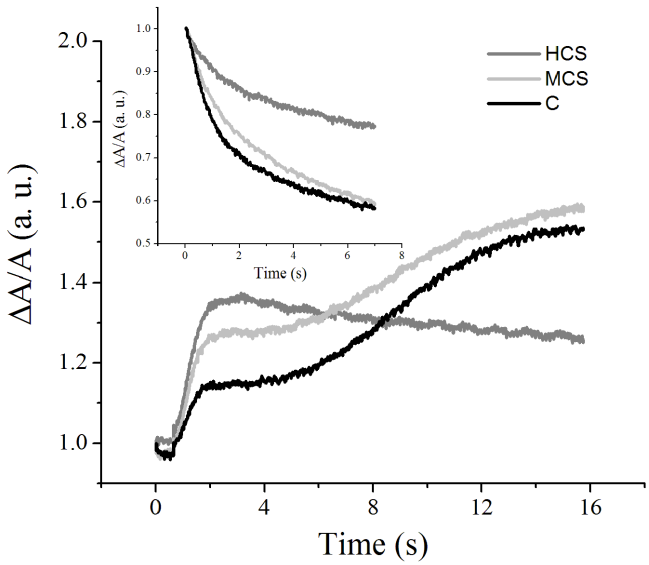

Figure 7. Far-red light induced changes in P700 oxidation and P700 reduction after far-red light switching off in sunflower plants grown in contaminated soils for 30 days in a growth chamber under controlled conditions. Soils were collected from three different locations of a polluted area near a non-ferrous metal smelter situated in Plovdiv region (Bulgaria). Analyses were performed on the 3rd differentiated leaf. Inset – the decrease in ΔA/A signal after switching off far-red light. HCS – highly contaminated soil; MCS – moderately contaminated soil; C – control. Data are means of 3 to 5 independent measurements.

In contrast, a lower maximal level of ΔA/A (i.e. P700 oxidation by far-red light) in HCS plants was found. The fast phase of reduction kinetics after switching off far-red light (reflecting the activity of FQR-dependent PS1 cyclic electron transport) was retarded in both HCS and MCS plants (Fig. 7, inset).

Activity of SOD

The activity of SOD as one of the main antioxidant enzymes responsible for mitigating oxidative stress (Gratao et al. 2005) was strongly increased (2-fold) in the roots of HCS plants compared with the control (Fig. 8b). SOD activity measured in the leaves of HCS plants was also enhanced although to a lower degree (by 30%) (Fig. 8a). The activity of SOD measured in leaves of MCS plants remained similar to the control (Fig. 8a).

Non-enzymatic antioxidant defense

Total antioxidant activity reflects the activity of low molecular weight non-enzymatic antioxidants protecting plants from damages caused by the heavy metal induced generation of ROS. The highest values for total antioxidant activity were measured in both leaves and roots of HCS plants (Fig. 9a, b). Antioxidant activity in roots was almost 2-fold higher compared with the control whereas in leaves it exceeded by 30% the control values. MCS plants also demonstrated higher total antioxidant activity when compared with controls but to a lesser extent (Fig. 9a, b). Similarly, the highest content of leaf

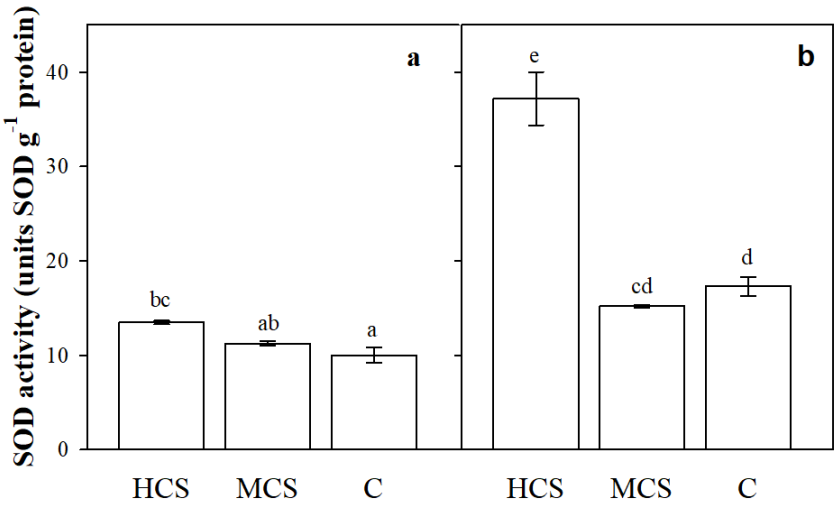

Figure 8. SOD activity in leaves (a) and roots (b) of sunflower plants grown in contaminated soils for 30 days in a growth chamber under controlled conditions. Soils were collected from three different locations of a polluted area near a non-ferrous metal smelter situated in Plovdiv region (Bulgaria). Analyses were performed on the 3rd differentiated leaf and intact root system. HCS – highly contaminated soil; MCS – moderately contaminated soil; C-control. Data are means ± SE (n=6). Different letters indicate significant differences assessed by the Fisher LSD test (P≤0.05) after performing ANOVA multifactor analysis.

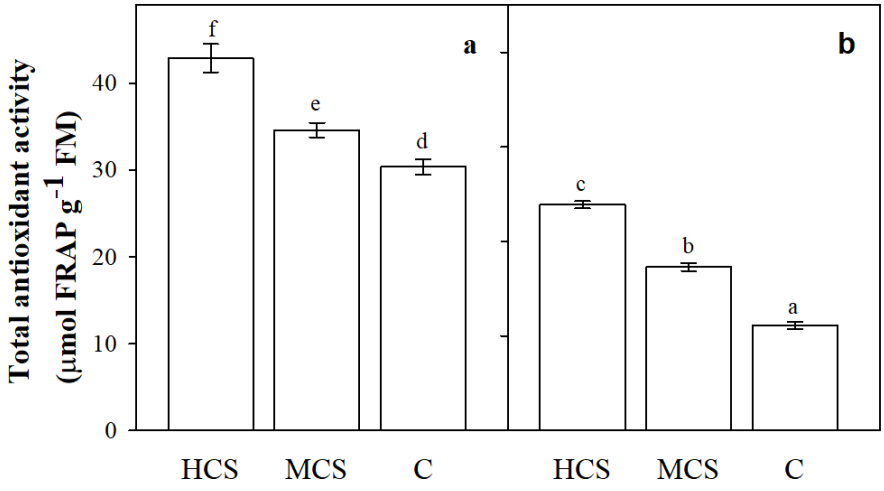

Figure 9. Total antioxidant activity assessed using the FRAP assay in leaves (a) and roots (b) of sunflower plants grown in contaminated soils for 30 days in a growth chamber under controlled conditions. Soils were collected from three different locations of a polluted area near a non-ferrous metal smelter situated in Plovdiv region (Bulgaria). Analyses were performed on the 3rd differentiated leaf and intact root system. HCS – highly contaminated soil; MCS – moderately contaminated soil; C – control. Data are means ± SE (n=6). Different letters indicate significant differences assessed by the Fisher LSD test (P≤0.05) after performing ANOVA multifactor analysis.

flavonoids was measured in the heavy metal stressed plants grown in HCS; it was by 80% higher than that of the control (Fig. 10). Although less affected, the content of flavonoids was also higher (by 28%) in the leaves of MCS plants when compared with the control.

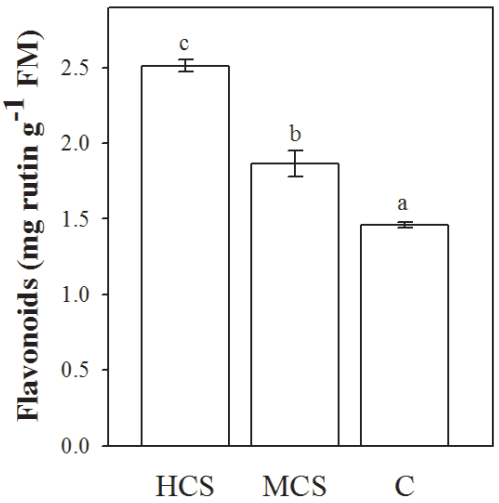

Figure 10. Total flavonoid content in leaves of sunflower plants grown in contaminated soils for 30 days in a growth chamber under controlled conditions. Soils were collected from three different locations of a polluted area near a non-ferrous metal smelter situated in Plovdiv region (Bulgaria). Analyses were performed on the 3rd differentiated leaf. HCS – highly contaminated soil; MCS – moderately contaminated soil, C-control. Data are means ± SE (n=6). Different letters indicate significant differences assessed by the Fisher LSD test (P≤0.05) after performing ANOVA multifactor analysis.

DISCUSSION

Heavy metal contamination of soils as a consequence of extensive industrial activities has become a major environmental problem of increasing significance from ecological, nutritional, and environmental points of view (Nagajyoti et al. 2010; Emamverdian et al. 2015). In the present study, we investigated the combined toxicity effects of several heavy metals present in soils collected from a polluted area near a non-ferrous metal smelter. The analysis of contaminated soils collected from three different locations showed the presence of P, Cd, Cu and Zn found at different concentrations depending on the distance from the non-ferrous metal smelter (Table 1). The experimental approach used in the present study allowed us to assess the response of plants under close to natural conditions of heavy metal pollution.

In general, it is known that the translocation of Cu, Cd and Pb from root to shoot in many plant species is very low (Doncheva et al. 2013; Meyer et al. 2015). We have previously shown that the exposure of sunflower plants from two genotypes (cultivated *H. annuus* cv.1114 and interspecific line *H. annuus* x *H. agrophyllus*) grown hydroponicaliy to Pb treatment resulted in higher Pb concentrations in the roots than in the above-ground parts of both genotypes (Doncheva et al. 2013). Nevertheless, the results from the present study showed well observable stress effects of the combined metal toxicity in the leaves of the cultivated *H. annuus* cv.1114, the most damaging effects being found in plants grown in HCS where the heavy metals tested were present in concentrations strongly exceeding the maximal content level (Table 1).

Heavy metals adversely affect the integrity of cell membranes which results in down-regulation of major metabolic processes (Hossain et al. 2012; Farid et al. 2013). Plasma membranes are considered

a primary target for metal toxicity in both leaves and roots. Estimated by EL used as a measure of membrane integrity both leaves and roots of HCS showed a higher degree of membrane damage when compared with MCS plants (Fig. 1).

Photosynthesis is one of the major metabolic processes which can be repressed by various stress factors. A reduction in chlorophyll content is the first symptom of deterioration of the photosynthetic activity representing one of the multiple toxic effects of heavy metals (Fig. 2). Similarly, the appearance of chlorosis mainly triggered by a preferential loss of chlorophylls, especially of chlorophyll b has been reported recently by D'Alessandro et al. (2013). The authors explained this result as being due to Cd toxicity effect on PS2 activity.

The fluorescence data reported in our study suggested some activation in the electron transport beyond the first stable quinone acceptor in PS2, Q_A. The retarded Q_A reduction was earlier suggested to be a regulatory mechanism (Stefanov et al. 2012) which could ensure better protection of PS2 in HCS plants. This suggestion is based on the decreased level of Q_B-non-reducing PS2 centers (inactive centers) estimated by the decreased IO/PO ratio (Fig. 4b). The results showing lower NPQ in MCS plants and slightly increased qP suggest higher rates of the linear photosynthetic electron transport that could not be down-regulated by enhanced non-radiative dissipation of excessive excitation energy (Fig. 5b, c). Accelerated FR light induced rate in P700 oxidation in plants grown in MCS was also observed (Fig. 7). Thus, some kind of stimulating effect of low doses of HMs on photosynthetic electron transport was found.

One of the most frequently observed common consequences of environmental stress is over-reduction of the photosynthetic electron transport chain which further causes damages to the photosynthetic machinery (Mittler 2002). It has been suggested that over-reduction of the electron transport chain should change the mechanisms of regulation of electron transport reactions. In such conditions, Bukhov and Carpentier (1996) and Stefanov et al. (2012) revealed an increased level of Q_A^- in the dark. This result correlated with the strongest decrease in the maximal PS2 quantum yield (Fv/Fm), i.e. PS2 centres with impaired function (Bukhov and Carpentier 1996; Stefanov et al. 2012). In our recent investigation, accelerated relaxation of variable fluorescence after 1 s saturated pulse and lower level of oxidation of the primary electron donor of PS1, P700, by actinic light were observed (Stefanov et al. 2014). In this case, activation of PTOX could be suggested (Shirao et al. 2013). A similar PTOX-dependent process was suggested in heavy metal stressed sunflower plants (Stefanov et al. 2014). PTOX caused an accelerated electron flow beyond Q_B but electrons could not reach P700. Our results showed an accelerated rate of the far-red induced P700 oxidation during the fast phase of A830 signal in heavy metal stressed sunflower plans (Fig. 7) which was in contrast with our recent study on actinic light induced A830 changes. Recently, Stefanov et al. (2014) suggested a retarded FNR linear electron transport in PS1. In this case lower FNR activity could induce a rapid depletion

of the pool of reduced P700, i.e. faster oxidation of P700 by far-red light (Fig. 7). In the present study, new evidence for accelerated electron flow beyond Q_A but not related to accelerated reduction of P700 is presented. Multiple alternative electron pathways have been identified or proposed including the water–water cycle, the malate shunt, the plastid terminal oxidase, and various types of cyclic electron flow around PS1 (Bukhov and Carpentier 2004). Strand et al. (2015) have proposed that the FQR and NADPH:plastoquinone oxidoreductase (NDH) pathways for CEF are activated under different conditions and play different roles in chloroplast energy balance. Participation of PSI CET can not be excluded during HM stress but the higher level of oxidized P700 after FR light excitation (Fig. 7) suggests that such cyclic electron transport was shuttled at PQ pool, i.e. a rise in PTOX activity.

The retarded rate of the IP increase (Fig. 3) together with the lower level of the Q_B-non-reducing PS2 reaction centers (Fig. 4b) in plants grown in HCS could suggest an accelerated rate of the electron flow in the PS2 acceptor side. This is in contrast to the accumulation of Q_A^- in the dark observed earlier (Stefanov et al. 2012). The lower maximal level of ΔA/A (i.e. P700 oxidation by far-red light) found in HCS plants (Fig. 7) suggested either some damage in PS1 and/or decreased FNR activity as was recently reported by Stefanov et al. (2014). In all heavy metal stressed plants an accelerated initial rate of oxidation was found as a result of insufficient electron flow coming from PS2 and the intersystem electron transport chain - donors of electrons from various alternative electron transport chains, described in details in the review of Bukhov and Carpentier (2004). Taking into consideration the lower PS1 activity (Fig. 7), it could be expected that activation of PTOX-induced alternative electron transport could be involved in a proton gradient activated mechanism of NPQ-enhancement that was shown by an accelerated rapid phase of NPQ accumulation in heavy metal stressed plants (Fig. 6). Other alternative electron transport sinks such as PS1 cyclic electron flow did not participate in the protection against heavy metal stress. The slower F_0' relaxation in HCS plants (data not shown) could be related to the lower rate of activation of NDH-dependent PS1 cyclic electron transport in these samples. The fast phase of reduction kinetics after switching off far-red light (reflecting the activity of FQR-dependent PS1 cyclic electron transport) was retarded in both HCS and MCS plants (Fig. 7, inset). These results suggested limited FQR-dependent PS1 cyclic electron transport. Relaxation kinetics reveals prevailing slow phase of A810/860 relaxation that reflects NDH-dependent PS1 cyclic electron transport and/or chlororespiration depending on environmental conditions (Peltier and Cournac 2002). Shirao et al. (2013) have recently suggested that the higher oxidation of P700 reflects accelerated PTOX activity. In the present study, enhanced P700 oxidation could be a result of a rise in PTOX activity. Thus, various types of PS1 cyclic electron transport could not be expected to participate in protection of the photosynthetic electron transport against deleterious effects of heavy metals. On the other hand, the residual presence of Q_A^- in the dark (Stefanov et al. 2012) indicated that

PTOX-dependent electron transport should not be considered insufficient for successful protection against over-reduction of the electron transport chain.

The main damages in PS2 could be related to inefficiency of the NPQ induced photoprotection during the late stages of the lag phase of photosynthesis. Thus, the steady state level of NPQ in HCS plants that was close to the control was not enough for effective photoprotection of PS2 (Figs. 5c, 6). Moreover, such a high level of NPQ was not reached during NPQ transient changes because of the visible decrease in NPQ during its second phase (Fig. 6). Our results showed that the main damages to the photosynthetic machinery due to heavy metal toxicity occurred in PS2. In HCS plants the main damages were found in the antenna (the F_0 increase suggested damages in PS2 antenna) (Fig. 4a). Besides, the lower fluorescence yield in HCS plants revealed decreased PS2 photochemical activity which appeared as a decrease in the apparent quantum yield (Fig. 5a). Thus, it could be suggested that PTOX-induced protection mechanisms alone could not ensure complete protection under heavy metal stress conditions.

One of the well established phytotoxic effects of heavy metals is the generation of ROS (Maksymiec 2007; Cuypers et al. 2011). These highly reactive molecules may cause oxidative damage to cellular components and at the same time they can act as important secondary messengers for inducing plant defense system which includes enzymatic and non-enzymatic scavengers of molecular species of active oxygen (Sytar et al. 2013). The responses of the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) or peroxidase (POD) can serve as indicators of oxidative damage caused by heavy metals. Literature data show that exposure of plants to high concentrations of redox active metals such as Cd and Cu results in oxidative damage (Cuypers et al. 2011). Accumulation of $O_2^{\cdot -}$ and H_2O_2 after short-term exposure to Cd and Cu ions was found in *Arabidopsis thaliana* (Maksymiec and Krupa 2006). The authors showed that only excess Cd induced SOD activity. Enhancement of SOD activity as a consequence of oxidative stress was found in wheat seedlings exposed to excess of Cd and Pb (Dey et al. 2007). The highest degree of enzyme activity enhancement was observed in the presence of Pb. The activity of SOD responsible for mitigating the oxidative stress was significantly enhanced in the leaves of sunflower plants after exposure to Co, Ni, Cd and Pb toxicity (Gopal and Khurana 2011). Zn is a non-redox metal but it can generate ROS indirectly, leading to defense responses including the induction of antioxidant enzymes such as SOD, CAT, and glutathione peroxidase (GPX) (Prasad et al. 1999; Chang et al. 2005). Our results showing enhancement of SOD activity in leaves and especially in roots only in plants grown in HCS (Fig. 8a, b) confirmed the role of this antioxidant enzyme in mitigating the oxidative damage caused by heavy metal toxicity.

Plant antioxidant defense system includes also non-enzymatic antioxidants such as ascorbate, glutathione, α-tocopherol, anthocyanins, carotenoids and phenolic compounds (flavonoids, tannins and lignin) (Sharma et al. 2012; Emamverdian et al. 2015). All these compounds can be easily oxidized.

Some of the biological molecules can be multifunctional and have antiradical, chelating or antioxidant activities. Phenolics and flavonoids are a large family of secondary metabolites that have been found to directly scavenge free radical ions (e.g. hydroxyl and superoxide anion) due to their ability to donate electrons or hydrogen atoms thus protecting plant cells from the adverse effects of abiotic stresses (Hernandez et al. 2009; Hossain et al. 2012). When plants are exposed to heavy metal stress, they can accumulate phenolics and flavonoids as an effective strategy against ROS formation (Michalak 2006; Okem et al. 2015). In the present study, the enhancement of total leaf antioxidant activity (Fig. 9a) accompanied with increased accumulation of flavonoids (Fig. 10) in the sunflower plants grown in HCS indicates the involvement of non-enzymatic antioxidants in the defense strategy operating in conditions of heavy metal stress. These metabolic changes reflect the ability of plants to modify metabolic processes by synthesizing and accumulating secondary metabolites with antioxidant activity when exposed to heavy metal toxicity (Michalak 2006; Okem et al. 2015).

In conclusion, the experimental approach used in the present work allowed us to study the response of plants under close to natural conditions of heavy metal pollution caused usually by the combined action of several heavy metals. The results showed that changes in thylakoid electron transport chain reactions accompanied with altered oxidative stress-related metabolic activity could be a mechanism to limit damages to the photosynthetic apparatus and protect plant cells against oxidative damage caused by combined heavy metal toxicity.

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