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Monitoring moderate Cu and Cd toxicity by chlorophyll fluorescence and P_{700} absorbance in pea leaves

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Abstract

We investigated the effect of moderate Cu^{2+} and Cd^{2+} stress by applying chlorophyll (Chl) fluorescence and P_{700} absorbance measurements to monitor the photosynthetic electron transport activity of 3-week-old *Pisum sativum* L. cv. Petit Provençal plants grown in a modified Hoagland solution containing 50 μM $CuSO_4$ or 5 μM $CdCl_2$. Both heavy metals caused a slight inhibition in PSII photochemistry as indicated by the decrease in the effective quantum efficiency of PSII (Φ_{PSII}), the maximum electron transport capacity (ETR_{max}), and the maximum quantum yield for electron transport (α). PSI photochemistry was also affected by these heavy metals. Cu^{2+} and Cd^{2+} decreased the quantum efficiency of PSI (Φ_{PSI}) as well as the number of electrons in the intersystem chain, and the Cu^{2+} treatment significantly reduced the number of electrons from stromal donors available for PSI. These results indicate that PSII and PSI photochemistry of pea plants are both sensitive to moderate Cu^{2+} and Cd^{2+} stress, which in turn is easily detected and monitored by Chl fluorescence and P_{700} absorbance measurements. Therefore, monitoring the photochemistry of pea plants with these noninvasive, yet sensitive techniques offers a promising strategy to study heavy metal toxicity in the environment.

Additional key words: Cd^{2+}; chlorophyll fluorescence; Cu^{2+}; heavy metal stress; P_{700} absorbance; photosynthetic electron transport; PSI photochemistry; PSII photochemistry.

Introduction

Heavy metals naturally occur in soils in trace amounts. Due to industrial and agricultural activities many of these compounds have gradually accumulated and reached toxic levels in our environment, thus now present increasing environmental and health risks (Hattab *et al.* 2009). Some of these metals, such as Cu, carry out important physiological functions, therefore they are indispensable for optimal plant development in small amounts, while other nonessential metals, like Cd, are potentially toxic even at low concentrations (Zhou *et al.* 2006). Toxicity symptoms caused by supraoptimal levels of heavy metals usually involve inhibition of plant growth, a decrease in photosynthetic activity and the induction of senescence (Hall 2002, Maksymiec 2007).

The photosynthetic apparatus, in particular, is a well-known target of heavy metals, including Cu and Cd. Uptake of these metals leads to a concentration-dependent loss of Chl and carotenoid pigments (Hattab *et al.* 2009), inhibition of Chl synthesis enzymes (Böddi *et al.* 1995) as well as Rubisco (Clijsters and Van Assche 1985), and alters the photosynthetic apparatus on the thylakoid and chloroplast level (Burzyński and Kłobus 2004, Maksymiec 2007). The primary Cu- and Cd-sensitive sites of the photosynthetic electron transport chain are the oxygen-evolving complex (OEC), NADP oxidoreductase and ATP-synthase (Van Assche and

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Abbreviations: AL – actinic light; Chl – chlorophyll; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ETR – apparent electron transport rate; ETR_{max} – maximum electron transport capacity; F_m – maximum fluorescence yield in the dark; F_m' – maximum fluorescence yield in the light-adapted state; F_o – minimum fluorescence yield in the dark; F_s – steady-state fluorescence yield during actinic illumination; F_v/F_m – maximum photochemical efficiency of PSII; MV – methyl viologen; NPQ – nonphotochemical quenching; OEC – oxygen-evolving complex; P_{700} – photosystem I reaction center; PPFD – photosynthetic photon flux density; PS – photosystem; α – maximum quantum yield for electron transport; Φ_{PSI} – quantum efficiency of PSI; Φ_{PSII} – effective quantum efficiency of PSII.
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Clijsters 1990). Inhibition of NADP oxidoreductase and ATP-synthase by Cu and Cd is explained by interaction of these metals with functional sulfhydryl groups found in these enzymes (Van Assche and Clijsters 1990). Through binding to crucial sites on donor and acceptor sides of PSII, Cd (Sigfridsson *et al.* 2004) and Cu (Jegerschöld *et al.* 1995, Yruela 2005) both inhibit electron transfer processes of PSII *in vitro*. Indeed, PSII rather than PSI is reported to be the main target of both Cd (Chugh and Sawhney 1999) and Cu (Ouzounidou *et al.* 1997). In *Chlamydomonas* and in isolated PSII, Cd, in the micromolar range, inhibits the photoactivation of PSII (that is, the assembly of the OEC) by competitive binding to the essential Ca^{2+} site in PSII (Faller *et al.* 2005). In addition, Cd decreases the Ca^{2+} content in pea plants by competitive binding to Ca transporters (Rodríguez-Serrano *et al.* 2009), and also tends to accumulate in chloroplasts (Van Assche and Clijsters 1990). Moreover, in experiments using epidermal strips, Cd has been shown to enter the cytosol of guard cells *via* Ca^{2+} channels and causes reduced stomatal opening (Perfus-Barbeoch *et al.* 2002), which also affects photosynthesis.

The majority of the described effects of Cd or Cu stress on photosynthetic electron transport were investigated *in vitro*, with only a limited number of *in vivo* studies, especially those directly addressing PSI electron transport. On the other hand, the targets of heavy metal action in the photosynthetic apparatus are in most cases studied using heavy metals in amounts that are well over the levels found even in polluted soils, which questions the ecophysiological relevance of these findings. In this study, we aimed to investigate the effect of environmentally relevant concentrations of Cd^{2+} and Cu^{2+} on the photosynthetic electron transport in pea plants using Chl fluorescence and P_{700} absorbance. Both methods are noninvasive, yet they provide direct information on the photochemical processes of PSII and PSI. In addition, any heavy-metal-induced changes detected in this study would indicate that monitoring the photosynthetic electron transport by using of these methods is a viable approach to study heavy metal toxicity in plants in future ecophysiological studies.

Materials and methods

Plant material and experimental solutions: Sterilized seeds of pea (*P. sativum* L. cv. Petit Provençal) were germinated for 3 d at 24°C, and the seedlings were grown in a semicontrolled growth chamber at a relative humidity of 55–60% for 3 weeks under a 12-h-light [150 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD)]/ 12-h-dark cycle and temperature of 22°C in a modified Hoagland solution containing: 2 mM $Ca(NO_3)_2$, 1 mM Mg SO_4, 0.5 mM KCl, 0.5 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 1 μM $MnSO_4$, 5 μM $ZnSO_4$, 0.1 μM $(NH_4)_6MO_7O_{24}$, 10 μM H_3BO_4, 0.1 μM $AlCl_3$ and 20 μM Fe-EDTA. For Cu and Cd treatments this solution was complemented with 50 μM $CuSO_4$ or 5 μM $CdCl_2$, respectively. Leaf disks of the youngest fully expanded leaves were prepared by a 15 mm diameter leaf punch and used for each measurement. Some experiments were performed in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and methyl viologen (MV), which were applied by vacuum infiltrating leaf disks with 200 μM DCMU, or 600 μM MV (or both) in the dark prior to measurements. All chemicals were purchased from *Sigma-Aldrich* (Hungary).

Pigment analysis: The youngest fully expanded leaves were homogenized in ice cold 100% (v/v) acetone (1.5 mL for a 250-mg sample) and extracted for 24 h. Samples were centrifuged at 5,000 × *g* for 15 min at 4°C. The pellet was extracted again with 80% (v/v) acetone (1.5 mL for a 250-mg sample) for 24 h. After centrifugation (5,000 × *g*, 15 min, 4°C), the supernatants were collected. The pigment composition was measured with a double-beam spectrophotometer using the method of Lichtenthaler and Wellburn (1983). This method involves measurement of the light absorbed in the plant extract at 470, 646.8, and 663.2 nm.

Chl fluorescence measurements: PSII Chl fluorescence of pea leaves was monitored with PAM fluorometers (*PAM-101* and *PAM-2000*, *Heinz Walz GmbH*, Effeltrich, Germany). Leaf disks were dark-adapted for at least 15 min for determination of minimum and maximum fluorescence yield in the dark (F_o and F_m, respectively). F_m was obtained by exposing the leaf sample to a high intensity (8,000 μmol m^{-2} s^{-1} PPFD) short pulse (0.8 s). Maximum photochemical efficiency of PSII (F_v/F_m) was calculated according to Kitajima and Butler (1975) where F_v equals $F_m - F_o$. Maximum fluorescence yield in the light-adapted state (F_m') was determined at the end of a 5-min white actinic light (AL) illumination of 220 μmol m^{-2} s^{-1} PPFD. The effective quantum efficiency of PSII ($\Phi_{PSII} = \Delta F/F_m'$) measured in the light-adapted state was determined according to Genty *et al.* (1989) where ΔF equals $F_m' - F_s$ and F_s is the steady-state fluorescence yield during actinic illumination. Steady-state nonphotochemical quenching (NPQ) was calculated as: $NPQ = (F_m - F_m')/F_m'$ (Bilger and Björkman 1990). The apparent electron transport rate (ETR) values were determined at 10 different light intensities (18, 34, 50, 120, 180, 420, 640; 1,300; and 2,600 μmol m^{-2} s^{-1} PPFD white AL) after Schreiber (2004) by multiplying $\Phi_{PSII} \times PPFD \times 0.5$ (two photons are used to release one electron, as we have assumed equal excitation between PSII and PSI) and × 0.84, which is considered the most common leaf absorbance coefficient for C_3 plants (Flexas *et al.* 1999). Light-response curves were obtained by plotting the ETR values as a function of light intensity. The maximum

electron transport capacity (ETR_{max}) was calculated according to Rascher *et al.* (2000) by fitting the light-response curves with the following function: $ETR = ETR_{max} [1 - e^{-kQ}]$, where k is fitting constant and Q is light intensity (PPFD). The maximum quantum yield for electron transport (α) was defined as the slope of the initial, linear part of the light-response curves (Schreiber 2004).

P_{700} absorbance measurements: The redox changes of P_{700} were monitored as the light-induced changes in absorption of the P_{700}^+ radical measured with a PAM fluorometer (*PAM-101, Heinz Walz GmbH*, Germany) equipped with a dual-wavelength emitter-detector unit (*ED-P700DW, Heinz Walz GmbH*). The difference signal is obtained from absorbance of measuring lights peaking at 810 and 870 nm. This ensures high selectivity for P_{700} absorbance by minimizing all nonspecific signal changes due to plastocyanin and light scattering. The signal was recorded with a computer connected to the PAM Data Acquisition System (*PDA-100, Heinz Walz GmbH*) and the sampling rate was set to 10 ms point^{-1}.

Leaf discs were illuminated by far-red light (FR, 735 ± 40 nm, 90 μmol m^{-2} s^{-1} PPFD, *102-FR, Walz*), actinic white light (AL, 220 μmol m^{-2} s^{-1} PPFD, *KL 1500 LCD, Schott,* Mainz, Germany) and saturating (> 8,000 μmol m^{-2} s^{-1} PPFD) single-turnover (ST, peak width at half-maximal intensity = 1.5 μs, *XE-STL/XE-STC, Heinz Walz GmbH*) and multiple turnover (MT, peak width = 50 ms, *XF-103/XMT-103, Heinz Walz GmbH*) flashes following a cycle of illumination described previously (Wodala and Horváth 2008) The quantum efficiency of PSI (Φ_{PSI}) was calculated according to Klughammer and Schreiber (1994) after a 5-min white AL illumination of 220 μmol m^{-2} s^{-1} PPFD. The pool size of electrons in the inter-system chain and the number of electrons from stromal donors per reaction center were determined as previously (Wodala and Horváth 2008) following Asada *et al.* (1992). Pathways of electron flow to PSI during steady-state illumination were dissected by fitting the rereduction kinetics of P_{700}^+ with three exponentials following Chow and Hope (2004).

Statistical analysis: Each experiment was repeated at least six times. Values are expressed as means ± SD. The statistical significance of differences between means of control and Cu- or Cd-treated samples was determined using the Student's *t*-test and significance levels are indicated by asterisks: $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

Results

To assess the effect of a mild Cu^{2+} and Cd^{2+} treatment on plant growth and senescence we measured the shoot length and root length of 3-week-old pea plants, and determined the Chl content of leaves. Treatment with either 50 μM $CuSO_4$ or 5 μM $CdCl_2$ led to a significant inhibition shoot as well as root growth (Fig. 1*A,B*), however Cu^{2+}- as well as Cd^{2+}-treated plants had a significantly higher Chl (*a* + *b*) content (Fig. 1*C*). This strongly suggests that despite the stunted growth neither of the heavy metals led to senescence within the 3-week period of the experiments.

As PSII is one of the primary targets of heavy-metal stress, we applied Chl fluorescence measurements in order to assess the level of stress exerted by 50 μM $CuSO_4$ and 5 μM $CdCl_2$ on fluorescence quenching parameters F_v/F_m, Φ_{PSII} and NPQ (Fig. 2). Although statistically significant in some cases, growing pea plants in 50 μM $CuSO_4$ and 5 μM $CdCl_2$ caused no considerable changes in F_v/F_m and NPQ values of leaf disks. However, the small yet distinct reduction in Φ_{PSII} suggests that Cu^{2+} and Cd^{2+} slightly inhibited PSII photochemistry.

The heavy metal-induced slight reduction in PSII photochemistry is reflected in ETR values and becomes more pronounced as light intensites increase (Fig. 3*A*). Decreased ETR_{max} and especially α values (Fig. 3*B,C*) indicate that Cu^{2+} and Cd^{2+}, even at low concentrations, moderately reduce the maximum yield as well as the maximum capacity of PSII electron production.

Hampered electron output by PSII implies that fewer electrons enter the electron transport chain, which in turn may cause a reduction in PSI photochemistry. Fig. 4 shows that Cu^{2+} and Cd^{2+} both slightly reduce Φ_{PSI} as well as the number of electrons per reaction center in the intersystem chain. Moreover, the Cu^{2+} treatment also leads to a considerable reduction in the number of electrons from stromal donors per reaction center, which correlates well with the fact that Φ_{PSI} values of Cu^{2+}-treated leaf disks are slightly lower than those treated with Cd^{2+} (Fig. 4*A*). The number of electrons from stromal donors in Cu^{2+}-treated plants is in fact well under the numbers estimated in Cd^{2+}-treated plants – let alone untreated plants (Fig. 4*C*). This might be due to the fact that unlike Cd, Cu is a redox-active metal, thus may directly oxidize potential electron donors in the chloroplast stroma. In addition, Cd and Cu may both trigger ROS production in chloroplasts (Sharma and Dietz 2009), which also leads to a reduced number of electrons from stromal donors available for the electron transport chain.

Analysis of the post-illumination kinetics of P_{700} absorbance is a powerful tool to monitor the different electron transport pathways toward PSI. According to Chow and Hope (2004), the rereduction kinetics of P_{700} absorbance following far-red illumination are best fitted by three exponentials, which correspond to three different sources of electron flow to PSI. The fast phase, which is inhibited by DCMU, is attributed to electron flow to P_{700}^+ from PSII. The intermediate phase, which is partially

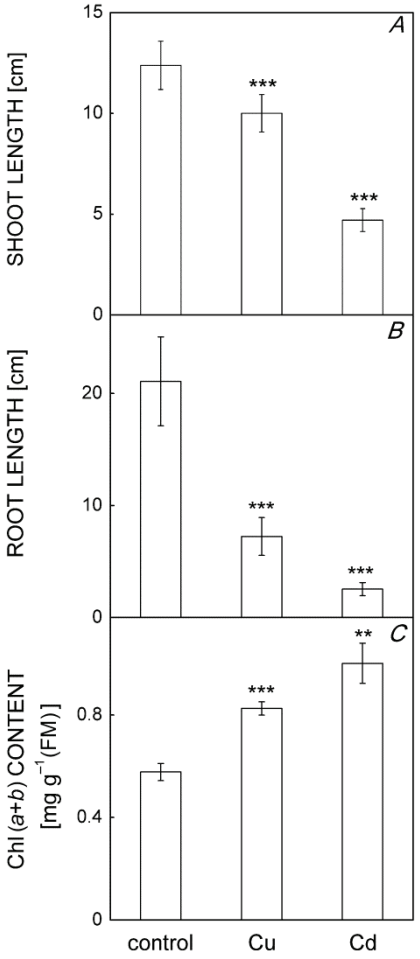

Fig. 1. The effect of Cu^{2+} and Cd^{2+} on (*A*) shoot length, (*B*) root length and (C) total chlorophyll content [Chl (*a*+*b*)] of leaves. Plants were grown in a modified Hoagland solution (control) complemented with 50 µM $CuSO_4$ (Cu), or 5 µM $CdCl_2$ (Cd). Bars represent means ± SD ($n \geq 6$). Asterisks indicate levels of significance by Student *t*-test: ** $P<0.01$ and *** $P<0.001$.

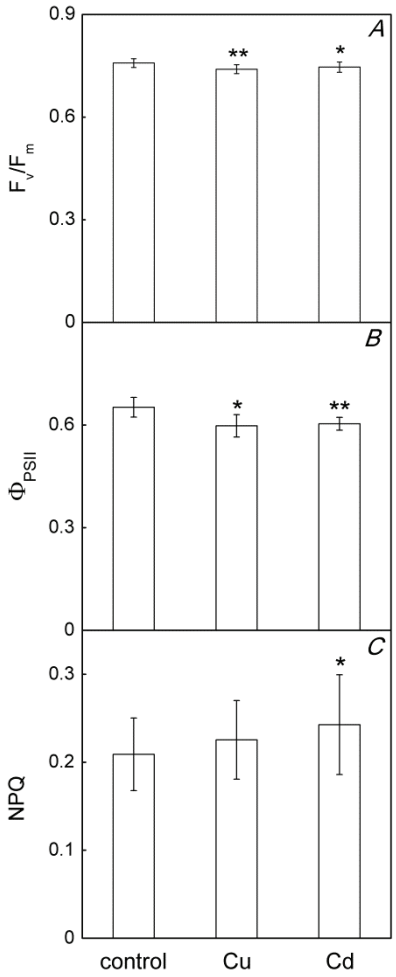

Fig. 2. The effect of Cu^{2+} and Cd^{2+} on maximum photochemical efficiency of PSII (F_v/F_m, *A*), effective quantum efficiency of PSII (Φ_{PSII}, *B*) and nonphotochemical quenching (NPQ, *C*) parameters of leaf disks from plants grown as described in Fig. 1. Φ_{PSII} and NPQ values were determined after a 5-min 220 µmol m^{-2} s^{-1} PPFD illumination with white actinic light. Bars represent means ± SD ($n \geq 6$). Asterisks indicate levels of significance by Student *t*-test: * $P<0.05$ and ** $P<0.01$.

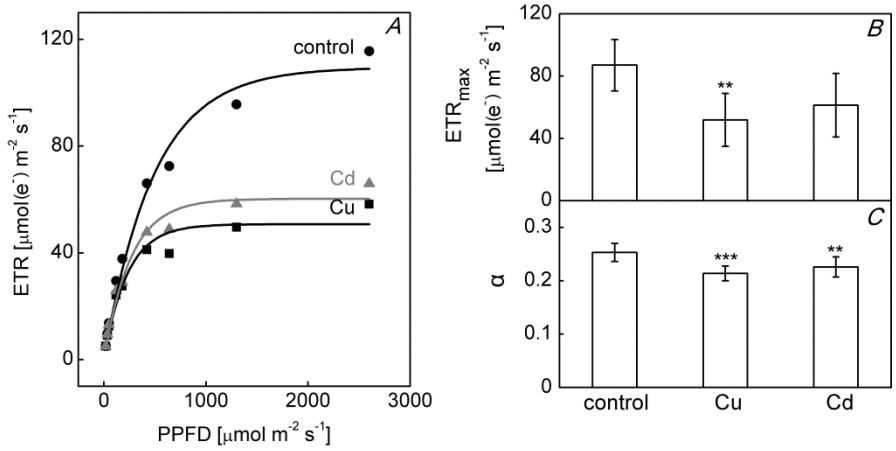

Fig. 3. The effect of Cu^{2+} and Cd^{2+} on leaf disks from plants grown as described in Fig 1. (*A*) light-response curves of control (●), Cu^{2+} (■)- and Cd^{2+} (▲)-treated samples, *solid lines* indicate fitting with an exponential function $ETR = ETR_{max} [1 - e^{-kQ}]$, where k is fitting constant and Q is light intensity (PPFD), which yielded maximum electron transport capacity (ETR_{max}, *B*) and maximum quantum yield for electron transport (α, *C*) values. Bars represent means ± SD ($n \geq 6$). Asterisks indicate levels of significance by Student *t*-test: ** $P<0.01$ and *** $P<0.001$.

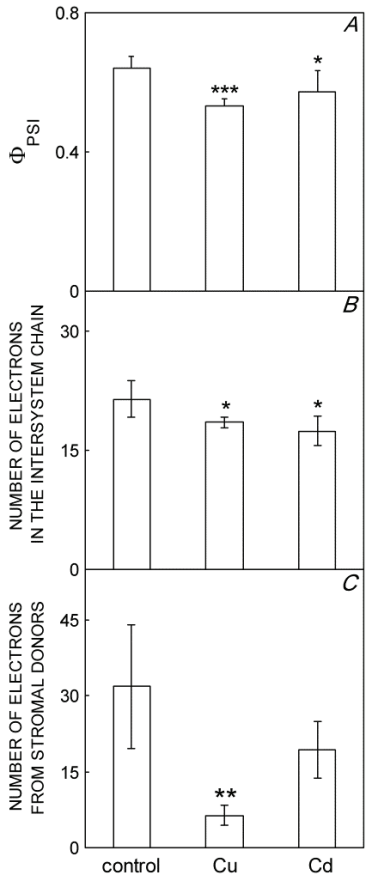

Fig. 4. The effect of Cu^{2+} and Cd^{2+} on quantum efficiency of PSI (Φ_{PSI}, *A*), the number of electrons in the intersystem chain per reaction center (*B*) and the number of electrons per reaction center from stromal donors (*C*) of leaf disks from plants grown as described in Fig 1. Φ_{PSI} values were determined after a 5-min 220 μmol m^{-2} s^{-1} PPFD illumination with white actinic light. Bars represent means ± SD ($n \geq 6$). Asterisks indicate levels of significance by Student *t*-test: * – $P<0.05$, ** – $P<0.01$ and *** – $P<0.001$.

Discussion

The aim of this study was to study the effect of a moderate Cu^{2+} and Cd^{2+} stress on the photosynthetic electron transport in pea leaves by directly monitoring the photosynthetic activity of PSII *via* Chl fluorescence and PSI *via* P_{700} absorbance measurements. Fig. 1 shows that low amounts of Cu^{2+} and Cd^{2+} already inhibit the growth of pea plants, but Chl levels, which are well above the levels measured in untreated leaves, indicate that neither of these heavy metals induces considerable senescence within the 3-week period of the experiments. PSII is one of the primary targets of Cu and Cd stress and Chl fluorescence is a widely used – and accepted – technique to monitor PSII photochemistry, so as the next step, we applied Chl fluorescence measurements to assess the effect of the mild Cu^{2+} and Cd^{2+} stress applied in this study. Growing pea plants in the presence of these metals resulted in a moderate but well-defined inhibition in photosynthetic performance: decreasing Φ_{PSII} and ETR values show that even these small amounts of Cu^{2+} and Cd^{2+} cause a reduction of electron output of PSII, which is in good agreement with earlier studies (Burzyński and Kłobus 2004, Hattab *et al.* 2009).

PSI is reported to be less sensitive to Cu and Cd; however, apart from a few exceptions (*e.g.* Ouzounidou *et al.* 1997), previous studies mostly used various artificial electron donors and inhibitors to isolate and study PSI activity (Chugh and Sawhney 1999, Zhou *et al.* 2006) under heavy-metal stress, which may themselves influence PSI activity. It is for these reasons that in this study we applied P_{700} absorbance measurements, which allow direct, noninvasive monitoring of PSI photochemistry in intact leaves without the need for articifial electron donors or inhibitors. Our results clearly indicate that Cu^{2+} and Cd^{2+} both decreased PSI photochemistry, blocked by MV, corresponds at least partly to cyclic electron transport: electron donation from PSI-reduced ferredoxin to the intersystem pool – and ultimately to PSI. Finally, the slow phase represents electron flow to PSI from stromal reductants. To test this argument we treated pea leaf disks with saturating concentrations of DCMU, MV, or both. Fig. 5*A* shows a typical P_{700} rereduction trace of a control sample with a three-exponential fit and Fig. 5*B* shows the averaged traces of control leaf disks and disks treated with DCMU, MV or both. Rereduction traces of untreated samples could be fitted well using three exponential components, DCMU- or MV-treated disks could be best fitted with two exponentials, and a single exponential yielded a good fit with samples treated with both inhibitors (Table 1). These data provide further evidence in support of three routes of electron transport contributing to the reduction of PSI reaction centres, which can thus be dissected using mathematical methods.

The Cu^{2+} and Cd^{2+} treatment caused only minor alterations in the electron transport pathways leading to PSI (Fig. 6*A*). The slightly decreased amplitude values (Fig. 6*B*) and unchanged time constants of the fast phase (Fig. 6*C*) indicate a small reduction in the linear electron flow to PSI, which is in good correlation with the moderately decreased Φ_{PSII} and electron transport capacity of PSII. Interestingly, the slight but statistically significant increase in the amplitude of the slow phase in Cu^{2+}-treated plants would suggest a greater volume of electrons from stromal donors (Fig. 6*B*); however, the corresponding time constant is remarkably slower (Fig. 6*C*), which suggests that Cu^{2+} ultimately led to a slower rate of electron transport from stromal donors to PSI. This finding correlates well with the Cu^{2+}-induced reduction of electrons from stromal donors (Fig. 4*B*). The same trend occurs in Cd^{2+}-treated plants, but the changes remain below the threshold of significance.

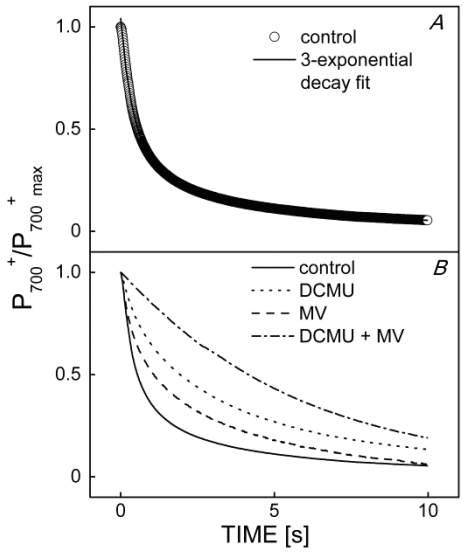

Fig. 5. Rereduction kinetics of P_{700} absorbance following cessation of illumination with FR light. (*A*) a three-exponential decay fit for the average of control traces and (*B*) average traces measured in control leaf disks and disks infiltrated with 200 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 600 μM methyl viologen (MV), or both (DCMU + MV) ($n \geq 4$). Leaf disks were taken from plants grown as described in Fig 1.

which was further hampered by the Cu^{2+}-induced decrease in electron flow to PSI from stromal donors. Asada *et al.* (1992) list peripheral ferredoxin and pyridine nucleotides, primarily NADH and NADPH as the potential source of electrons from the stroma. The pool of triose phosphates in the stroma may also supply electrons *via* these pyridine nucleotides (Asada *et al.* 1992). Chow and Hope (2004) suggest that electrons from pyridine nucleotides may then be channeled into the plastoquinone pool by NAD(P)H dehydrogenase, and also mention ascorbate as an additional potential electron source. Cu^{2+} may indeed decrease the number of electrons from stromal donors available for PSI by directly reducing stromal donors, thus competing with this slow pathway of electron flow.

A moderate inhibition of PSII and especially PSI photochemistry ultimately results in a reduced number of electrons for CO_2 fixation and other assimilation processes, which probably contributes to inhibition of growth (Fig. 1*A,B*) and other heavy metal stress-induced symptoms (Maksymiec 2007).

It is important to note that $CdCl_2$ and $CuSO_4$ already damaged photosynthetic electron transport at relatively low concentrations of 5 μM and 50 μM, respectively. This indicates that pea plants are remarkably sensitive

Table 1. Decay kinetics of P_{700} absorbance traces in Fig. 5*B*. Traces measured in untreated (control) leaf disks were fitted with three exponentials. Leaf disks infiltrated with either 200 μM DCMU, or 600 μM MV were fitted with two exponential components, while leaf disks infiltrated with both chemicals were fitted with a single exponential component. Values in parentheses are relative amplitudes as a percentage of total P_{700} absorbance. Values represent means ± SD of calculated parameters and R^2 values of each fit ($n \geq 4$). τ_1, τ_2, τ_3 – time constants; A_1, A_2, A_3 – amplitude values. (The numbers 1, 2 and 3 in subscript indicate fast, middle, and slow phases).

Treatment	Fast Phase τ_1 [s] (A_1 [%])	Intermediate Phase τ_2 [s] (A_2 [%])	Slow Phase τ_3 [s] (A_3 [%])	R^2
control	0.28 ± 0.11 (34 ± 8.4)	1.37 ± 0.45 (31 ± 5.3)	7.63 ± 1.72 (19 ± 5.8)	0.999 ± 0.000
DCMU	- (0)	0.71 ± 0.32 (58 ± 19)	5.28 ± 4.48 (50 ± 13)	0.999 ± 0.000
MV	0.56 ± 0.16 (44 ± 11)	- (0)	3.75 ± 0.47 (61 ± 13)	0.999 ± 0.000
DCMU + MV	- (0)	- (0)	4.02 ± 1.81 (109 ± 14)	0.997 ± 0.003

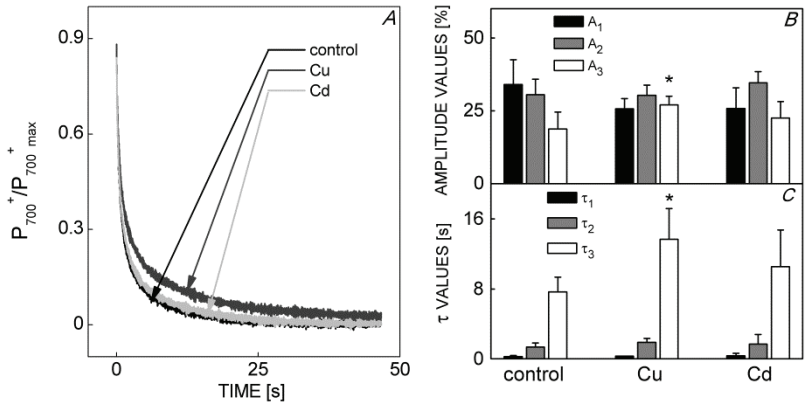

Fig. 6. Rereduction kinetics of P_{700} absorbance measured in leaf disks from plants grown as described in Fig 1. (*A*) average traces. The amplitude values – A_1, A_2, and A_3 (*B*), and time constants – τ_1, τ_2 and τ_3 (*C*) – were obtained by fitting traces with three exponential components. (The numbers 1, 2 and 3 in subscript indicate fast, middle, and slow phases). Bars represent means ± SD ($n \geq 6$). Asterisks indicate the level of significance by Student *t*-test: * – $P<0.05$.

to the presence of heavy metals, which make them suitable candidates for biomonitoring heavy metal toxicity in the environment. On the other hand, PAM fluorometry and especially P_{700} absorbance – two noninvasive, yet photosynthetically informative measuring techniques – detected the photochemical changes induced by Cu^{2+} and Cd^{2+} with appreciable sensitivity. These findings indicate that both methods are indeed viable tools to study heavy metal toxicity in photosynthetic organisms. Moreover, P_{700} absorbance measurements may well provide additional *in vivo* data to expand our knowledge on the effect of heavy metals on PSI photochemistry in intact plants.

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