Multiple Effects of Cadmium on the Photosynthetic Apparatus of *Avicennia germinans* L. as Probed by OJIP Chlorophyll Fluorescence Measurements

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The toxic effects of cadmium on the photosynthetic apparatus of *Avicennia germinans* were evaluated by means of the chlorophyll fluorescence transient O–J–I–P. The chlorophyll fluorescence transients were recorded *in vivo* with high time resolution and analyzed according to the OJIP-test that can quantify the performance of photosystem II. Cadmium-treated plants showed a decrease in yield for primary photochemistry, TR_0/ABS. The performance index of photosystem II (PSII), PI_{ABS}, decreased due to cadmium treatment. This performance index is the combination of the indexes of three independent parameters: (1) total number of active reaction centers per absorption (RC/ABS), (2) yield of primary photochemistry (TR_0/ABS), and (3) efficiency with which a trapped exciton can move an electron into the electron transport chain (ET_0/TR_0). Additionally, the F_0/F_v registered the highest sensitivity to the metal, thus indicating that the water-splitting apparatus of the oxidizing side of PSII is the primary site of action of cadmium. In summary, cadmium affects several targets of photosystem II. More specifically the main targets of cadmium, according to the OJIP-test, can be listed as a decrease in the number of active reaction centers and damage to the activity of the water-splitting complex.

Key words: Avicennia germinans, Chlorophyll Fluorescence, Heavy Metals, Photosystem II

Introduction

The mangrove ecosystems, although possessing enormous ecological and commercial importance, are often subjected to effluent discharges, urban and agricultural runoff and solid waste dumping, due to their proximity to urban development. Among the main anthropogenic impacts in mangrove systems from these sources are heavy metals, due to their affinity to and immobilization within anaerobic sediments (Cuong *et al*., 2005; Defew *et al*., 2005). For example, cadmium often occurs in high contents in mangrove forests, due to its prevalence in mangrove sediments, up to 200 mg/kg (Nascimento *et al*., 2006). Mangroves are important primary producers in estuarine systems, and appear to possess a tolerance to high levels of heavy metal pollution. For instance several researchers have found high concentrations of accumulated metals in the tissues of a variety of mangrove species, *Avicennia* spp., *Kandelia* spp., and *Rhizophora* spp. (Peters *et al*., 1997; De Lacerda, 1998; MacFarlane *et al*., 2003).

Although considered tolerant, uptake of heavy metals in excess by mangroves can induce a disruption of many physiological functions which can cause damage at the cellular level and effects on the photosynthetic activity and respiration (Meharg, 1994; Boucher and Carpentier, 1999). In recent years chlorophyll (Chl) fluorescence meanly quantum yield of photosystem II (F_v/F_m) has been used in mangrove species to evaluate their response to exposure to diverse xenobiotic agents (MacFarlane, 2003; Bell and Duke, 2005). Variable Chl fluorescence is related to the redox state of the electron acceptor (plastoquinone A, Q_A) and can be used as a probe to study photosynthetic

Abbreviations: Chl, chlorophyll; F_0, F_m, minimum and maximum dark-adapted fluorescence yield; F_v/F_m, quantum yield of photosystem II photochemistry in the dark-adapted state; OJIP-test, analysis of parameters derived from the fluorescence transient; PSII, photosystem II; Q_A, plastoquinone A carrier of electrons from P680; Q_B, plastoquinone B carrier of electrons from Q_A.

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activity (Krause and Weiss, 1991). The change in fluorescence from minimum (F_0) to maximum (F_m) dark-adapted fluorescence is the variable component. The ratio of F_v/F_m is highly correlated with the quantum yield of net photosynthesis of intact leaves and usually falls between the values of 0.75 and 0.85 (Bolhar-Nordenkampf *et al.*, 1989; Jones *et al.,* 1999).

Additionally, advances in Chl fluorescence measuring techniques had allowed the development of the OJIP-test as a tool for rapid screening of many samples *in vivo*, providing adequate and detailed information about the structure, conformation, and function of the photosynthetic apparatus (Strasser and Strasser, 1995). Therefore, the study presented in this paper was undertaken to provide a first-hand *in vivo* measurement of the impact and the mode of action of Cd^{2+} on the photosynthetic apparatus of *Avicennia germinans* (black mangle).

Materials and Methods

Field collection and germination of plant material

Black mangle seeds were collected from native populations from Chabihau Bay, Yucatan, Mexico, and transported to the laboratory for further examination. The disinfection process was achieved by sequential steps starting with 1% NaOCl (Clorox) for 5 min, followed by a rinse with deionized sterile water. The propagules were then peeled with a sterile scalpel and the pericarp discarded. The naked propagules were further disinfected with 0.1% NaOCl and finally rinsed with deionized sterile water six times. All these steps were carried out under sterile conditions. On the other hand, 150 propagules were germinated in sand (grain size 3–6 mm) inside a greenhouse under the following conditions: temperature range 28–35 °C during the day, 24–26 °C during the night, 12-h light/dark photoperiods and 60% relative air humidity.

Cadmium exposure

Twenty individual, three-month-old *Avicennia germinans* seedlings were randomly allocated (n = 4) and transferred to individual plastic containers holding 500 ml Hoagland's solution prepared with 0.054 and 0.267 M cadmium chloride ($CdCl_2$). Control plants were transferred to plastic containers with 500 ml Hoagland's solution without $CdCl_2$. Treated and control plants were exposed during a period of 30 h to hydroponics conditions. These exposures were performed in quadruplicates. Treatments started at 9 a. m. and the measures taken at 16 h were made during the dark period.

Gas exchange

The net photosynthetic rate (P_n), and stomatal conductance (g_s) were measured at 4, 8, 16, 24 and 30 h after exposure to treatments with heavy metal using a portable infrared gas analyzer (LI-Cor Model 6200, Lincoln, NE, USA). It was equipped with a clamp-on leaf cuvette that exposed 6 cm^2 of leaf area. Light, temperature and humidity were 400 μmol m^{-2} s^{-1}, (23 ± 1) °C and 60%, respectively. The measurements were made on four randomly selected plants of each treatment.

Chlorophyll fluorescence

The determination of chlorophyll fluorescence was carried out using a portable fluorometer (Plant Efficiency Analyzer-MK2–9600-Hansatech, Norfolk, UK) on completely expanded leaves of appropriate phytosanitary condition. The data were collected at 4, 8, 16, 24 and 30 h after exposure to heavy metals using 4 plants per treatment. The selected leaves were subjected to a 5 min period of adaptation to darkness, sufficient for complete oxidation of the reaction centers. Induction kinetic curves were recorded for period of 2 s at actinic irradiance of 2100 μmol m^{-2} s^{-1} and data obtained were processed using the Biolyzer 2.5 software (Maldonado-Rodriguez, 2002). Photosynthetic activity was monitored by Chl fluorescence parameters provided by the OJIP-test (Strasser and Strasser, 1995; Strasser *et al.*, 2000).

Analysis of fluorescence: the OJIP-test

The OJIP-test was performed on all measured fluorescence transients and is based on a simple model of how photon flux absorbed by the photosynthetic antenna pigments (ABS^0) is dissipated as heat (DI^0) and fluorescence, or channeled as trapping flux (TR^0) to the reaction centers to be converted to redox energy by reducing plastoquinone (Q_A to Q_{A-}). Q_{A-} is then re-oxidized to Q_A and creates electron transport (ET) that leads to CO_2 fixation (Strasser *et al.*, 2000). These fluxes are expressed as specific energy fluxes (per reaction center) or as proportions of flux ratios or

Table I. Summary of the OJIP-test formulae using data extracted from the fast Chl fluorescence transient. ABS, absorption energy flux; CS, excited cross section of leaf sample; DI, dissipation energy flux at the level of the antenna chloophylls; ET, flux of electrons from Q_{A-} into the electron transport chain; ET_0/ABS, probability that an absorbed photon will move an electron into electron transport further than Q_{A-}; TR_0/ABS, maximum quantum yield of primary photochemistry; PI, performance index; ET_0/TR_0, efficiency by which a trapped exciton, having triggered the reduction of Q_A to Q_{A-}, can move an electron further than Q_{A-}, into the electron transport chain; RC, reaction center of PSII; RC/CS, concentration of reaction center per excited cross section of a given leaf area; TR, excitation energy flux trapped by a RC and utilized for the reduction of Q_A to Q_{A-}.

Technical fluorescence parameters	
Quantum efficiencies	
TR_0/ABS	$= F_v/F_m$
ET_0/ABS	$= F_v/F_m * (1 - V_J)$
ET_0/TR_0	$= (1 - V_J)$
Specific fluxes	
ABS/RC	$= M_0 * (1/V_J) * [1/(TR_0/ABS)]$
TR_0/RC	$= M_0 * (1/V_J)$
ET_0/RC	$= M_0 * (1/VJ) * (ET_0/TR_0)$
DI_0/RC	$= (ABS/RC) - (TR_0/RC)$
Phenomenological fluxes	
ABS/CS_0	$= ABS/CS_{Chl} = Chl/CS_0$ or $ABS/CS_0 = F_0$ or $ABS/CS_m = F_m$
TR_0/CS_0	$= ABS/CS_0 * TR_0/ABS$
ET_0/CS_0	$= ABS/CS_0 * (TR_0/ABS) * (ET_0/TR_0)$
Density of PS II reaction center	
RC/CS_0	$= (ET_0/TR_0) * (V_J/M_0) * ABS/CS_0$
RC/CS_m	$= (ET_0/TR_0) * (V_J/M_0) * ABS/CS_m$
Performance indexes	
PI_{ABS}	$= (RC/ABS) * [(ET_0/ TR_0)/ 1 - (ET_0/TR_0)] * [ET_0/ABS/ 1 - (ET_0/TR_0)]$
PI_{CS_0}	$= (ABS/CS_0) * [(ET_0/ TR_0)/ 1 - (ET_0/TR_0)] * [ET_0/ABS/ 1 - (ET_0/TR_0]$
PI_{CS_m}	$= (ABS/CS_m) * [(ET_0/ TR_0)/ 1 - (ET_0/TR_0)] * [ET_0/ABS/ 1 - (ET_0/TR_0)]$

yields. Fluorescence values at time intervals corresponding to the steps O–J–P were recorded and used as original data in the OJIP-test including: the maximum fluorescence intensity (F_m), the fluorescence intensity at 50 μs (F_0), 300 μs, and 2 ms (F_J). The derivation of specific energy fluxes and flux ratios have been comprehensively explained by Strasser and co-workers (Strasser and Strasser, 1995). Table I lists all OJIP-test parameters, their biophysical or biochemical meanings and how they are calculated from original data (F_m, F_0, and F_J) extracted from fluorescence transients. In addition to the specific energy fluxes and flux ratios or yields (presented in this paper), it is possible to calculate phenomenological energy fluxes (expressed per leaf cross section) and various other vitality indices (Strasser *et al.*, 2000). The F_v/F_m, F_v/F_0 and F_0/F_v ratios were calculated from the measurement of F_v, F_m and F_0. The ratio of variable fluorescence to maximal fluorescence (F_v/F_m) is an indicator of the efficiency of the photosynthetic apparatus, while the ratio of variable fluorescence to unquenchable portion of fluorescence (F_v/F_0) is an indicator of the size and the

number of active photosynthetic reaction centers, and F_0/F_v represents the efficiency of the water-splitting apparatus (Kriedemann *et al.*, 1985).

Statistical analysis

All data presented are the mean values. Statistical analysis was carried out by one-way ANOVA using Student's *t*-test to test the significance of the difference between means. Means were considered significantly different for $p \leq 0.05$.

Results and Discussion

Analysis of chlorophyll fast fluorescence transients

Fig. 1 shows the polyphasic rise of Chl fluorescence transients determined from control plants and from plants treated for 30 h with 0.054 and 0.267 M Cd^{2+}. No differences were found between control plants and Cd^{2+}-treated plants during the first stage (O). However, significant reduced fluorescence levels were found in Cd^{2+}-treated plants with both concentrations throughout the stages J, I and P compared to control plants. Plants exposed to the highest Cd^{2+}concentrations had the lowest fluorescence values at the end of the P stage.

Changes of chlorophyll fluorescence parameters and OJIP-test

In the case of F_0/F_v, values for control plants dropped from 4 to 8 h followed by a progressive rise from 8 to 24 h towards the end of the dark hours, and finally drop again the following day to values similar found the previous day (Fig. 2a). On the contrary Cd^{2+}-treated plants also showed diurnal fluctuations during the first 24 h but after 30 h of exposure to the metal, significantly higher F_0/F_v values were recorded in treated plants relative to those of control plants (Fig. 2a).

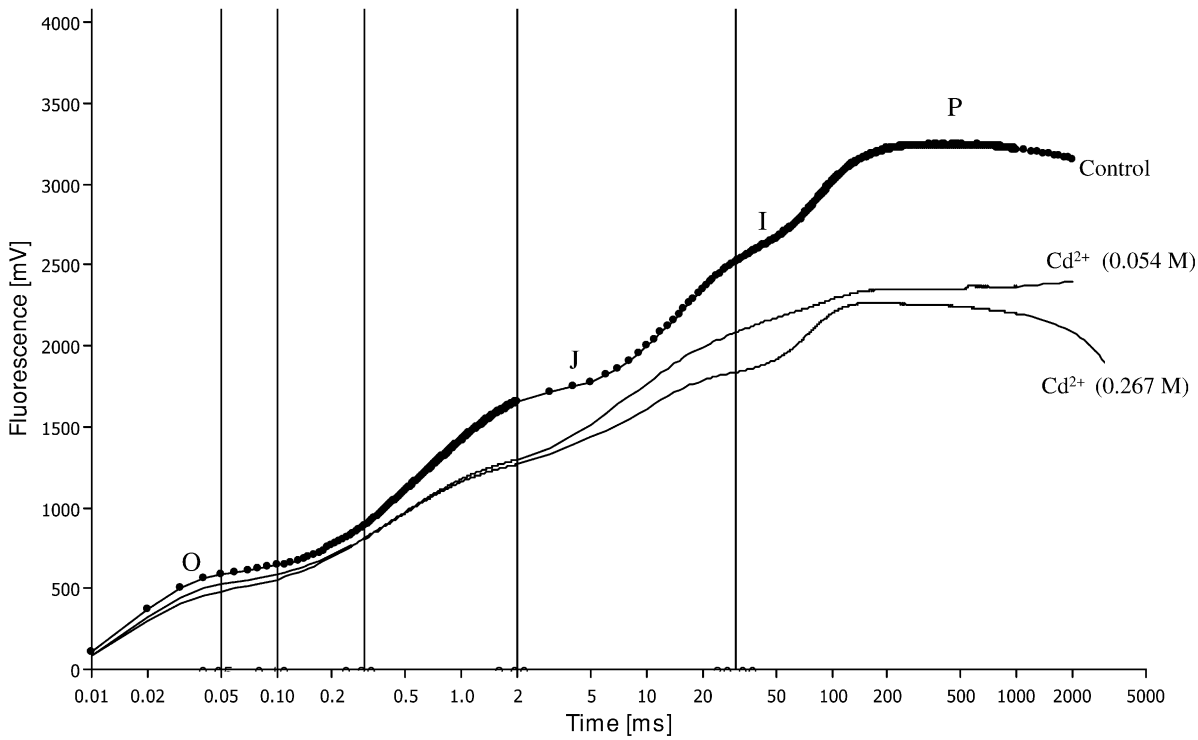

Fig. 1. Polyphasic Chl fluorescence transients of untreated plants (control) of *A. germinans* or plants after 30 h exposure to 0.054 and 0.267 M Cd^{2+}, respectively. Leaves were dark-adapted for 5 min. The vertical lines represent the fluorescence intensity at particular time spans. The first, second, third, fourth, and fifth lines (left to right) represent the fluorescence intensities at 50 μs, 100 μs, 300 μs, 2 ms, and 30 ms, respectively. The point where the fluorescence curve meets at 50 μs, 2 ms, and 30 ms are known as O-, J- and I-phase, respectively. The highest peak in the curve represents F_m (P).

In the case of F_v/F_m and F_v/F_0, values for control plants rose slightly from 4 to 8 h followed by a progressive drop from 8 to 24 h towards the end of the dark hours, and finally rise again the following day to values similar found the previous day (Figs. 2b and c). On the contrary, Cd^{2+}-treated plants with both concentrations also showed diurnal fluctuations in F_v/F_m and F_v/F_0 values during the first 24 h. However, after 30 h of exposure to the metal, significantly lower F_v/F_m and F_v/F_0 values were recorded in treated plants compared to control plants (Figs. 2b and 2c). These changes of chlorophyll fluorescence parameters (F_v/F_m, F_v/F_0 and F_0/F_v) by Cd^{2+} concentrations demonstrated a rapid inactivation of photosystem II (PSII). The fluctuations in these parameters can be the result of a Ca^{2+} substitution by Cd^{2+} in the catalytic center of PSII during photoactivation. Another possibility is that manganese (Mn^{2+}) is replaced by Cd^{2+} from the water-splitting apparatus at the oxidizing side, resulting in a disruption of photosynthetic reactions (Ouzounidou *et al.*, 1997; Kajsa *et al.*, 2004; Faller *et al.,* 2005). Similar results were observed by Mallick and Mohn (2003) and Plekhanov and Chemeris (2003) who reported that higher concentrations of heavy metals induced one rapid inactivation of PSII in *Scenedesmus obliquus* and *Chlorella pyrenoidosa*, respectively.

On the other hand, the performance indexes (PI_{CS0}, PI_{CSm}, and PI_{ABS}) of plants treated with 0.054 and 0.267 M Cd^{2+} were significantly different to those of controlplants after 30 h exposure (Table II). The significantly lower performance index values of the plants exposed to both concentrations of this not essential metal were the result of lower quantum yields (TR_0/ABS), lower efficiencies at which a trapped electron can move beyond Q_{A^-} in the electron transport chain (ET_0/TR_0) and the lower quantum yield of electron transport chain (ET_0/ABS) (Table II). In contrast, heat dissipation (DI_0/RC), energy absorption and trapping per reaction center (ABS/RC and, TR_0/RC) and the electron transport flux per reaction center (ET_0/RC) were significantly higher than in control plants ($p < 0.05$, Student's *t*-test) (Table II). Additionally, the derived phenomenological fluxes per excited cross section of leaf sample, estimated from F_0, were affected by both Cd^{2+} doses. ABS/CS_0 (photons absorbed at F_0 by antenna molecules associated with all PSII RCs per cross section of the leaf), TR_0/CS_0 (maximal trapping rate of excitons that will lead to Q_A reduction per cross sec-

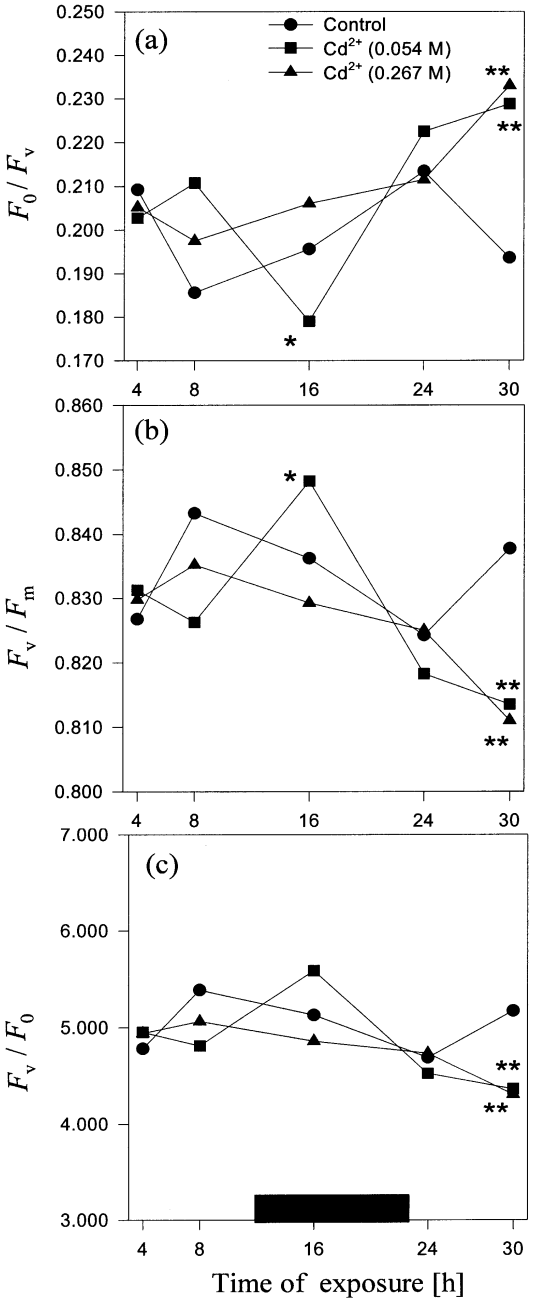

Fig. 2. Time course response of various fluorescence parameters for untreated *A. germinans* plants or plants after 30 h exposure to 0.054 and 0.267 M Cd^{2+}: (a) water-splitting apparatus of PSII, F_0/F_v; (b) the maximal photochemical yield of PSII in dark-adapted leaves, F_v/F_m; and (c) the ratio of variable fluorescence to unquenchable portion of fluorescence, F_v/F_0. The black bar indicates the dark hours. Each point is the mean of 4 replications. Significant differences among treatments are indicated by: * $p < 0.05$; ** $p < 0.01$.

Table II. Measured fluorescence variables for leaves of *A. germinans* treated with different doses of Cd^{2+} at 30 h after exposure.

	Control		0.054 M		0.267 M	
Quantum efficiencies						
TR_0/ABS	0.820 ±	0.007[a]	0.780 ±	0.008[b]	0.772 ±	0.119[a]
ET_0/ABS	0.511 ±	0.037[a]	0.453 ±	0.300[a]	0.448 ±	0.390[a]
ET_0/TR_0	0.600 ±	0.060[a]	0.580 ±	0.110[a]	0.586 ±	0.039[b]
ET_0/DI_0	2.741 ±	0.341[a]	2.09 ±	0.153[b]	2.08 ±	0.231[ab]
Specific fluxes						
ABS/RC	1.403 ±	0.118[a]	1.955 ±	0.136[b]	2.114 ±	0.127[ab]
TR_0/RC	1.150 ±	0.087[a]	1.531 ±	0.116[b]	1.650 ±	0.128[ab]
ET_0/RC	0.691 ±	0.121[a]	0.891 ±	0.144[b]	0.963 ±	0.029[ab]
DI_0/RC	0.253 ±	0.032[a]	0.424 ±	0.320[b]	0.464 ±	0.610[ab]
Phenomenological fluxes						
ABS/CS_0	585.250 ±	42.035[a]	517.666 ±	26.080[b]	497.250 ±	10.012[ab]
TR_0/CS_0	479.527 ±	29.040[a]	405.206 ±	16.308[b]	388.336 ±	5.863[ab]
ET_0/CS_0	286.802 ±	33.694[a]	235.097 ±	28.674[b]	277.723 ±	21.183[ab]
Density of PS II reaction center						
RC/CS_0	417.991 ±	23.94[a]	265.605 ±	22.154[b]	236.441 ±	20.573[ab]
RC/CS_m	2324.330 ±	67.475[a]	1223.383 ±	71.789[b]	1084.308 ±	137.304[ab]
Performance indexes						
PI_{ABS}	51.260 ±	15.102[a]	25.786 ±	4.06[b]	25.283 ±	9.865[ab]
PI_{CS_0}	29625.894 ±	7757.761[a]	13366.563 ±	2427.348[b]	12550.071 ±	4833.839[ab]
PI_{CS_m}	165746.060 ±	47091.126[a]	61606.337 ±	10680.403[b]	57957.861 ±	24542.091[ab]

Data are means ± S.D. ($n = 4$). Those with different superscript letter (a and b) in the same column are significantly different ($p < 0.05$, tukey multiple comparison).

tion of the sample) and the ET_0/CS_0 (maximal electron transport rate per cross section of sample) tended to decrease as the concentrations of Cd^{2+} increase (Table II). Additionally, the estimated number of active PSII reaction centers per sample cross section using F_0 and F_m, RC/CS_0 and RC/CSm, were significantly lower ($p < 0.05$, Student's *t*-test) in plants treated with both Cd^{2+} concentrations than those of control plants (Table II). Our results showed that quantum efficiency parameters were all clearly decreased with lower and higher Cd^{2+} concentrations. In contrast, the specific flux parameters were increased by both Cd^{2+} concentrations. This is probably due to the much higher dissipation flux of untrapped excitons in the active PSII RC (DI_0/RC) in plants exposed to Cd^{2+}. Similar results were found by Zhou and Qiu (2005), where the specific fluxes parameters tended to increase in the presence of Cd^{2+}, and their increases could be due to the inactivation of some RCs in *Sedum alfredii* plants. On the other hand, the integration of these differences per active PSII RC leads to a decrease in the derived parameter PI_{ABS} (performance index based on equal absorption), the density of PSII RCs per excited cross section of leaf sample and phenomenological fluxes in plants treated, indicating a less effective processing per PSII RC in these plants.

Photosynthesis parameters

In the case of stomatal conductance (g_s), and net photosynthesis (P_n), control plants showed a progressive decline in g_s (Fig. 3a) and P_n (Fig. 3b) towards the end of the light hours. Both g_s and P_n values increased again the following morning to values similar to those at the previous day. On the other hand, Cd^{2+}-treated plants had a rapid drop in both g_s and P_n from 4 to 8 h of exposure, followed by a progressive rise in g_s (Fig. 3a) but keeping close to 0 in the case of P_n during the dark hours. The following morning, g_s values dropped to 0 in plants treated for 30 h with both concentrations of the metal. In the case of P_n values plants treated with Cd^{2+} were able to photosynthesize although not to the extent that the control plants did, but those plants treated with high Cd^{2+} concentrations were unable to photosynthesize showing the lowest P_n values of all treatments (Fig. 3b). In the case of net photosynthesis values one possible explanation for the reduction is that Cd^{2+} reduces stomatal opening (reductions in stomatal conductance), thereby reducing mesophyll CO_2 availability. Alternatively, Cd^{2+} could have induced a reduction in the synthesis or activity of Calvin cycle enzymes reducing the demand for CO_2 and, resulting in reductions in stomatal conductance (Krupa *et al.*, 1993; Dong *et al.*, 2005). Similar results were found by Chaffei *et al.* (2004) where *Lycopersicum esculentum* plants, exposed for one week to Cd^{2+}, showed a decrease in the rate of net photosynthesis due to stomata closure, Rubisco inactivation and chlorophyll degradation.

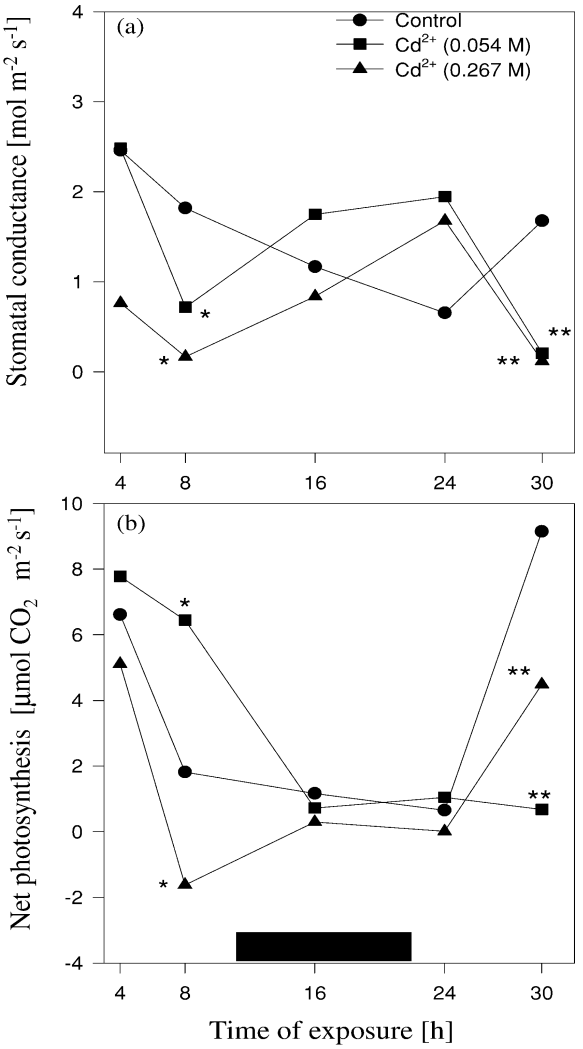

Fig. 3. Time course response of stomatal conductance (a) and net photosynthesis (b) of untreated plants or plants treated with 0.054 M and 0.267 M Cd^{2+} during an exposure period of 30 h. The black bar indicates the dark period and each point is the mean of 4 replications. Significant differences among treatments are indicated by: * $p < 0.05$; ** $p < 0.01$.

Conclusion

Our results suggest that one of the main targets of cadmium, according to the OJIP-test, can be listed as a decrease in the number of active reaction centers and inactivation of the water-splitting complex that finally results in a decrease in the net photosynthetic range. Additionally, the method of chlorophyll fluorescence and OJIP-test may be used as a tool to understand the primary mode of action of heavy metals on the photosynthetic apparatus of mangrove plants.

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