Ultrastructural and Photosynthetic Response of *Populus* 107 Leaves to Cadmium Stress

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Abstract

As *Populus* 107 is an efficient phytoextraction plant, it was used in the present investigation in order to better understand the mechanisms of detoxification and tolerance of Cd. Cd-induced impacts on photosynthetic parameters (chlorophyll content, soluble protein, and chlorophyll fluorescence) and ultrastructural changes in leaves of *Populus* 107 exposed to 50 μM, 100 μM, and 500 μM for 40 days were carried out. The results showed that in the Cd-treated cells, almost all the chloroplasts seemed to be affected. Cd induced several significant ultrastructural changes, including swollen chloroplast thylakoids, dissolved thylakoid grana, disintegrated chloroplasts, and numerous plastoglobuli in chloroplasts. Data from chlorophyll fluorescence showed that F_v/F_m, F_v'/F_m', ΦPSII, ETR, and qP decreased while qN increased in leaves of *Populus* 107 exposed to Cd when compared to control. The content of soluble protein increased with increasing Cd concentration and declined with prolonged duration of treatment. The soluble protein content in leaves treated with 50 μM Cd reached the maximum, which was 14.29% more than that of control. The content in leaves exposed to 500 μM Cd were only 61.76% of control. At the end of the experiment the contents of chlorophyll *a*, *b*, and *a+b* of *Populus* 107 treated with 500 μM Cd decreased to the minimum, which were 47.69%, 37.10%, and 45.49% of control, and respectively, and significantly (P < 0.05) lower than control. The toxic mechanisms of Cd are briefly explained.

Keywords: chlorophyll fluorescence parameters, chloroplast ultrastructure, photosynthesis pigments, soluble protein

Introduction

Cadmium (Cd) naturally found in low concentrations in the environment tends to accumulate to high and toxic concentrations in connection with mining and the excessive use of sewage sludge in agriculture [1]. Cd is thought to be a particularly dangerous pollutant due to its high toxicity and large solubility in water [2]. It is well known that Cd is one of the most important factors limiting normal plant growth and can be absorbed readily by plants and accumulated in the human body through the food chain [3]. At a morphological level, an excessive amount of Cd causes stunted growth, root browning, leaf epinasty, chlorosis, and necroses [4]. At a cellular level, intoxication by Cd induces oxidative stress as evidenced by enhanced lipid peroxidation, hydrogen peroxide generation, and ion leakage [5].

As Cd is a highly reactive heavy metal and shows a high affinity toward functional groups of biomolecules [6], it interacts with various physiological processes that are

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essential for plant metabolism [1, 7, 8]. Cd has been shown to be one of the most effective inhibitors of photosynthetic activity. Some investigations have been reported that plant growth is affected under Cd stress, resulting in alteration of chlorophyll synthesis [9], transpiration and respiration processes [10], and stomatal opening [11], thus reducing photosynthesis [12, 13]. The chloroplast is a large, complex double membrane organelle that performs the function of photosynthesis within plant cells and contains the substance chlorophyll that is essential for this process. Cd convincingly resulted in marked distortion of chloroplast ultrastructure leading to disturbed shape and inflated thylakoids [14]. Once Cd enters chloroplasts, it can disturb chloroplast function by inhibiting the enzymatic activities of chlorophyll biosynthesis, pigment – protein complexes, O_2-evolving reactions of photosystem II (PSII), electron flow around photosystem I (PSI), and chloroplast structure [15-18].

Phytoremediation by using vegetation to remove, detoxify, or stabilize heavy metals is an acceptable tool for decontamination of polluted soil and water. Our previous study indicated that *Populus* 107 was an efficient phytoextraction plant as it had considerable ability to accumulate Cd (Cd concentration in shoots exceeding 0.01% (w/w)) [11]. However, few studies parallel analysis of chlorophyll fluorescence and photosynthesis parameters under Cd stress, and research related the effect of Cd on ultrastructural changes in leaves. To gain a deeper insight into Cd-induced impacts on photosynthetic parameters (chlorophyll content, soluble protein, and chlorophyll fluorescence) and ultrastructural changes in leaves of *Populus* 107 grown in the presence of different concentrations of Cd, our investigation was carried out.

Material and Methods

Culture Condition and Cadmium Treatment

Populus 107 (*Populus*×*euramericana* cv.'Neva') was selected in the present investigation. Woody cuttings (15 cm in length and 1.5 cm in diameter) from 1-year old shoots were rooted in vermiculite for a month. Then they were selected for uniformity of roots and new shoots, and transferred to 1/2-strength Hoagland nutrient solution spiked with different concentrations of Cd (50 μM, 100 μM, and 500 μM) and grown for 40 days. Cadmium was provided as cadmium chloride ($CdCl_2$). The Hoagland nutrient solution consisted of 0.75 mM K_2SO_4, 0.65 mM $MgSO_4$, 0.01 mM KCl, 0.25 mM KH_2PO_4, 2 mM $Ca(NO_3)_2$, 100 μM FeEDTA, 10 μM H_3BO_3, 1 μM $MnSO_4$, 0.1 μM $CuSO_4$, 0.05 μM $(NH_4)_6Mo_7O_4$, and 1 μM $ZnSO_4$ [19], adjusted to pH 5.5. The experiments were conducted in a greenhouse under a 14 h photoperiod at 20/18ºC (day/night), irradiation of 360 W·m^{-2} and 65-75% humidity. The solutions were constantly aerated and replaced every 10 days. All treatments were done in 5 replicates.

Measurement of Soluble Protein Contents

Measuring soluble protein content in this investigation was carried out according to Bradford's method [20] using bovine serum albumin (BSA) as a standard. The fresh roots and leaves from each treatment (6 seedlings) were washed in distilled water, dried, and put in a mortar with 5 mL 0.05 M phosphate buffer solution (PBS) (pH 7.8) at the end of each time interval (10 d) of the Cd treatment. The homogenate was centrifuged at 10,000×g for 20 min and the supernatant was used for analyzing soluble protein content. The soluble protein content was expressed as mg per g fresh weight.

Leaf Pigment Measurements

Each leaf sample from each treatment was homogenized in 5 ml of 80% acetone at 4ºC, and adding 5 ml acetone to a total of 10 ml in each tube. Tubes were stored in the dark at 4ºC for 12 h prior to spectrophotometer measurements. Pigmentation of the sample was centrifuged (4,000 g) for determination. Absorbance was measured at 646 and 663 nm with a UV-Vis spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan). Chlorophyll *a* (C_a) and chlorophyll *b* (C_b) concentrations were calculated according to Zarco-Tejada et al. [21]:

$$C_a \text{ [mg/g fresh matter]} = [12.21 \cdot A_{663} - 2.81 \cdot A_{646}] \cdot \text{mL Acetone/mg fresh matter}$$

$$C_b \text{ [mg/g fresh matter]} = [20.13 \cdot A_{646} - 5.03 \cdot A_{663}] \cdot \text{mL Acetone/mg fresh matter}$$

Measurements of Chlorophyll Fluorescence

Chlorophyll fluorescence quenching analysis was carried out at room temperature with a portable fluorometer (LI-6400, LI-COR, Lincoln, USA). Leaves were darkened for 12 h prior to measurement. The initial Chl fluorescence intensity of the dark-adapted samples (F_0) was obtained upon excitation with a weak measuring beam (0.08 μmol photons m^{-2}·s^{-1}). Maximum fluorescence (F_m) was determined following a saturating red light (7200 μmol photons m^{-2}·s^{-1}). Yield of variable fluorescence (F_v) was calculated as F_m-F_0, and maximum efficiency of PSII photochemistry in the dark-adapted state as $F_v/F_m = (F_m - F_0/F_m)$. Efficiency of energy harvesting by open reaction centers of photosystem II for light-adapted leaves were calculated as: $F_v'/F_m' = (F_m' - F_0')/F_m'$, where F_0' is minimal fluorescence of a momentarily darkened leaf, and F_m' is maximal fluorescence during a saturating flash light of >7 mmol m^{-2}·s^{-1}. Photochemical quenching (q_p) was calculated as indicated by the manufacturer's manual for the LI-6400 leaf chamber fluorometer, $q_p=(F_m'-F_s)/(F_m'-F_0')$, where F_s is the Chl fluorescence yield during actinic illumination. Other fluorescence parameters were measured on light-adapted leaves using the equations of Genty et al. [22]. The quantum yield of PSII electron transport, $\Phi PSII =(F_m'-F_s)/F_m'$. The appar-

ent electron transport rate, $ETR=(F_m'-F_s)/F_m'\times PPFD\times0.5\times0.84$ [23], Non-photochemical quenching, $qN=1-(F_m'-F_0')/(F_m-F_0)$. For the calculation of ETR, PPFD was the photosynthetic photon flux density of actinic illumination, 0.5 was assumed as the fraction of the excitation energy distributed to PSII, and 0.84 as the fractional light absorption of the leaf. These data were collected every 10 days.

Transmission Electron Microscopy

The leaf samples of the middle area of leaf blades from control and the treated groups grown for 40 days were hand cut with a razor blade into 1 mm² pieces and fixed in a mixture of 2% formaldehyde and 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h and then thoroughly washed with the same buffer three times. This was followed by post-fixation with 2% osmium tetroxide in the same buffer for 2 h. They were dehydrated in an acetone series, and embedded in Spurr's ERL resin [24]. For ultrastructural observations, ultrathin sections of 75-nm thickness were cut on an ultramicrotome (Leica EM UC6, Germany) with a diamond knife, and were mounted in copper grids with 300 square mesh. The sections were stained with 2% uranyl acetate for 50 min. and lead citrate for 15 min. Observation and photography were accomplished by transmission electron microscopy (JEM-1230, Joel Ltd, Tokyo, Japan).

Statistical Analysis

Analysis of variance of the data was done with SigmaPlot 8.0 software. For statistical analysis, one-way analysis of variance (ANOVA) and t-test were used to determine the significance at P<0.05. All treatments were done in five replicates.

Results

Effects of Cd on Soluble Protein Contents

The effects of Cd on soluble protein contents are presented in Fig. 1. The results indicated that soluble protein contents in leaves increased significantly (P<0.05) on the day 20 The soluble protein content in leaves treated with 50 μM Cd reached the maximum, which was 14.29% more than that of control. The soluble protein contents in leaves exposed to 500 μM were observed to decline significantly (P<0.05) after 30 d treatment. At the end of the experiment soluble protein contents in leaves exposed to Cd treatment were significantly lower (P<0.05) when compared with control. The soluble protein content in leaves exposed to 500 μM Cd were only 61.76% of control.

Effects of Cd on Content of Leaf Pigments

The contents of chlorophyll *a*, *b*, and *a+b* of *Populus* 107 after 10 to 40 day Cd treatments were presented in Fig. 2.

The contents of leaf pigments expose to Cd decreased significantly (P<0.05) when compared with control, except for the group exposed to 50 μM Cd on day 10. The contents of chlorophyll of poplar 107 exposed to 50 μM Cd increased during 30 days and then declined with prolonging the treatment time (Figs. 2a-c). At the end of the experiment the contents of chlorophyll *a*, *b*, and *a+b* of *Populus* 107 treated with 500 μM Cd decreased to the minimum, which were 47.69%, 37.10%, and 45.49% of control, respectively, and significantly (P<0.05) lower than control.

Effects of Cd on Chlorophyll Fluorescence

F_v/F_m in *Populus* 107 exposed to Cd was significantly lower (P < 0.05) in comparison with control and represented a decreased tendency with prolonging the treatment time (Fig. 3). Fig. 4 showed the changes of fluorescence parameters in light-adapted leaves. The decrease tendency in photochemical efficiency of PSII(F_v'/F_m'), actual photochemical efficiency of PSII (PSII), electronic transfer rate (ETR), and photochemical quenching (qP) were observed, whereas qN had increased tendency. F_v'/F_m' of *Populus* 107 exposed to Cd was significantly lower (P<0.05) during the whole treatment in comparison with control, except for the group treated with 50 μM and 100 μM Cd on day 10. Significant difference (P<0.05) was shown in PSII and ETR of *Populus* 107 exposed to Cd when compared with control, except for ETR on day 10. qP in control was significantly higher when compared with the treatment groups. A significant increase of qN (P<0.05) was noted after 20 days of treatment as compared to control plants.

Effect of Cd on Subcellular Structures of Leaves

Electron microscopic observations of mesophyll chloroplasts from poplar leaves treated with Cd revealed distinct differences between the samples examined compared to control (Fig. 5). Under natural conditions, the
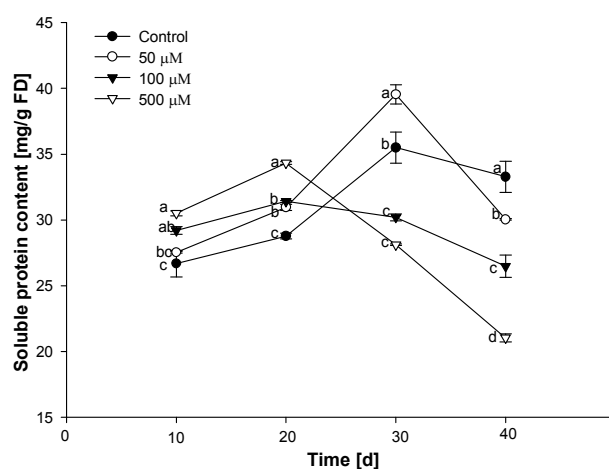

Fig. 1. Effects of different Cd concentrations on the contents of soluble protein in leaves of *Populus* 107 exposed to Cd stress in 40 days. Vertical bars denote SE (n=3). Values with different letters differ significantly from each other (p<0.05, t-test).

cytoarchitecture of *Populus* 107 leaves was intact with typical organelles. The shuttle-like or spheroid chloroplasts were abundant in normal leaf cells. Chloroplasts with a well-developed lamellar system and the grana layers were arranged in an orderly fashion. Some plastoglobuli
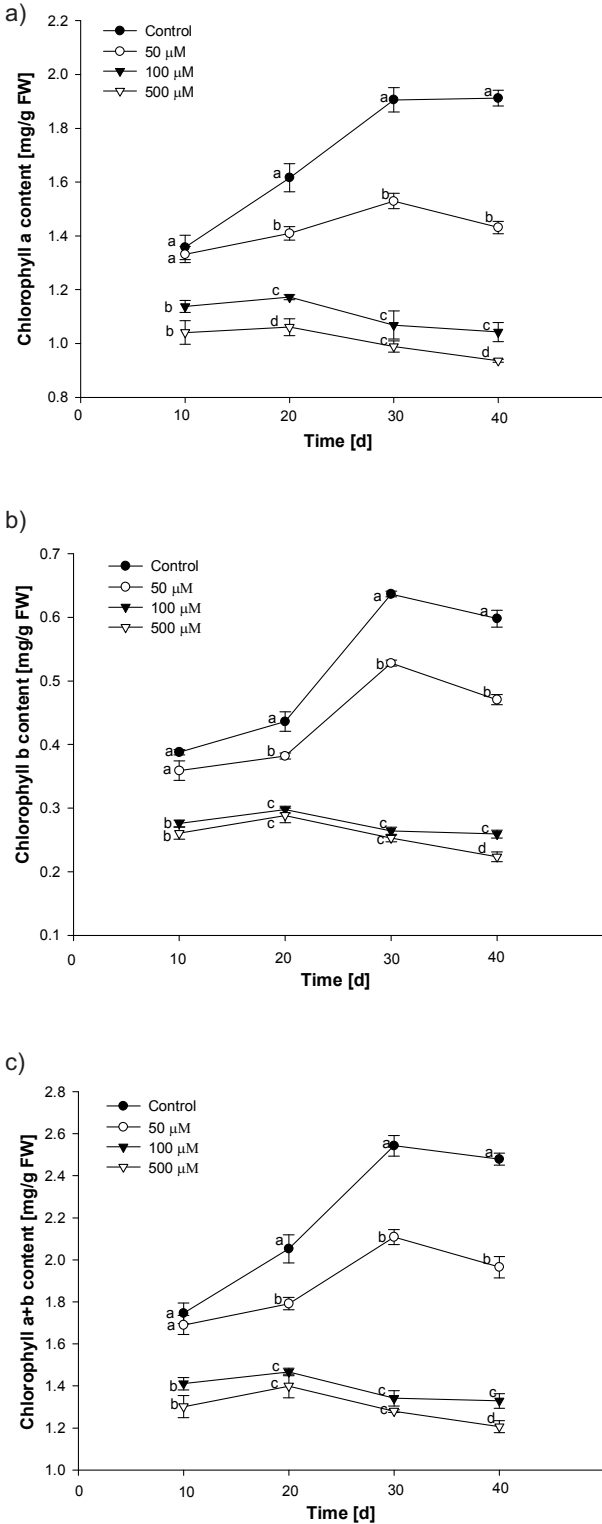

Fig. 2. Chlorophyll *a*, *b*, *a*+*b* contents of *Populus* 107 (a, b, c) exposed to Cd stress in 40 days. Vertical bars denote SE (n=3). Values with different letters differ significantly from each other (p<0.05, t-test).
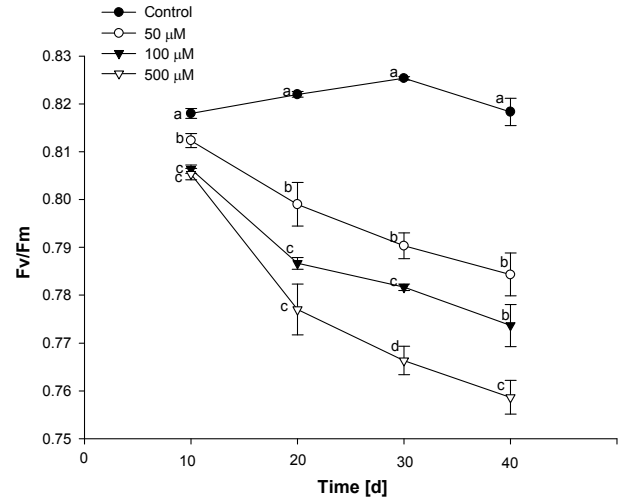

Fig. 3. Effects of Cd on changes of F_v/F_m in *Populus* 107 treated with different concentrations of Cd in 40 days. Vertical bars denote SE (n=3). Values with different letters differ significantly from each other (p<0.05, t-test).

appeared in the chloroplasts (Fig. 5a). In the Cd-treated cells, almost all the chloroplasts seemed to be affected, but to different degrees. At 50 µM Cd, most of the chloroplasts were either undamaged or only slightly damaged in comparison with control. Generally, a small proportion of thylakoid membranes were slightly disintegrated in some cells (Fig. 5b). Occasionally the outer membrane of the chloroplasts envelope was disrupted. The main alterations observed in chloroplast appearance, in 100 µM Cd concentrations, were that distorted chloroplasts were more common and they were rounded in shape and irregular in outline. Some cells showed strongly swollen chloroplast thylakoids (Fig. 5c). The thylakoid grana were dissolved and were hardly visible in some cells (Figs. 5d). With the further increase in Cd concentration (500 µM), the toxic symptoms of chloroplasts were more significant because chloroplasts in the cell decreased obviously in number and the breakages occurred in the membrane of some chloroplasts (Fig. 6a). The thylakoid grana were damaged and disappeared, and chloroplasts disintegrated (Fig. 6b). Plastoglobuli were more numerous in chloroplasts and large inter-thylakoidal spaces in the stroma were lightly stained (Fig. 6c).

Discussion

Soluble protein content in organisms is an important indicator of reversible and irreversible changes in metabolism and responds to a wide variety of stressor [25, 26]. The results indicated that the soluble protein content in leaves of *Populus* 107 exposed to Cd increased with increasing Cd concentration and declined with prolonging duration of treatment. We think that there are two reasons why Cd induces the high soluble protein content. First of all, Cd induces the expression of several genes and increases the synthesis of several proteins. In addition, a high concentra-

tion of heavy metal treatment induces the production of heavy metal-resistance proteins. They could resist the toxicity of heavy metal [27]. During the later period of treatment, the decline of soluble protein contents could be explained that the Cd-induced oxidative stress appeared

[26] and the protein synthesizing machinery was impaired due to the Cd effect [28]. Reactive oxygen species (ROS) can affect protein functions through multiple mechanisms, including regulation of protein expression, post-translational modifications, and alteration of protein stability [29]. Cd
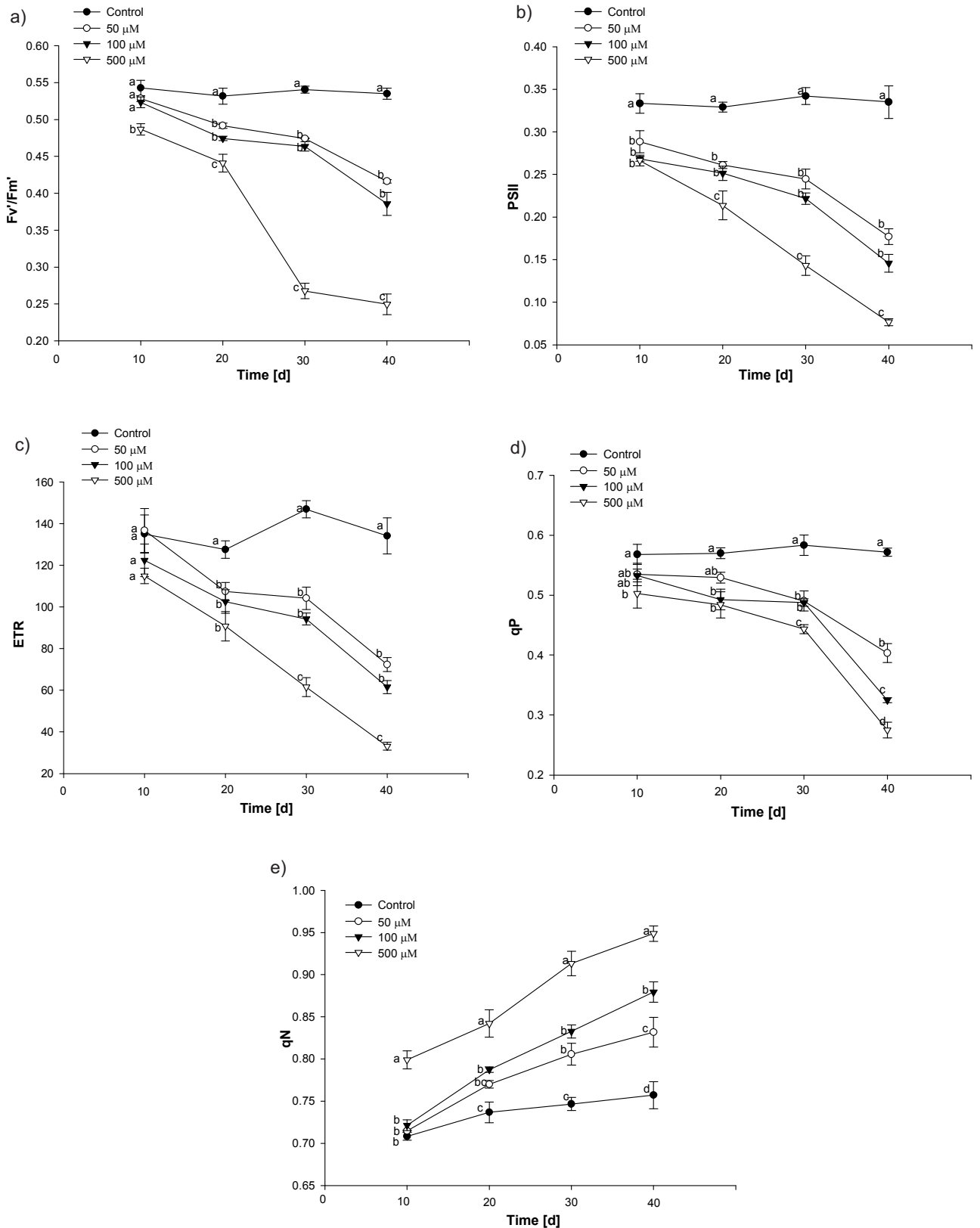

Fig. 4. Effects of Cd on changes of F_v'/F_m', PSII, ETR, qP, qN in *Populus* 107 treated with different concentrations of Cd in 40 days. Values with different letters differ significantly from each other (p<0.05, t-test).

can affect the absorption of several important nutrition elements and stop some significant physiological material from synthesizing, so some physiological index declined and the biomass decreased (data unpublished).

Chlorophyll is a major pigment in the photosynthesis process, whose content and a/b value are an indication of the activity of photosynthesis. It is well known that photosynthesis is especially sensitive to the presence of Cd in chloroplasts [30, 31]. Cd could interfere with the whole photosynthetic process, from chlorophyll biosynthesis and degradation, assembly of pigment protein complexes and thylakoids, to the electron transport chain, the Calvin cycle enzymes, and sugar transport and consumption [32]. It has long been known that interveinal chlorosis of leaves is one of the firstly visible symptoms of Cd toxicity and that it is due to decreased rate of chlorophyll biosynthesis and chlorophyll content caused by damages to thylacoid membranes. The results from the present investigation indicated that the amounts of chlorophyll *a*, chlorophyll *b*, and, consequently, total chlorophylls were lower than those in control plants, which is in agreement with the findings of Pietrini et al. [33] and Shen et al. [34] in poplar. Nada et al. indicated that Cd could induce Fe deficiency in almond seedlings exposed to 25, 50, 100, 150 μM Cd, which resulted in the decrease in ferredoxins necessary for the light-induced oxido-reduction process and reduction in chlorophyll content [35]. We assume that in the present investiga-

tion Cd induced Fe deficiency and resulted in the decrease of chlorophyll content. However, more work needs to be done in this direction. In recent years the technique of chlorophyll fluorescence has become ubiquitous in plant ecophysiology studies. Chlorophyll fluorescence depends to a great extent on pigment content and a leaf's ability to photosynthesize. Chlorophyll fluorescence parameters such as ΦPSII, F_v/F_m, F_v'/F_m', ΦPSII, ETR, and qP have been established as a reliable indicator of the intensity of stress [36]. Data from the present investigation showed that F_v/F_m, F_v'/F_m', ΦPSII, ETR, and qP decreased, while qN increased. The decrease could be explained by the fact that Cd destroyed the reaction center of photosynthesis and restrained the photosynthesis [37]. It is commonly accepted that Cd ions and other heavy metals are highly reactive and affect various aspects of plant physiology [38]. Khan and Khan indicated that although Nickel and zinc were metals considered nutrients essential for plant growth and development, at high concentrations they became phytotoxic, and photosynthetic parameters were reduced significantly in plants treated with Ni and Zn when compared with control plants [39]. In different plant species heavy metals decrease carbon assimilation and can generate oxidative stress [40], which ultimately results in wilting and affects the respiration rate [41]. Pietrini et al. found that the peculiar Cd accumulation in small necrotic areas close to the main vein in willow and poplar clones is associated with
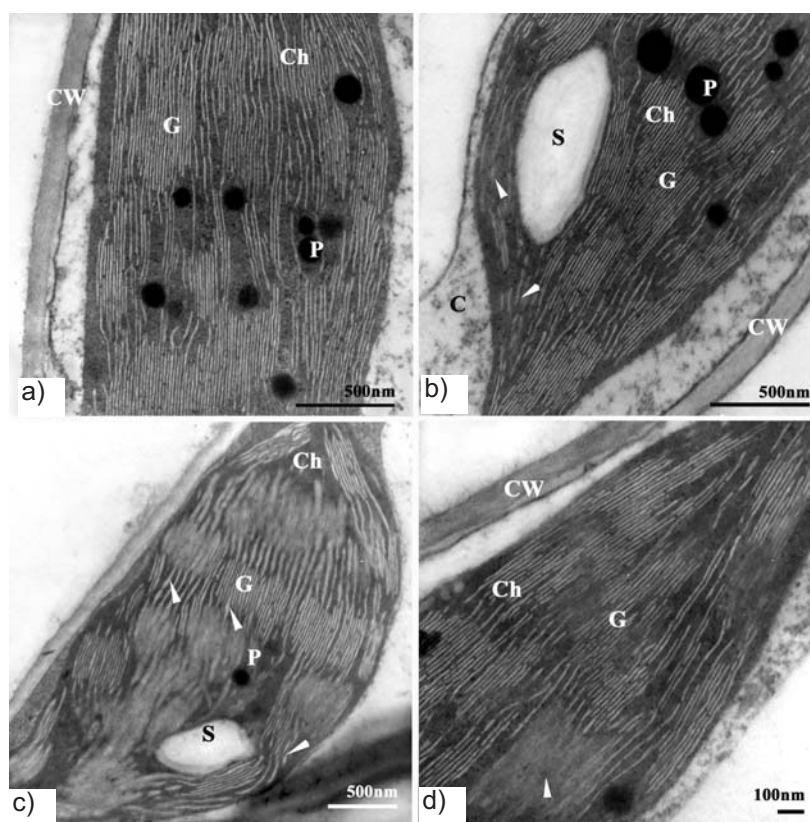

Fig. 5. TEM micrographs of chloroplasts from control and Cd-treated *Populus* 107. a) Chloroplasts with a well-developed lamellar system and the grana layers in control cell. b) Slightly changed chloroplast in the plant exposed to 50 μM Cd. Note slightly disintegrated thylakoid membranes (arrows). c) Strongly swollen chloroplast thylakoids in plants treated with 100 μM Cd (arrows). d) Dissolved thylakoid grana (100 μM Cd; arrow).
Abbreviations: C – cytoplasm, Ch – chloroplast, CW – cell wall, G – grana stack, P – plastoglobuli, S – starch grain.

low values of the chlorophyll fluorescence parameters (F_0, F_m, F_v/F_m, ΦPSII, qP, and NPQ) after the investigation, where willow and poplar clones grown in the presence of 50 μm $CdSO_4$ for 3 weeks under hydroponic conditions were used to examine leaf gas exchange, chlorophyll fluo-
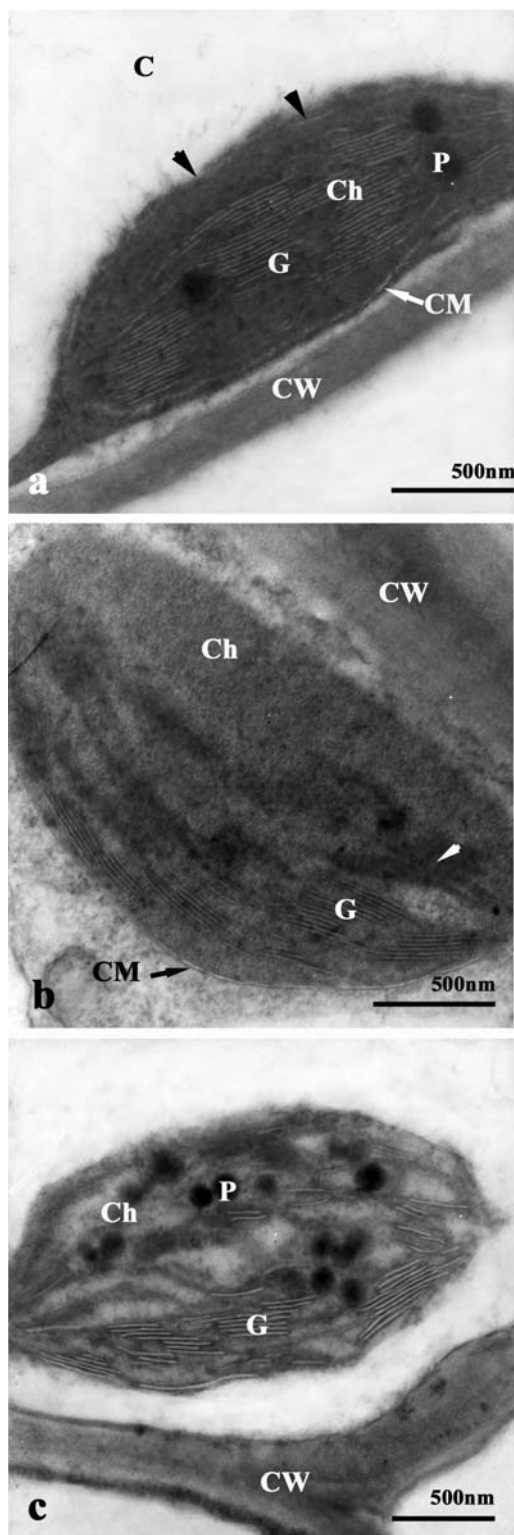

Fig. 6. TEM micrographs of chloroplasts from Cd-treated of *Populus* 107. a) Breakages in the membrane of chloroplasts (500 μM Cd, arrows). b) The damaged thylakoid grana and disintegrated chloroplasts (500 μM Cd, arrows). c) Damaged disintegrating chloroplasts and increased plastoglobuli (500 μM Cd).

rescence parameters and images, and for Cd detection using energy dispersive X-ray fluorescence (ED-XRF). The findings supported the results in the present investigation. High fluorescence parameters (F_v/F_m, FPSII, and qP values) confirmed the absence of detectable Cd [42].

Choudhury and Panda [43] and Bi et al. [44] indicated that the alterations observed in the ultrastructure of the chloroplasts in leaves of plants exposed to Cd might be due to an increase in the production of reactive oxygen species (ROS), which in high concentration in the cellular environment can cause oxidative damage to cellular structure and function. The ultrastructural effects found in *Populus* 107 exposed to Cd, even at high concentrations and prolonged periods, are mainly confined to the chloroplasts structure. Chloroplasts looked misshapen and Cd treatment interfered with the correct organization of the inner membrane system. The thylakoid system appeared incorrectly shaped: grana and stroma thylakoids were not clearly distinguishable. Numerous plastoglobuli distributed in the stroma under Cd stress is usually considered a symptom of senescence because these globules were regarded as lipid droplets from thylakoid degradation [45, 46]. Senescence induced by Cd was given by the swollen thylakoids. Gallego et al. [47] indicated that senescence was induced by oxidative stress, contributing to aging of whole cells and chloroplasts, suggesting the possible peroxidation of thylakoid membranes by the lipoxygenase system. However, the effects of Cd on chloroplast structure differ from those induced by other heavy metals. Heavy metals were shown to induce starch accumulation, probably by inhibition of vein loading [48-50]. In the present study, the absence of starch accumulation in chloroplast stroma may be explained by the inhibition of photosynthesis by Cd [51-53].

Conclusion

Alterations in ultrastructure and photosynthetic parameters would occur before any visible symptom of toxicity appears, and the endpoint based on these parameters might be more sensitive or indicative than morphological observation in revealing eco-toxicity of Cd. Our previous results indicated that *Populus* 107 tolerated high concentrations of Cd and have an ability to take up it from treatment medium [54]. In view of their fast growth, high biomass, and adequate Cd tolerant system, *Populus* 107 appears to have great potential for remediation purposes. Future research is needed to analyze the potential of *Populus* 107 to accumulate Cd from contaminated soils in non-laboratory environments. Besides, a better understanding of the mechanistic details of Cd detoxification in *Populus* 107 may lead to engineering of these plants to enhance their Cd phytoremediation capacity.

Acknowledgements

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