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BRIEF COMMUNICATION

Cadmium effects on growth and antioxidant enzymes activities in *Miscanthus sinensis*

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Abstract

Plants of *Miscanthus sinensis* (cv. Giganteus) were grown in hydroponics for three months in nutrient solution with 0, 2.2, 4.4 and 6.6 μM $CdNO_3$. Growth parameters, catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX) and superoxide dismutase (SOD) activities were analysed in leaves and roots collected after 1- and 3-month exposure. Dry biomass of all miscanthus organs was affected by Cd concentration both after 1- and 3-month exposure. No visible symptoms of Cd toxicity were observed in shoots and rhizomes of plants grown in presence of Cd. In contrast, roots became shorter and thicker and the whole root system more dense and compact already after one month of treatment with 6.6 μM Cd. The lower Cd concentration increased the enzymes activities after 3 months in leaves and only after 1-month in roots, while a decrease in activity was observed at higher Cd concentrations.

Additional keywords: catalase, heavy metals, oxidative stress, peroxidases, superoxide dismutase.

Cadmium (Cd) is a trace pollutant toxic for plants, animals and humans. In plants, exposure to Cd causes inhibition of growth and even plant death owing to its influence on photosynthesis, respiration, water and nutrient uptake (Baszynski *et al.* 1980, Sanità di Toppi and Gabbrielli 1999). Although not essential for plants, in several species Cd is easily taken up by roots and readily translocated and accumulated in shoots (Hardyman and Jacoby 1984, Wagner 1994). At the molecular level, Cd injury has been attributed to *1*) blocking of essential functional groups in biomolecules (Schützendübel and Polle 2002), *2)* displacement of essential metal ions from biomolecules (Rivetta *et al.* 1997) and *3*) production of reactive oxygen species (ROS) by autoxidation and Fenton reaction (Van Assche and Clijsters 1990, Gallego *et al.* 1996, Chaoui *et al.* 1997). Plants have developed a defence system, which is mainly composed of metabolites and enzymes scavenging ROS. In several plants, Cd-induced changes in activities of ROS-scavenging enzymes, including superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX), have been demonstrated (Shaw 1995, Lozano-Rodrigez *et al.* 1997, Gallego *et al.* 1996, Chaoui *et al.* 1997, Wu *et al.* 2003).

Miscanthus is a vigorous perennial *Gramineae* species that reproduces by rhizomes and performes high growth rate and biomass production. It is characterised by wide adaptability to different environmental conditions and in the last decade its cultivation has been promoted in Europe to replace fossil energy sources (Schwarz 1993, Himken *et al.* 1997, Ercoli *et al.* 1999). Miscanthus cropping has also been suggested as an economically viable system to extract and dispose heavy metals from soil, because its potentially contaminated biomass, when burnt for energy production, may enable heavy-metals concentration and recovery into ashes (Arduini *et al.* 2003).

Arduini *et al.* (2003, 2004) found that the highest Cd accumulation in the aerial part of miscanthus was

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; POD - guaiacol peroxidase; SOD - superoxide dismutase.
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achieved with long time exposure to low concentrations of Cd (4.4 μM), which enhanced plant growth and Cd translocation. In order to elucidate the physiological and biochemical responses of *M. sinensis* to low Cd stress (0 up to 6.6 μM Cd in nutrient solution), we determined plant growth patterns and the activity of the antioxidant enzymes SOD, APX, CAT and POD.

Plants of *Miscanthus sinensis* Greef *et* Deu (cv. Giganteus) with shoots of about 20 cm height were transferred to an open-air NFT (nutrient film technique) – hydroponic installation (16 plants per hydroponics line placed at a distance of 50 cm). A modified Clark nutrient solution was used (Clark 1982). Ion concentrations in the final solution were: 25.1 mM NO_3-N, 9.1 mM NH_4-N, 7.5 mM Ca, 8 mM K, 6 mM S, 1.8 mM Cl, 1.6 mM Mg, 0.2 mM Na, 71.6 μM Fe, 64.6 μM P, 17.7 μM Mn, 49.6 μM B, 4.6 μM Zn, 1.2 μM Cu, 1.6 μM Mo. The pH was 7.5 and conductivity 3.8 mS cm^{-1}. Evaporated and transpired water was continuously replaced with tap water and the nutrient solution was completely renewed every two weeks. After one-month plant acclimation, Cd was added to the nutrient solution as $CdNO_3$ to final concentrations of 0, 2.2, 4.4 and 6.6 μM. Clark nutrient solution without Cd addition was used as a control.

After 1 and 3 month of Cd exposure, four plants per treatment were randomly harvested from hydroponics lines for biomass analysis. Plants were divided into leaves, culms, rhizomes and roots and dry mass was determined on plant organs oven-dried at 70 °C to constant mass. Biomass data were corrected for fresh leaves and roots sampled for biochemical analysis.

Samples were immediately frozen in liquid nitrogen and stored at -80 °C until protein extraction. Frozen tissues were ground in liquid nitrogen with mortar and pestle and the powder was suspended in 0.5 cm^3 0.1 M Tris pH 8.0, 1 mM PMSF, 1 % (m/v) polyvinylpyrrolidone, 1 % (m/v) sodium ascorbate and 1 % (v/v) β-mercaptoethanol. Extracts were centrifuged twice at 26 000 *g* for 20 min (4 °C), to discard the cellular debris and the clarified supernatant was used for determination of enzyme activity. Protein concentration was determined using a protein assay kit (*Bio-Rad Laboratories*, Hercules, CA, USA) with bovine serum albumin as a standard.

Enzyme activities were measured at 25 °C using a termostated spectrophotometer (*Lambda 6 UV-vis*; *Perkin-Elmer*, Beaconsfield, UK). SOD (E.C.1.15.1.1) activity was measured and expressed according to Madamanchi *et al.* (1994). Different amounts (0.005, 0.010, 0.020 and 0.040 cm^3) of crude extract were added to a reaction mixture containing 50 mM sodium phosphate buffer pH 7.8, 0.1 mM ethylenediaminetetraacetic acid, 13 mM methionine, 2 μM riboflavine and 75 μM 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT). The reaction was started by exposing the mixture to cool white fluorescent light for 15 min. After this period the light was switched off, the tubes were stirred and the blue colour was measured at 560 nm. CAT (EC 1.11.1.6) activity was assayed in a reaction mixture composed of 50 mM potassium phosphate buffer pH 7.0 to which 30 % (m/v) H_2O_2 was added to reach, at 240 nm, an absorbance value of 0.550. Measurements were started by adding the reaction solution to 0.010 cm^3 of crude extract and the activity followed by monitoring the decrease in absorbance at 240 nm as a consequence of H_2O_2 consumption (Aebi 1984). POD (E.C.1.11.1.7) activity was assayed in a reaction mixture containing 10 mM potassium phosphate buffer pH 7.0, 10 mM H_2O_2 solution, 20 mM guaiacol and 0.01 cm^3 of crude extract (Chance and Maehly 1955). The reaction was started by adding at the same time H_2O_2 and guaiacol solution and the activity was determined by monitoring the increase of absorbance at 470 nm, as a result of guaiacol oxidation. APX (EC 1.11.1.11) activity was determined, according to Chen and Asada (1989), in a reaction mixture containing 100 mM potassium phosphate buffer, pH 7.5, 0.5 mM ascorbate, 0.2 mM H_2O_2 and the crude extract, following the decrease in absorbance at 290 nm.

The experiment was set up in a completely randomised design with four replicates. Data from the two harvest periods were subjected to analysis of variance to test the effects of Cd concentrations on the different organs. Mean values were separated by Fisher's LSD test at 0.05 probability level. Statistical analysis was conducted using a statistical package (*SYSTAT*, *SPSS Inc.*, Evanston, IL, USA).

No visible symptoms of Cd toxicity (chlorosis and leaf senescence) were observed in shoots and rhizomes of plants grown with all Cd concentrations at both harvests. In contrast, roots underwent no visible modification up to 4.4 μM Cd concentration, whereas with 6.6 μM Cd roots appeared shorter and thicker and the whole root system more dense and compact already after 1-month treatment.

The increase of the Cd concentration in the nutrient solution greatly affected dry biomass of all miscanthus organs both after 1- and 3-months exposure (Fig. 1). After 1-month exposure, biomass of rhizomes, shoots and especially roots was higher than in control plants up to 4.4 μM Cd, whereas with 6.6 μM Cd it was lower. At the second harvest, similar response to Cd concentration was evidenced for roots and shoots, whereas rhizome biomass was negatively affected by Cd and progressively decreased with increasing Cd concentrations.

Cadmium effects on antioxidant enzyme activities were similar for all the enzymes analysed, except for SOD. After 1-month exposure (Table 1) no effects on the activities of these enzymes were observed in leaves at the lowest Cd concentration (2.2 μM), while a decrease was induced by the highest concentration (6.6 μM). Moreover,

for CAT and POD the decrease in activity was also observed at 4.4 μM. Stronger effects were instead observed after 3-months of exposure; the lowest Cd concentration, 2.2 μM, induced a significant increase in all the analysed enzyme activities except in SOD, while 4.4 and 6.6 μM had no or less remarkable effect compared to the control (Table 1).

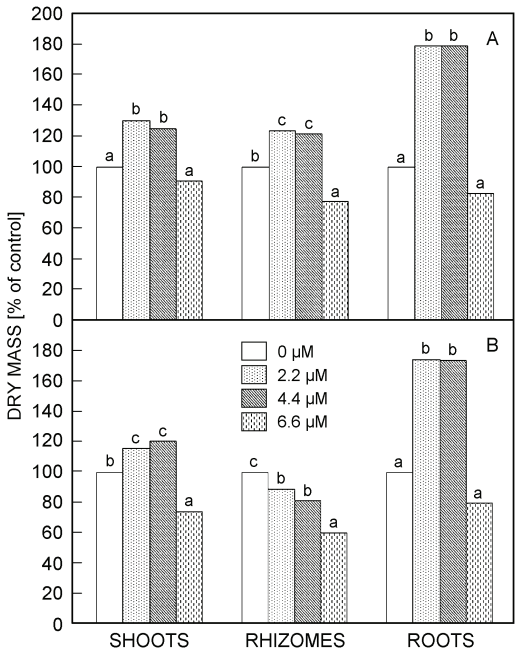

Fig. 1. Effect of increasing cadmium concentrations (0, 2.2, 4.4 and 6.6 μM) on dry mass (% of control) of shoot, rhizomes and roots of miscanthus plants after 1 (*A*) and 3 (*B*) months of exposure. Values are the means (n = 4 individuals) of each cadmium treatment. Within the same sampling time, dry mass change of shoot, rhizomes and roots were analysed independently by *ANOVA*. Means within each graph and organ followed by the same letters do not differ statistically according to Fisher's LSD test ($P \leq 0.05$).

In roots, the Cd concentration modified enzymes activities already after one month of metal exposure, in that the lowest Cd rate increased enzymes activities while the higher rates decreased values to comparable or lower values than control. After three months of exposure, 2.2 and 4.4 μM Cd increased enzymes activities, while at the highest Cd concentration roots showed slightly lower enzyme activity values, but higher or comparable than control samples. Therefore roots seem to respond more rapidly to Cd and, with the increase of the duration of exposure, to enhance antioxidant enzymes reaction to higher metal concentrations.

SOD showed a trend that differed from those of the other enzymes. In leaves, both after 1- and 3-month exposure, no clear effect was observed with the concentration 2.2 μM, while in the presence of 6.6 μM Cd and especially of 4.4 μM Cd a significant decrease was observed (Table 1). In roots a similar trend was noticed after 1-month, even if the decrease induced by the concentration 6.6 μM was higher than that induced by 4.4 μM. After 3-months exposure, no significant effect was observed at the dose 4.4 μM, but a significant increase in activity in comparison to control was observed both at the 6.6 and 2.2 μM Cd concentration. Therefore for both leaves and roots, and for both exposure periods, the critical dose, which induces a decrease in enzyme activity, was 4.4 μM.

Table 1. Catalase (CAT), peroxidases (POD), ascorbate peroxidase (APX), and superoxide dismutase (SOD) activity in leaves and roots of miscanthus plant after 1 and 3 months of exposure to increasing (0, 2.2, 4.4 and 6.6 μM) cadmium concentrations. Activity was expressed as [U mg^{-1}(protein)], where U were: μmol H_2O_2 decomposed per min (CAT); μmol guaiacol oxidized per min (POD); μmol ascorbate oxidized per min (APX); extract volume able to induce 50 % of MTT reduction (SOD). Within the same type of enzyme and sampling time, activities in leaves and roots were analysed independently by *ANOVA*. Means within time and organ followed by the same letters do not differ statistically according to Fisher's LSD test ($P \leq 0.05$). n.d. - not-detectable.

Enzyme	Cd [μM]	Leaves 1 month	Leaves 3 months	Roots 1 month	Roots 3 months
CAT	0	0.010a	0.011b	0.055ab	0.017d
	2.2	0.012a	0.055a	0.072a	0.059b
	4.4	n.d.	0.024b	0.016b	0.064a
	6.6	n.d.	0.016b	0.014b	0.045c
POD	0	0.045a	0.039b	0.264b	0.207b
	2.2	0.034a	0.099a	0.974a	0.202b
	4.4	0.004c	0.113a	n.d.	0.502a
	6.6	0.012b	0.007b	0.130b	0.174b
APX	0	0.094a	0.322b	0.372b	0.268bc
	2.2	0.112a	0.438a	0.692a	0.346b
	4.4	0.070a	0.317bc	0.225b	0.646a
	6.6	0.036b	0.274c	0.055c	0.226c
SOD	0	95.7a	96.3a	97.3b	58.3b
	2.2	101.2a	119.6a	192.6a	84.4a
	4.4	43.2b	53.3b	64.9b	43.4b
	6.6	55.0b	86.7ab	52.9b	87.9a

Our data showed that Cd enhanced miscanthus growth up to 4.4 μM Cd and decreased it with 6.6 μM Cd both after 1- and 3-month exposure. These results are also consistent with the findings of Wu *et al.* (2003) and Chen *et al.* (2003) who found a positive effect of Cd on plant growth at low Cd concentrations. However, after 3-months only root and shoot biomass was higher than control, whereas rhizome biomass decreased. The lower allocation of photosynthetates to rhizomes during longer Cd exposure may therefore determine negative consequence on plant resources. The negative effects on

the growth traits observed at higher Cd concentrations are in agreement with others studies performed on woody (Šottníková *et al.* 2003) and herbaceous species (Astolfi *et al.* 2004).

Many studies showed significant changes in reactive oxygen species content and activity of antioxidant enzymes during Cd exposure. The decline in CAT (Chaoui 1997, Gallego *et al.* 1996, Streb *et al.* 1993, Skórzyńska-Polit *et al.* 2003/4) and the dose- and time-dependent changes in POD activity (Astolfi *et al.* 2004, Skórzyńska-Polit *et al.* 2003/4) have been associated with Cd toxicity in several herbaceous species. Indeed, in our experiments, the activity of antioxidant enzymes underwent significant changes. However, cadmium differently affected leaves and roots and their response depended both on the concentration and the duration of exposure. In leaves, an increase in CAT, POD and APX activities was noticed especially after 3-months exposure at the lower (2.2 μM) Cd concentration. In roots this effect was more evident and they responded to low Cd stimulation more rapidly (after 1-month). This reaction may be explained by the fact that roots is the first organ coming in contact with Cd. Moreover, the different enzyme activities in leaves might be also due to the lower Cd concentration in the aerial than in the hypogeal part as observed in miscanthus (Arduini *et al.* 2004) and other species (Kevrešan *et al.* 2003).

Since the generation of Cd-induced oxidative stress in plant has been assumed to be an early symptom of Cd toxicity (Sandalio *et al.* 2001), the observed enzyme activity stimulation at low-Cd, could plays a central role in cellular protection against the Cd induced oxidative stress. However, it cannot be excluded that in some cases the increase in activity might represent toxic effects itself.

In conclusion, the lower Cd concentrations (2.2 - 4.4 μM) applied during the first year of growth affected miscanthus physiological processes and metabolism without inducing visible injuries and growth reductions. Enzyme activities showed that miscanthus plants were still able to adapt their antioxidant system to overcame Cd oxidative stress, whereas at the highest Cd concentration (6.6 μM) significant changes in morphological parameters and enzymes activity occurred. Cd-induced growth reductions could however be expected in the following years even at the lower metal concentrations, as a consequence of the lower biomass allocation to rhizomes in the first year of growth.

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