Effects of Cd^{2+} on the physiological state and photosynthetic activity of young barley plants

A. VASSILEV*, I. YORDANOV**,+ and T. TSONEV**

*Agricultural University - Plovdiv, BG-4000 Plovdiv, Bulgaria**
*M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Street, Bl. 21, BG-1113 Sofia, Bulgaria***

Abstract

Barley plants (*Hordeum vulgare* L. cv. Obzor) were grown as a water culture in a climatic room. One part of them was subjected to a long-term Cd^{2+} stress - 12 d with 5.4×10^{-5} M Cd. The Cd^{2+} stress inhibited formation of the photosynthetic apparatus and its capacity for ^{14}C photoassimilation, decreased the content of soluble proteins, increased the dark respiration rate and the free amino acids content, disturbed plant water relations, as well as the distribution of ^{14}C within primary photoproducts of the treated barley plants.

Additional key words: amino acids; ^{14}C photoassimilation; dark respiration rate; growth; *Hordeum vulgare*; photoassimilates; proteins; transpiration rate; water relations.

Introduction

Over the last few years heavy metals have received considerable attention as a consequence of the increased environmental pollution from industrial, agricultural, energetic, and municipal sources (Adriano 1986). They function in the soil as stress factors causing physiological disorders after having been absorbed by the root system which results in decreased vigour of a plant and retardation of its growth (Clijsters and Van Assche 1985, Ouzounidou 1993, Moustakas *et al.* 1994). Cadmium (Cd) is a major environmental heavy metal contaminant (Friberg *et al.* 1974). In high concentrations, this metal manifests considerably more phytotoxicity than other heavy metals (Baszyński 1986).

Physiological response of plants to a toxic Cd-treatment is not only growth inhibition, but also changes in various biochemical characteristics, such as higher

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+Author for correspondence: fax: 359 2 73-99-52; e-mail: ifr@bgcict.acad.bg
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activity of dark respiration (Lee *et al.* 1976) and hydrolytic enzymes (Van Assche *et al.* 1988), decreased content of soluble proteins (Stiborová *et al.* 1986a, Sheoran *et al.* 1990), enlarged content of free amino acids in their organs (Costa and Morel 1994), *etc.*

In treatments *in vivo* the metal toxicity causes multiple direct and indirect effects on almost all physiological functions. In higher plants, roots are the first organs to contact the toxic metal concentrations, and roots usually accumulate significantly higher amounts of metal than shoots (Breckle 1991). Cadmium disrupts the plant water relations and its negative effect can be observed in the uptake, transport, and transpiration of water in plants (Barceló and Poschenrieder 1990, Costa *et al.* 1994). Water use efficiency in plants treated with heavy metals declines just as well (Carlson *et al.* 1975). Cadmium inhibits the photosynthetic process (Clijsters and Van Assche 1985): this effect is related to disorders in chlorophyll biosynthesis (Stobart *et al.* 1985, Abdel-Basset *et al.* 1995, Böddi *et al.* 1995), photosynthetic electron transport around photosystem (PS) 1 and mainly PS2 (Tukendorf and Baszyński 1991, Siedlecka and Baszyński 1993, Becerril *et al.* 1988), as well as in the ultrastructure of chloroplasts (Baszyński *et al.* 1980, Barceló *et al.* 1988, Stoyanova and Chakalova 1990). It is also connected with changes in the composition of fatty acids and with decreased content of acyl lipids and proteins in thylakoid membranes (Krupa and Baszyński 1985, Maksymiec and Baszyński 1988). Cd may accelerate ageing of the photosynthetic apparatus (Skorzyńska *et al.* 1991, Krupa and Baszyński 1995).

Some studies, performed mainly *in vitro*, have shown disturbances in the so-called dark reactions of photosynthesis (Krupa and Baszyński 1995). In isolated intact chloroplasts, Cd inhibited to a greater extent processes connected with the regeneration of the Calvin's cycle (Weigel 1985). Results of the *in vitro* experiments cannot be compared with those received *in vivo* where Cd affects the photosynthetic process directly and/or indirectly (Krupa and Baszyński 1995). In this aspect, the *in vivo* investigations are focused on enzymes of the Calvin's cycle, mainly on the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO). Heavy metals inhibit its *de novo* synthesis, and decrease the activity of an already functioning enzyme (Stiborová *et al.* 1986a,b, 1988, Moustakas *et al.* 1994). According to Sheoran *et al.* (1990) and Malik *et al.* (1992), Cd inhibits the activity of almost all enzymes of the Calvin's cycle.

Hence, the photosynthetic rate is decreased as a result of the negative multiple Cd effect upon separate units of the integral photosynthetic process (Baszyński 1986, Sheoran *et al.* 1990, Costa *et al.* 1994, Prasad 1995). Inhibition of the photosynthetic process is probably also connected with the toxic effect of Cd on other basic physiological processes.

In our previous investigations with winter barley cultivars we found that the rate of CO_2-fixation in young plants treated with cadmium decreased by about 20 %, and the ultrastructural organization of the chloroplasts was partly disordered. At the same time, the changes in functional activity of PS2 in Cd-treated barley plants were insignificant. They were within the limits of the norm, and obviously at this phase of

plant development they were not the reason of the established tendency to a decrease in the photosynthetic rate (Vassilev *et al.* 1995).

The goal of this investigation was to check the integral physiological response of young barley plants to Cd, evaluated by parameters of growth, photosynthesis, water relations, and some other biochemical characteristics.

Materials and methods

Plants: Barley plants (*H. vulgare* L. cv. Obzor) were grown as water culture in a climate room at 120 μmol m^{-2} s^{-1} photon flux density, temperature 22±2/18±2 °C day/night, and photoperiod of 14/10 h. Seeds were germinated for 2 d on a wet filter paper in a thermostat at 25 °C. The seedlings were then transferred to a 1/2 strength Knop nutrient solution, enriched with microelements according to Hoagland and with 0.54 μM Cd ions in the form of 3 $CdSO_4 \times 8\ H_2O$. 15-d-old barley plants were analysed.

Growth: Dry mass was determined after drying the plant material at 105 °C. The leaf area was measured using an electronic area meter *NEO-2* (TU, Sofia, Bulgaria).

Water relations: The relative water content (RWC) in leaves was determined according to Morgan (1986). The water potential (ψ) in stems (leaf sheaths) of 5-10 plants was measured with a pressure chamber (*ELE-International*, England).

Gas exchange was determined by a closed gas-analytical system *LI 6000* (*Li-Cor*, U.S.A.). For each measurement the first fully developed intact leaves of 5-6 plants were put in the leaf chamber giving a total of 10 cm^2 leaf area. Leaf temperature was 28 30 °C, CO_2 concentration in the system was *ca.* 400 cm^3 m^{-3}, and photon flux density was 800 μmol m^{-2} s^{-1} (PAR). Before measurements of dark respiration rate (R_D) the plants were dark adapted for 30 min.

Radiometric investigations: The incorporation of ^{14}C in primary products in the first fully developed leaves of control and Cd-treated 15-d-old barley plants was determined. 3 cm long leaf segments, taken from the middle part of leaf, were exposed in a 5 000 cm^3 chamber at CO_2-concentration of 400 cm^3 m^{-3} and ^{14}C with a radioactivity of 740 MBq m^{-3}. The chamber was put under the conditions described above. The pulse-chase labelling technique was also used. Duration of the pulse was 1 min, and the tracing of ^{14}C in the main fractions and in individual compounds of the methanol-water fraction was made after 0, 1, and 10 min. The material was fixed, and the compounds were extracted according to Manolov *et al.* (1978). The methanol-water fraction was separated into basic fractions by passing through ion-exchange resins *Dowex 1* (anionit) and *Dowex 50* (cationit). Individual compounds were fractionated and identified using paper chromatography in the presence of marker compounds. For fractionating the amino acids and sugars the system butanol - acetic acid - water (4:1:1), and for the organic acids 1 M ammonium acetate - 0.1 M EDTA (70:35:1) was used. The radiochromatograms were exposed on X-ray films.

the radioactive zones of individual compounds were drawn and determined quantitatively using the radiometric technique.

Other determinations: The soluble protein content was determined according to Lowry *et al.* (1951). The free amino acids of leaves were extracted in 80 % ethanol. The next step was passing through the ion-exchange resin *Dowex 50,* and elution using 1 M ammonium solution. Due to the presence of the amides asparagine and glutamine in the eluent, a partial hydrolysis with 2 M HCl was performed. The determination was made after digesting the dry residue with 0.125 M HCl using an automatic amino analyser *AAA-801.*

Statistics: Altogether three independent experiments were carried out. The variants were analysed in five replications (pots). There were 6 plants in each pot. The results shown are mean values ± SE. Significant differences were determined by the Student's *t* criterion.

Results and discussion

The term "toxic concentration" is used in the literature for a heavy metal concentration that significantly inhibits the metabolic activity without inducing plant death (Clijsters and Van Assche 1985). The long-term 54 μM Cd-treatment resulted in symptoms of phytotoxicity and in a considerable inhibition of the initial growth of young barley plants. There was chlorosis and necrosis of leaf tips, as well as necrosis and reduction of the number of ramifications in the root systems of treated plants.

Table 1. Influence of Cd on growth parameters, and some biochemical characteristics of young barley plants. Means of 3 separate experiments ± S.E. ($n = 5$); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Parameter	Control plants	Cd-treated plants	[%] of control
Plant			
Shoot height [cm]	25.15 ± 1.14	14.65 ± 0.51***	58
Root length [cm]	9.67 ± 0.97	4.30 ± 0.26***	44
Dry mass [mg plant^{-1}]	59.01 ± 0.99	38.73 ± 2.01***	66
Leaf			
Leaf dry mass [mg]	30.51 ± 1.06	20.48 ± 1.26***	67
Leaf area [cm^2]	18.2 ± 0.7	9.9 ± 0.9***	54
LMR [kg kg^{-1}(plant)]	0.517 ± 0.020	0.529 ± 0.029	102
LAR [m^2 kg^{-1}(plant)]	30.84 ± 1.25	25.56 ± 1.13*	83
SLA [m^2 kg^{-1}(leaf)]	59.65 ± 2.61	48.34 ± 2.11*	81
Rate of dark respiration [μg(CO_2) kg^{-1} s^{-1}]	124.9 ± 8.8	154.7 ± 6.5*	123
Soluble protein [g kg^{-1}(d.m.)]	155.1 ± 7.8	126.1 ± 7.3*	81
Free amino acids [μmol kg^{-1}(f.m.)]	4971.0 ± 256.5	6080.4 ± 378.5	122

In our experiments (Table 1) the accumulation of biomass in Cd-treated plants was inhibited by 44 %, and the linear growth of the organs by 42 to 54 %. The leaf area

and its mass decreased by 46 and 33 %, respectively, but the relative share of leaves in the total leaf mass of plants (Leaf Mass Ratio - LMR) was not changed considerably. The leaf area ratio (LAR) and the specific leaf area (SLA) decreased significantly in the Cd-treated plants. This can be explained by a stronger Cd effect upon the linear leaf growth than on the accumulation of biomass. The symptoms of phytotoxicity together with increased rates of R_D and increased contents of the free amino acids and proteins in leaves correspond to the idea of accelerated leaf ageing in Cd-treated plants (Skorzyńska *et al.* 1991). Much more significant changes of parameters in roots than in leaves of the Cd-treated plants confirm the indirect effect of this heavy metal upon the photosynthetic apparatus in *in vivo* experiments (values not shown).

Water balance in plants is determined by three interconnected processes - uptake, transport, and transpiration (guttation) of water. With the water import being effected by roots and because of its fairly fast translocation to the above-ground organs, Cd may practically affect each one of them. Water potential (Ψ), basic thermodynamic parameter indicating water relations and water activity in plants, decreased to about one half of the normal value in the Cd-treated plants (Table 2). Changes in

Table 2. Influence of Cd stress on water relations (E - transpiration rate; r_s - stomatal resistance; RWC - relative water content; WC - water content; Ψ - water potential) and the ratio of net photosynthetic rate to transpiration (P_N/E) in young barley plants. Values are means of 3 separate experiments ± s.e. ($n = 5$); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Parameter	Control plants	Cd-treated plants	[%] of control
WC [%]	92.27 ± 0.56	89.55 ± 0.63**	97
RWC [%]	96.22 ± 0.12	94.46 ± 0.62**	98
E [mg(H_2O) m^{-2} s^{-1}]	31.6 ± 2.2	26.0 ± 1.3*	82
r_s [s cm^{-1}]	9.397 ± 0.311	16.895 ± 1.304***	179
P_N/E [g(CO_2) kg^{-1}(H_2O)]	7.073 ± 0.445	7.520 ± 0.409	106
ψ [kPa]	-230 ± 20	-730 ± 20***	213

the absolute values of 'Ψ' were within 500 kPa, and thus there was a mild water stress (Pil'shchikova 1993). Our results correspond to those of Costa *et al.* (1994) on Cd treatment of plants of *Lupinus albus*. The induced Cd stress decreased the absolute (WC) and relative (RWC) water contents in leaves by *ca.* 2-3 %, the transpiration rate (E) by 18 %, and it increased the stomatal resistance (r_s) by 79 %.

Our results on the effect of Cd upon WC and RWC correspond to those of Barceló *et al.* (1986a) and Costa *et al.* (1994) with bean and lupine plants. According to Barceló *et al.* (1986) the import of water in plants decreases under these conditions, and this results in a decreased turgor potential of tissues leading to formation of small cells with filled intercellular space area. This statement is confirmed by our findings of significant inhibition of root growth and of decreased SLA in leaves of Cd-treated plants (Vassilev *et al.* 1993). On the other hand, the decreased water content of photosyntetic tissues is probably a result of the accelerated leaf ageing in Cd-treated plants.

The continuous Cd treatment inhibits the transpiration in young barley plants. In this aspect we confirmed the results of Bazzaz *et al.* (1974) and Costa *et al.* (1994), but there are also some contradictory results (Hagemaer and Waisel 1989). Generalizing the known data, Barceló and Poschenrieder (1990) hypothesize that under a long-term treatment with toxic Cd concentrations, the stomata close either hydroactively due to the influence of abscisic acid, or hydropassively because of loss of stomata control. Since the RWC in leaves is not changed considerably because of plant adaptation to the disturbed water regime, it is likely that the induced Cd stress causes a hydroactive decrease of stomata aperture resulting in an increased r_S.

Our results show that the long-term Cd treatment negatively affects the water relations in young barley plants. The major effects of this heavy metal are related to decreased WC of the photosynthetic apparatus and to the inhibition of E. The steady values of the P_N/E ratio indicated that the water use efficiency was not changed significantly in Cd-treated plants. The decreased WC of photosynthetic tissues may be a significant cause of biochemical changes during the dark phase of photosynthesis.

The changes in dark metabolism of the assimilated CO_2 could be assessed to a great extent by the incorporation of ^{14}C into individual compounds of the methanol-water fraction which includes 80-85 % of all labelled photoproducts (Table 3). The rate of ^{14}C incorporation in the fraction of organic phosphates was lower in Cd-treated plants than in the controls. It was reduced almost by half after a 10 min metabolism of ^{14}C in the atmosphere of CO_2 without ^{14}C. This indicated a relatively smaller pool of the labile phosphorylated compounds (mainly from the Calvin's cycle), and corresponded to the decreased protein content and decreased P_N in leaves.

Organic acids were represented mainly by malate, citrate, glycerate, and glycollate. By extending the time of dark metabolism in Cd-treated plants the relative part of radioactivity of the first two compounds increased which characterised them as end metabolites. Thus, the induced Cd stress did not activate photorespiration in the leaves of young barley plants, a fact established by Weigel (1985) in his *in vitro* experiments.

Also the amino acids (glycine, serine, alanine, and aspartate) were labelled. The relative parts of ^{14}C-glycine and serine in control and Cd-treated plants were fairly similar in the variants 1 min in ^{14}C and 1 min in ^{14}C + 1 min without ^{14}C atmosphere, although in the second case their share increased significantly both in the control and Cd-treated plants. In the case of prolonged (10 min) metabolism in atmosphere without ^{14}C, however, the relative part of ^{14}C-glycine + serine decreased more than 4-fold in the control and only two-fold in the Cd-treated plants. The behaviour of alanine and aspartate was similar. While this is the natural performance of glycine and serine as compounds of the glycollate pathway, the decreased radioactivity of alanine after 10 min without ^{14}C could be related to their partial participation in the metabolism of organic acids (*e.g.*, in the considerable increase of malate radioactivity). Organic acids (malate especially) protect plants against stresses (Rajmane and Karadge 1986). We found a significant retardation of the metabolic rate of this pool in Cd treated plants in comparison to the controls. One of the probable causes of this Cd effect may be the inhibition of protein synthesis. Our

supposition is based on the decreased content of soluble protein in leaves, as well as on the decreased WC in photosynthetic tissues as characteristic features of ageing leaves.

Table 3. Distribution of ^{14}C in the primary individual photoassimilates extracted in methanol-water fraction from control and Cd-treated young barley plants (% from total radioactivity). Mean values ± s.e. (n=5); $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001.

Organic compound	Exposure in ^{14}C at light [min] + dark metabolism [min]					
	1		1 + 1		1 + 10	
	control	Cd-treated	control	Cd-treated	control	Cd-treated
Organic phosphates	33.09	30.50	11.93	9.79	4.58	2.59
3-PGA	20.14±0.91	18.67±1.03	3.94±0.26	3.08±0.22	0.35±0.04	0.19±0.03*
Sugar phosphates	2.15±0.17	1.90±0.27	1.30±0.10	0.93±0.06	0.89±0.07	0.45±0.10**
Hexose monophos.	10.80±0.59	9.93±0.41	6.69±0.47	5.78±0.31	3.34±0.27	1.95±0.12**
Organic acids	20.16	17.89	18.81	17.34	23.13	18.30
Malate	10.70±0.57	9.89±0.59	11.91±0.54	9.85±0.50	16.93±0.94	13.39±0.62*
Citrate	-	-	-	-	2.82±0.14	1.45±0.09**
Glycerate+glycolate	8.44±0.51	7.45±0.50	6.09±0.32	6.86±0.42	2.51±0.15	2.49±0.21
Unidentified	1.02±0.08	0.55±0.09**	0.81±0.07	0.63±0.05	0.87±0.06	0.97±0.11
Amino acids	19.48	18.86	30.18	27.09	7.55	15.95
Glycine+serine	15.79±0.93	14.57±1.06	23.39±1.19	21.67±0.93	5.51±0.39	12.62±1.03***
Alanine	2.54±0.18	3.23±0.26	5.85±0.36	4.60±0.32	0.82±0.09	1.70±0.11***
Aspartate	1.15±0.11	1.06±0.10	0.94±0.07	0.82±0.06	1.22±0.13	1.63±0.14**
Sugars	27.27	32.75	39.08	45.78	64.74	63.16
Sucrose	26.40±1.37	30.69±1.23*	36.74±1.29	42.11±1.67*	60.63±2.12	60.24±1.63
Glucose	0.62±0.06	1.05±0.09	1.12±0.08	1.52±0.12*	1.45±0.08	1.30±0.11
Fructose	0.25±0.03	0.75±0.11**	0.79±0.12	1.20±0.17*	1.44±0.14	0.88±0.07*
Maltose	traces	0.26±0.08	0.43±0.07	0.95±0.10**	0.68±0.04	0.74±0.05
Unidentified					0.54±0.06	

The sugars labelled with ^{14}C included mainly sucrose and glucose, the contents of fructose and maltose were much lower. The labelling of these final products increased by extending the duration of dark metabolism. The effect of Cd stress on these sugars was a result of a clearly expressed multiple effect. The relatively slighter ^{14}C labelling of other groups of compounds, especially of the phosphorylated ones (which is an indicator of smaller pool of the primary photoproducts) is obviously a result of faster metabolization to the final compounds, such as sugars. The relative share of glucose and fructose in total sugar radioactivity increased by extending the duration of the dark metabolism for the control plants, while this tendency was not so clearly expressed in the Cd-treated plants. Partitioning of radioactivity in individual sugars in the controls was characteristic for young, active leaves, while in the Cd-treated plants it was more similar to the ageing leaves. This corresponds to the physiological state of the photosynthetic apparatus in Cd-treated plants (Table 1) and

agrees to the idea of Skorzyńska *et al.* (1991) about the accelerated ageing of plants under a metal stress.

Our results may be summarized as follows: A long-term treatment with 54 μM Cd inhibits the formation of photosynthetic apparatus in young barley plants. There are symptoms of phytotoxicity manifested by the necrosis of leaf tips. The inhibitory effect of Cd is more clearly expressed in the linear growth of plants than in the accumulation of biomass. The cadmium imported by roots in concentrations toxic for the growth decreases Ψ and WC of the photosynthetic apparatus and induces disorders in *E* of young barley plants. Incorporation of ^{14}C into the main biochemical groups and in individual compounds of the methanol-water fraction reveals a tendency towards decreasing the functional activity of the photosynthetic apparatus in Cd-treated barley plants. This statement was proved by the relatively smaller pool of the compounds labelled with ^{14}C, as well as by the nature of their partitioning. The symptoms of toxicity together with the number of biochemical characteristics, such as decreased content of soluble proteins, increased R_D and increased content of free amino acids, as well as the partitioning of ^{14}C in the primary photoproducts, show that the long-term Cd treatment results in an accelerated ageing of the photosynthetic apparatus.

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