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Effects of cadmium on the metabolic activity of *Avena sativa* plants grown in soil or hydroponic culture

S. ASTOLFI*, S. ZUCHI and C. PASSERA

Dipartimento di Agrobiologia e Agrochimica, Università degli Studi della Tuscia, via S.C. de Lellis, I-01100 Viterbo, Italy

Abstract

Oat (*Avena sativa* L.) plants cultured in soil and hydroponic culture were treated with cadmium [0.154 mg g^{-1}(dry soil) and 100 μM $CdSO_4$, respectively] for 21 d and growth rate and various biochemical processes were studied. Applied cadmium reduced plant growth and chlorophyll content. Changes in activity of enzymes involved in C, N and S metabolism and in guaiacol peroxidase activity were observed. In particular, *O*-acetylserine sulphydrylase (OASS; EC 4.2.99.8) activity was increased by Cd exposure in both growth conditions, probably as a resistance mechanism to cadmium based on the production of phytochelatins. Results show that both field and hydroponic conditions represent suitable systems for investigating Cd effects on plant growth and metabolism.

Additional keywords: carbon metabolism, nitrogen metabolism, sulphur metabolism, guaiacol peroxidase activity.

Introduction

Cadmium is released into the environment as a result of rock mineralization processes but mostly by industrial processes (Sanità di Toppi and Gabrielli 1999). Cd is taken up readily and accumulated by plants despite being non-essential element (Prasad 1995), thus increasing the potential for contamination of the food chain.

The toxic effects of Cd on plant growth, metabolism and enzyme activity are well documented: Cd inhibits plant growth and also disturbs photosynthesis, sugar metabolism, sulphate assimilation, and several enzyme activities even at low concentrations (Van Assche and Clijsters 1990, Sanità di Toppi and Gabbrielli 1999, Kevrešan *et al*. 2003, Šottníková *et al.* 2003).

Some plant species have developed heavy metal adaptation which enables them to survive and reproduce in such unfavourable conditions. Heavy metal tolerance may results from different strategies including the formation of complexes with phytochelatins (PCs), cysteine-rich peptides (Rauser 1990, 1995).

Most of the studies aiming to improve our understanding of toxic effects of Cd on plant metabolism in higher plants have been performed on hydroponic cultures because of their easiness and suitability. Very few studies of plant responses to Cd exposure have been undertaken using soil as growth substrate, mainly because of the technical difficulties of measuring exactly metal bioavailabilty. In fact, soil Cd concentrations may increase steadily as result of soil type and properties, such as pH and cation exchange capacity (CEC) and, on the other hand, plants may influence metal solubility and uptake directly, through exudation of organic compounds by roots, or indirectly, by affecting microbial activity. In the same way, soil microorganisms may affect metal bioavailabilty through acidification or chelation processes (Marschner 1995). Despite this, when assessing such responses it could be important to consider the plant and the soil, with all its components, as one body with their complex interactions to gain a better understanding of how the different component may interact each other.

Aim of this work was *1*) to investigate the effects of Cd on metabolic activity of oat plants and *2*) to provide a valuable link between hydroponic and field studies. For this purpose we investigated changes in activity of enzymes involved in C, N and S metabolism and in peroxidase activity during the experimental period (21 d) in soil and hydroponically cultured oat plants.

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Abbreviations: ATPS - ATP sulphurylase; BSA - bovine serum albumine; d.s. - dry soil; DTT - DL-dithiothreitol; CEC - cation exchange capacity; EDTA - ethylenediaminetetracetic acid; f.m., fresh mass; GDH - NAD-dependent glutamate dehydrogenase; GPX - guaiacol peroxidase; GS - glutamine synthetase; HEPES - 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid); OASS - *O*-acetylserine sulphydrylase; PCs - phytochelatins; PEPC - phosphoenolpyruvate carboxylase; PMSF - phenylmethylsulphonyl fluoride; PVP - polyvinylpyrrolidone; RUBPC - ribulose-1,5-biphosphate carboxylase.

* Fax: (+39) 0761 357242, e-mail: sastolfi@unitus.it

Materials and methods

Soil culture: Soil used in this experiment was taken from a ploughed agricultural plot, 0 - 15 cm layer, in the Research Area of the University of Viterbo, Viterbo, Italy. The soil had the following chemical properties: pH 7.6, CEC 420 $mmol_c$ kg^{-1}(d.s.), total C 14.8 g kg^{-1}, organic C 25.5 g kg^{-1}, total N 1.7 g kg^{-1}. It was sieved (< 2 mm), homogenized and air-dried. Before use, Hoagland and Arnon (1950) solution was added to the soil to reach the soil moisture level close to field capacity (about 25 % m/m) and to provide optimum rates of nutrients for plant growth. Soil was kept indoors at 25 °C for two weeks to stabilize microbial activity. Pots were filled with 1 kg soil (bulk density 1.6 kg dm^{-3}), planted with 25 pre-germinated oat (*Avena sativa* L. cv. Argentina) seeds and kept in a climate chamber for 44 d (26/20 °C day/night temperature, relative humidity 70/80 %, 14-h photoperiod, irradiance of 200 μmol m^{-2} s^{-1} PAR at leaf level). Twenty-three d after planting, 0.154 mg g^{-1}(d.s.) $CdSO_4$ were added to half of the pots. Cd salt was dissolved in 10 cm^3 distilled water and added drop wise to the soil. Plant harvesting was carried out after 7 and 21 d from the beginning of Cd treatment (30 and 44 d from planting, respectively). Leaves were excised, frozen in N_2 and stored at -80 °C until used.

Hydroponic culture: Oat seeds were allowed to germinate in moistened paper in the dark at 26 °C for 3 d. Seedlings with roots were transferred to plastic pots (about 30 seedlings in each pot) containing 1.3 dm^3 of nutrient solution (Hoagland and Arnon 1950), continously aerated and changed every 2 d. Plants were grown in a climatic chamber under the same conditions as those in soil. After 7 d $CdSO_4$ (final concentration 100 μM) was added to half of the plants. Plant harvesting was carried out after 7 and 21 d from the beginning of Cd treatment (14 and 28 d from planting, respectively). Leaves were excised, frozen in N_2 and stored at -80 °C until used.

Enzyme extraction and assays: Frozen tissue (*ca.* 1 g of fresh mass) was ground to a fine powder in a pre-chilled mortar under liquid N_2. Cold extraction buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES)-KOH (pH 7.4), 5 mM $MgCl_2$, 1 mM ethylenediaminetetracetic acid (EDTA), 10 % (v/v) glycerol, 0.1 % (v/v) *Triton X-100*, 5 mM DL-dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1 % (m/v) polyvinylpyrrolidone (PVP) was added in a ratio of 1:7 (m/v). The brei was filtered through four layers of cheesecloth and the homogenate was centrifuged at 1000 *g* for 5 min at 4 °C. The resulting supernatant was desalted at 4 °C on a *Sephadex G-25* column (*PD-10*, *Pharmacia*, Uppsala, Sweden) pre-equilibrated with extraction buffer minus *Triton X-100*. The desalted extract was then centrifuged at 30 000 *g* for 5 min at 4 °C. The supernatant was divided into 0.3 cm^3 aliquots which were frozen in liquid N_2 and stored at -80 °C until analysis.

The NAD-dependent glutamate dehydrogenase (GDH; EC 1.4.1.2) and phosphoenolpyruvate carboxylase (PEPc; EC 4.1.1.31) activities were assayed as described in Astolfi *et al*. (2001). Ribulose-1,5-biphosphate carboxylase (RUBPc; EC 4.1.1.39) and glutamine synthetase (GS; EC 6.3.1.2) activity were determined as described in Lea *et al*. (1990). ATP sulphurylase (ATPs; EC 2.7.7.4) activity was assayed by the bioluminescence technique (Schmutz and Brunold 1982) and *O*-acetyl-serine sulphydrylase (OASs; EC 4.2.99.8) following the procedure described by Ferretti *et al*. (1993). Guaiacol peroxidase (GPX; E.C. 1.11.1.7) activity determination was performed as described in Rugini *et al*. (1997) using guaiacol as hydrogen donor.

All reported activities were measured in triplicate and were linear with time and proportional to the amount of extract used.

Other measurements and statistics: Protein content was determined according to Bradford (1976) using bovine serum albumine (BSA) as standard. Chlorophyll content was measured in filtered homogenate by spectrophotometer (*Lambda 3*, *Perkin Elmer*) at 649 and 665 nm according to Wintermans and De Mots (1965).

Data given are means from triplicate assays representative of four independent experiments. S.D. did not exceed 5 % of the means.

Results

In this work, a non-lethal Cd concentration was applied which induced similar decrease of the plant fresh mass in both growth conditions. From first to second harvest (7 and 21 d from Cd addition, respectively), shoot fresh and dry mass of oat control plants increased in both soil (+42 and +129 %, respectively) and hydroponic (+63 and +85 %, respectively) cultures (Table 1), showing that both experimental systems allowed plant growth.

After exposure to Cd, plants showed a significant decrease in shoot growth with increasing time. In soil grown plants, fresh mass decreased by 32 and 48 % and similarly dry mass decreased by 33 and 43 % (7 and 21 d after Cd application, respectively) (Table 1). Also hydroponically cultured plants showed similar growth reduction. After 7 and 21 d from treatment, fresh mass of treated plants was 30 and 49 %, respectively, lower than that of controls, whereas dry mass was 21 and 29 %, respectively. Parallel reductions in fresh and dry mass

caused by Cd application suggest a complex interference with plant metabolism.

Chlorophyll content was severely decreased by 7 and 21 d of Cd treatment, and it was 26 and 32 % in soil cultured plants, and 27 and 13 % in hydroponic grown plants, lower than the respective control (Table 1). In contrast to growth and chlorophyll content, protein content was not different in shoots of control and Cd exposed oat plants in both growing conditions (Table 1).

Cd only slightly influenced the activity of two enzymes involved in C metabolism, PEPC and RUBPC (Table 1). Cd treatment caused an 11 and 18 % decrease of PEPC activity (after 7 and 21 d, respectively) in soil cultured plants, whereas the same treatment had no negative effect on hydroponic grown plants. On the other hand, in soil cultured plants RUBPC activity was initially increased (+11 %) but then decreased (-28 %) as leaf aged and Cd effect proceeded.

Subsequently, we examined Cd effects on N metabolism determining GS and GDH activity (Table 1). GS showed scant sensibility to Cd application, in both growth conditions. On the other hand, GDH exhibited a different behaviour that was dependent to growth condition. In particular, this enzyme was almost unaffected by Cd exposure in soil grown plants, but it was significantly increased in hydroponic cultured plants (+30 and +26 % after 7 and 21 d, respectively).

Since the most prevalent mechanism of adaptation to toxic concentration of heavy metals is the chelation by PCs, our research has focused on sulphur metabolism determining the effects of Cd on activity of ATPS and OASS, the first and the last enzyme of sulphate assimilation pathway, respectively (Schwenn and Trebst 1976). The obtained results (Table 1) indicate that Cd application differently affected the two enzymes. Exposure to Cd caused no significant effect on ATPS activity in both growth conditions. On the other hand, OASS activity showed a substantial increase after Cd treatment (53 and 40 %, in soil cultured plants, and 15 and 11 %, in hydroponically grown plants, after 7 and 21 d, respectively).

One possible mechanism by which heavy metals may damage plant tissues is the stimulation of free radical production, so we finally examined whether the guaiacol peroxidase (GPX), an enzyme controlling oxidative stress, plays a role in the response mechanism towards Cd. Plants exposed to Cd in both growth conditions showed an initial increase of GPX activity compared to control, that was followed by a decrease (Table 1). Cd-induced stimulation of GPX activity was particularly evident for hydroponically grown plants.

Table 1. Growth parameters [g plant^{-1}], chlorophyll and protein contents [mg g^{-1} (f.m.)], and activities of PEPC [mmol g^{-1}(protein) s^{-1}], RuBPC [mol g^{-1}(protein) s^{-1}], GS [mol g^{-1}(protein) s^{-1}], GDH [mol g^{-1}(protein) s^{-1}], ATPS [mmol g^{-1}(protein) s^{-1}], OASS [mol g^{-1}(protein) s^{-1}], and GPX [ΔA_{470} g^{-1}(protein) h^{-1}] of soil and hydroponic cultured oat plants grown for 7 and 21 d in the presence of Cd. Values in parenthesis are % of control (100 %). Data given are means from triplicate assays representative of four independent experiments. S.D. did not exceed 5 % of the means.

Parameter		Soil culture		Hydroponic culture	
		7 d	21 d	7 d	21 d
Fresh mass	control	0.626	0.888	0.271	0.443
	treated	0.427 (68)	0.460 (52)	0.191 (70)	0.226 (51)
Dry mass	control	0.055	0.126	0.020	0.037
	treated	0.037 (67)	0.072 (57)	0.016 (79)	0.026 (71)
Chlorophyll content	control	0.596	0.570	0.333	0.339
	treated	0.443 (74)	0.390 (68)	0.245 (73)	0.298 (87)
Protein content	control	4.323	4.177	2.600	2.470
	treated	4.123 (95)	4.462 (106)	2.854 (109)	2.279 (92)
PEPC	control	6.66	5.76	5.36	5.22
	treated	5.94 (89)	4.79 (82)	5.79 (108)	5.51 (105)
RuBPC	control	12.89	12.85	19.15	11.01
	treated	14.29 (111)	9.25 (72)	18.97 (99)	10.87 (98)
GS	control	21.87	19.64	8.75	8.80
	treated	22.20 (101)	18.97 (96)	10.07 (115)	9.09 (103)
GDH	control	1.99	2.06	1.27	1.33
	treated	1.64 (82)	2.07 (100)	1.65 (130)	1.67 (126)
ATPs	control	411.5	395.6	652.3	725.8
	treated	369.7 (89)	375.5 (95)	546.5 (84)	552.9 (76)
OASs	control	373.7	315.4	855.4	915.8
	treated	573.8 (153)	444.2 (140)	982.4 (115)	1017.4 (111)
GPX	control	57.4	58.9	98.4	78.6
	treated	70.6 (122)	33.5 (57)	164.4 (167)	73.2 (93)

Discussion

Cd-exposure [0.154 mg g^{-1}(d.s.) and 100 μM $CdSO_4$, respectively, in soil and hydroponic culture] for 7 and 21 d caused reduction of plant growth independently on growth condition: this finding let us to relate obtained results from the above mentioned approaches (soil and hydroponic culture). Observed decrease in chlorophyll content in response to Cd supply may be due to disorders in chlorophyll biosynthesis (Prassad and Prassad 1987) as well as to speeding up ageing of the photosynthetic apparatus (Krupa and Baszynski 1995).

Obtained results show Cd-induced changes in investigated enzyme activities and illustrate that *in vivo* Cd uptake was positively correlated with some enzyme activities and negatively correlated with other ones. The significance of the former response has not been elucidated (Mattioni *et al*. 1997). It is reasonable to think that enzyme stimulation plays a role in the cellular protection against metal toxicity, but it cannot be excluded that in some cases the increased enzyme activity might represent a toxic effect. Moreover, most of enzyme activities were similarly affected in both culture conditions. An exception was represented by PEPC and RUBPC activities, that were differently influenced by Cd exposure depending to growth condition. In particular, PEPC activity was slightly inhibited in soil cultured plants, but its activity was nearly not affected in hydroponically cultured plants. Also RUBPC activity was not affected by Cd exposure in hydroponic culture, but we reported that soil cultured plants showed an initial stimulation of the enzyme activity followed by its inhibition. It is well known that the inhibitory effect of Cd on photosynthesis is due to inhibition of different reaction steps of the Calvin cycle (Prasad 1995). The unexpected pattern of RUBPC might be explained by a transient response mechanism of plants.

As regards nitrogen metabolism, the finding that GS showed scant sensibility to Cd application might be explained taking into account that GS functions as the major assimilatory enzyme for ammonia and it is distributed in different subcellular locations and in different tissues and organs. The enzyme is present in the chloroplasts and in the cytosol in two physically distinct forms: G1 and G2, respectively (Marttila *et al.* 1993). The subcellular distribution could allow GS being less susceptible to Cd and remaining active longer than other enzymes. Then again, GDH is present in abundance in plant tissues but the physiological role of the enzyme remains undefined (Lam *et al.* 1995, Oaks 1995, Pahlich 1996). Results obtained by Robinson *et al.* (1991) and later by Fox *et al*. (1995) showed that GDH plays no role in ammonium assimilation and it is active in the catabolism of glutamate in higher plants in response to a deficiency of carbon. The particular pattern of GDH activity might be elucidated assuming that the different growth conditions (soil and hydroponic culture) could influence leaf senescence program. Senescence is a highly regulated, ordered series of events involving a decline in photosynthesis, dismantling of chloroplasts, degradation of macromolecules such as proteins, nucleic acids and lipids, loss of chlorophyll, and mobilization of nutrients to developing parts of the plant (Smart 1994, Buchanan-Wollaston 1997). Chlorophyll catabolism is the most visible symptom of leaf senescence, but in general, yellowing and protein N remobilisation are well correlated (Thomas *et al*. 2002). GDH is one of the few enzymes capable of releasing amino nitrogen from amino acids to give a keto-acid and NH_3 that can be separately recycled to be used in respiration and amide formation, respectively. GDH may be expected to function in the deaminating direction in tissues that are converting amino acids into transport compounds with a low C/N ratio, for example senescing leaves (Miflin and Habash 2002).

Concerning sulphur metabolism, our data report that the activity of OASS was more affected by Cd than ATPS, probably for the role of this enzyme in the production of cysteine. In fact, OASS activity is required in the final step of inorganic sulphide assimilation in plants and reduced sulphur in the form of cysteine is needed for many different functions in plant cell (Rennenberg *et al*. 1990). On the other hand, Nussbaum *et al.* (1988) reported an increase in maize leaf ATPs activity when plants were exposed to 5 mM Cd, whereas with higher concentrations the enzyme was strongly inhibited. The overall results indicate that Cd influenced ATPs activity to different extents, depending on the plant species, cultivars, or experimental condition. On the other hand, increase of the OASs activity was common to both soil and hydroponically cultured plants and, in particular, the values of stimulation in soil cultured plants were higher than those recorded for the hydroponically cultured plants. As above discussed, the main role of this enzyme is the production of cysteine and it is well known that cysteine is also essential as a precursor for glutathione and the glutathione derivatives that respond to plant stress as PC (Rauser 1990, 1995). Kuske *et al*. (1996) found that Cd-tolerant cell cultures contain 1.8 times as much constitutive OASS activity as the wild-type cell line, and 2.9 times more than the Cd-hypersensitive cell line and this allowed a faster cellular response to Cd exposure. Our results show that OASS activity was increased by Cd exposure, and this is consistent with the general premise that the availability of reduced sulphur is an essential term for PC synthesis (Rennenberg *et al.* 1990). PC have been extensively considered as the most prevalent mechanism of adaptation to toxic concentration of heavy metals. Thanks to their ability to form complexes with heavy metals such as Cd, it is generally accepted that they can maintain internal concentrations of these metals below their toxicity threshold so reducing their negative effects in plants (Rauser 1995, Sanità di Toppi and Gabbrielli 1999). Research is needed to understand if this finding is accomplished by increase of glutathione and PC synthesis. It is however reasonable to suppose that OASS

may play an important role in the resistance mechanisms involved in Cd detoxification.

Taken together, results show several Cd-induced effects that resemble the ageing phenomena, *i.e*. the lower chlorophyll and protein content and the higher GPX activity (Buchanan-Wollaston 1997). Peroxidase induction is a general response of higher plants to uptake of toxic amounts of metals and it is likely to be related to oxidative reactions corresponding to an increase in peroxides and free radicals in the plant cells (Van Assche and Clijsters 1990). Our finding confirm that oxidative disorder is part of the overall expression of Cd toxicity in oat leaves.

In this way, it is interesting to consider the evidence that both approaches used in this work allow to investigate Cd effects on metabolic activity of oat plants. In particular, the hydroponic culture provides a representative system of field conditions, but it is noteworthy that leaves from hydroponic culture present significant indicators of senescence initiation (*i.e.* decrease in chlorophyll content accomplished by stimulation of GDH and GPX activity) thus suggesting that hydroponic system could speed up leaf senescence.

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