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Cd-induced membrane damages and changes in soluble protein and free amino acid contents in young barley plants

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Abstract: Barley (*Hordeum vulgare* L., cvs. Obzor and Hemus) plants were grown in a Knop nutrient solution with and without 54 µM Cd^{2+} for 12 days. Some data concerning the ability of Cd to induce membrane damages as well as changes in protein and free amino acids contents of barley plants from the same cultivars are presented in the this work. The Cd treatment enhanced both membrane lipid peroxidation and leakage of K^+ from the tissue as well as decreased chlorophyll content. Cd also diminished the content of soluble protein and glutamic acid in leaves and increased that of aspartic acid and several sress-related free amino acids. The negative effects were stronger expressed in the less tolerant barley cv. Hemus.

Keywords: barley, cadmium, soluble protein, amino acids, and chlorophyll.

استحثاث عنصر الكادميوم للاضرار فى بروتين قابل للذوبان و محتويات الاحماض الامنينة الحرة في بادرات نبات الشعير

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الملخص: تم زراعة نباتات الشعير (*Hordeum vulgare* L., cvs. Obzor and Hemus) مع وبدون ($Cd2^+$ 54 M) لمدة 12 يوم. وتم الحصول على بعض النتائج الخاصة بقدرة الكادميوم على استحثاث اضرار في الأغشية وأيضاً في البروتين والأحماض الأمينية فى نباتات الشعير من الاصناف نفسها من خلال هذه التجربة.رفعت المعاملة بالكادميوم من عملية الأكسدة(peroxidation) وتسرب أيونات البوتاسيوم K^+ من الانسجة وكذلك تناقص محتوى الكلورفيل.أيضاً خفض الكادميوم محتوى البروتينالذائب وحمض الجلوتاميك فى الاوراق وسبب ذيادة حمض الاسبرتيك وعدة احماض امينية حرة ذات صلة بالضغوطات. . التأثير السلبي كان اكثر وضوحا في صنف الشعير الاقل تحملا cv Hemus.

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Introduction

Cd is widely spread environmental pollutant. It has no biological function, but is easily assimilated by plants being toxic at even low concentrations. Cd phytotoxicity has been studied for a long time (Foy et al., 1978; Milone et al., 2003), but some unclear aspects still remain (Vassilev and Yordanov, 1997; Krupa, 1999). For example, the nature of the so-called Cd-induced "premature senescence" (Baszynski et al., 1980; Skorzynska et al., 1991) is not completely understood. This syndrome may be recognised by the degradation of the lamellar structure of the chloroplasts (Barcelo et al., 1988; Vassilev et al., 1995); loss of chlorophyll (Malik et al., 1992; Vassilev et al., 1998); similar pattern of ^{14}C incorporation in the primary photoproducts with that in ageing leaves (Vassilev et al., 1997), etc. These negative Cd effects may be provoked by different mechanisms, which finally are multiplied and leading plant performance to premature senescence. For example, the loss of chlorophyll in Cd-exposed plants may be due to an inhibition of its biosynthesis (Stobart et al., 1985), enhanced enzymatic degradation (Abdel-Basset et al., 1995), oxidative damage (Somashekaraiah, 1992) as well as a decrease of chloroplast density per cell (Baryla et al., 2001). Also, the mechanism of Cd-induced degradation of chloroplast membrane may be a consequence of lipoxygenase induction resulting in the oxidation of polyunsaturated fatty acids (Somashekariah et al., 1992), increased galactolipase activity leading to monogalactolipids hydrolysis (Krupa and Baszynski, 1995), oxidative damage (Hendry et al., 1992, Sandalio et al., 2001), etc.

The integral senescence process is tightly related to nitrogen nutrition status of plants (Hőrtensteiner and Feller, 2002). It has been shown that Cd may interfere with nitrogen metabolism in plants (Kastori et al., 1997). In fact, Boussama et al. (1999) showed that Cd declined in the total protein content with a progressive increase of the protease activity in the tissue, which is characteristic for ageing leaves. In addition, it was found that it retarded glutamine synthetase activity (Hsu and Kao, 2003a) and increased free amino acids content in Cd-exposed rice plants (Hsu and Kao, 2003b). The changes appearing in free amino acids as a response to different stress factors are important for plant metabolism as they are precursors of both proteins and nucleic acids, but in Cd-exposed plants this aspect has not been often addressed. More of the data available showed only the changes of proline (Alia and Saradhi, 1991; Kastori et al., 1992), but some other amino acids, especially those derived by aspartic acid, including asparagines, isoleucine, leucine, methionine, and valine may also respond to environmental stresses (Fukutoki and Yamada, 1981). In fact, Costa and Morel (1994) found that Cd-exposed lettuce proline level was not changed while asparagines, methionine and lysine increased.

We have already reported several aspects of Cd toxicity in two barley cultivars *cv.* Obzor and *cv.* Hemus (Vassilev et al., 1995, 1997, 1998). Briefly, c*v*. Hemus accumulated significantly higher Cd levels than *cv*. Obzor and therefore expressed less metal tolerance as judged by both growth and photosynthetic performance. Here, we present some data concerning the ability of Cd to induce membrane damages as well as changes in protein and free amino acids contents of barley plants from the same cultivars.

Material and Methods

Plant material and growth conditions

Seeds of barley (*Hordeum vulgare* L., *cvs.* Obzor and Hemus) were germinated for 2 days on a wet filter paper at 25°C. The seedlings were transferred to ½ strength Knop nutrient solution, enriched by micronutrients according to Hoagland, and with or without 54 μM Cd^{2+} in the form of $3CdSO_4.8H_2O$ as described elsewhere (Vassilev et al., 1997). Plants were grown for 12 days at photon flux density of 120 μM m^{-2} s^{-1} (PAR), day/night temperature of 22 ± 2 / 18 ± 2°C, and a 14 hours photoperiod. The nutrient solution was aerated automatically and changed weekly.

Lipid peroxidation

The lipid peroxidation in plant tissues was determined as 2-thiobarbituric acid (TBA) reactive substances (TBARS) as described previously (Heath and Packer, 1968). Plant material (0.5 g) was extracted in mortar with sand and 5 ml of 0.5 trichloric acid (TCA). After centrifugation at 20 000 g for 10 min at 20°C, 1 ml of the supernatant was added to 4 ml of 0.5% TBA made in 20% TCA. This solution was boiled in a bath at 95°C during 30 min and then quickly cooled in ice for 5 min. After centrifugation at 10 000 g for 10 min at 20°C, the absorbance was measured with spectrophotometer (UV 1601 PC, Shimadzu, Japan) at 532 nm. Correction of non-specific turbidity was made by subtracting the absorbance value taken at 600 nm. The level of lipid peroxidation was expressed as nM g^{-1} FW formed using an extinction coefficient of 155 mM cm^{-1}.

Leakage of K+ ions

The leakage of potassium (K^+) ions was performed as described by Milone et al. (2003) with slight modifications. The samples from roots and leaves were excised and washed to remove the contents of the cut cells. Samples were incubated in 25 ml of double distilled water, shaken at 21°C for 24 h and aliquots for K^+ determination were taken. Samples were then heated at 90°C for 15 min, shaken for one hour prior to the measurement of total K^+ leakage from the killed cells. Potassium released was measured by atomic absorption spectrophotometry with hollow-cathode lamp.

Soluble protein, free amino acids and chlorophyll contents

The soluble protein content was determined by a method of Lowry et al. (1951). The free amino acids were extracted from leaves in 80% ethanol, the extract was passed through the ion-exchange resin *Dowex 50X2-200* and eluated using 1m NH_4OH. Due to presence of glutamine and asparagine amides, a hydrolysis by 0.2 n HCl was performed. The content of several amino acids was determined using an automatical amino analyzer *AAA-881* (*Microtechna, Praga, Czechoslovakia*).

Chlorophyll content was extracted in acetone, measured spectrophotometrically and calculated according to Lichtenthaler (1987).

Statistical analysis

The data shown in the present work are from a representative experiment. Values shown in the tables are means of 3 replicates. Significant differences were determined by the Student's *t*-test.

Results and Discussion

Cd-exposed barley plants from both cultivars were distinguished by their inhibited growth and the presence of toxicity symptoms, such as spot chlorosis, turning to yellowing and complete dying of leaf tips as well as browning of the roots accompanied by reduction in the primary root branches as described elsewhere (Vassilev et al., 1997, 1998). In this study we found these apparent toxicity symptoms were accompanied by damages to membrane lipids and protein turnover. We have measured Cd-induced changes in membrane integrity by two ways: by lipid peroxidation as well as through K^+ leakage from the plant tissue. The content of MDA equivalents in both roots and leaves increased 2-3 fold (Table 1) indicating oxidative damage. The negative Cd effect was stronger expressed in *cv*. Hemus and was higher in the roots, where MDA equivalents increased by 328 and 342% than in leaves (182 and 246%) for the *cv*. Obzor and *cv*. Hemus, respectively. The values of K^+ leakage, a common consequence of membrane damage, were also significantly higher in Cd-exposed plants showing similar trends with membrane lipid peroxidation. The results obtained were in line with those recently found in Cd-treated barley (Metwally et al., 2003) and wheat (Milone et al., 2003) plants. The 12 days of Cd exposure diminished the chlorophyll content (chl a and chl b) by about 20%, but it was clear even earlier by the developing chlorotic symptoms. All together the obtained results gave evidence that Cd toxicity in barley plants was performed, instead of other ways, by oxidative related damages.

Table 1. Lipid peroxidation (MDA equivalents; nmol g^{-1} FW), K^+ leakage (% of total leakage) and chlorophylls content (mg g^{-1} DW) of two barley cultivars after 12 days exposure to Cd.

Parameters	Treatments	
	control - (0 Cd)	54 μM Cd L^{-1}
	***cv*. Obzor**	
MDA equivalents in roots	10.5 ± 1.1	34.5 ± 1.9 (328)**
MDA equivalents in leaves	12.7 ± 1.3	23.1 ± 2.1(182)**
K^+ leakage in roots	25.6 ± 1.3	45.5 ± 0.4 (178)**
K^+ leakage in leaves	17.5 ± 1.5	28.6 ± 1.2 (163)**
Chlorophyll *a* content	7.64 ± 0.45	6.24 ± 0.24 (83)**
Chlorophyll *b* content	4.29 ± 0.25	3.83 ± 0.28 (77)**
	***cv*. Hemus**	
MDA equivalents in roots	13.2 ± 1.2	45.2 ± 0.1 (342)**
MDA equivalents in leaves	12.9 ± 1.4	31.7 ± 0.3 (246)**
K^+ leakage in roots	27.2 ± 0.8	52.1 ± 1.7 (192)**
K^+ leakage in leaves	21.5 ± 0.5	38.4 ± 1.1 (179)**
Chlorophyll *a* content	7.90 ± 0.38	6.27 ± 0.39 (80)**
Chlorophyll *b* content	4.43 ± 0.15	3.64 ± 0.17 (82)**

**P<0.01

Cd has low redox potential and therefore it cannot participate in biological redox reactions as, for example Cu does (Lidon, 1999), but there are some evidence that it could perform oxidative related disturbances, including lipid peroxidation (Hendry et al., 1992; Sandalio et al., 2001; Vassilev et al., 2004). This effect might be explained, at least partially, by the known high affinity of Cd ion to the functional groups of proteins, which may affect their functional properties (Vangronsveld and Clijsters, 1994). In fact, Cd was found to increase, but also to decrease activities of several antioxidative enzymes (Sandalio et al., 2001; Milone et al., 2003). Thus, we may suggest when plant cells are not able to keep low mesophyll Cd level through efficient detoxifying mechanisms, this may lead to depletion of cell defense network and as consequence to Cd-provoked oxidative damages to important molecules, including lipids. As the lipids are main membrane constituents, it is not surprising that Cd disturbed membrane permeability allowing high level of K^+ leakage (Table 1) with is in agreement with the results shown previously by Milone et al. (2003). Cd may decrease chlorophyll content by inhibition of its biosynthesis (Stobart et al., 1985) as well as diminished chloroplast density per cell (Baryla et al., 2001), but in our study, where apparent severe toxicity was relatively quickly achieved, it more likely was due to oxidative damage as well as enhanced enzymatic degradation as found by Abdel-Basset et al. (1995).

Table 2 presents data about Cd-induced changes in the contents of both soluble protein and selected free amino acids (proline, leucine, isoleucine, methionine, valine as well as glutamic and aspartic acid polls in barley leaves. In general, the protein level decreased by 19 – 22%, whereas the presented amino acids showed significantly higher levels excepting the glutamic poll (glutamic acid + glutamine). The levels of the measured amino acids were higher in Cd-exposed plants from *cv*. Hemus than those in *cv*. Obzor, with small exceptions. The highest increase was found in proline levels – 193 and 251% in *cv*. Obzor and *cv*. Hemus, respectively. The glutamic poll (glutamic acid + glutamine) decreased in Cd-exposed plants by 7 – 30%, whereas that of aspartic poll (aspartic acid + asparagine) increased significantly by 23 – 30%.

Table 2. Soluble protein content (mg g DW^{-1}) and free amino acids content (nmol g FW^{-1}) in the leaves of two barley cultivars after 12 days exposure to Cd.

Parameters	Treatments control - (0 Cd)	54 μM Cd L^{-1}
	***cv*. Obzor**	
Soluble protein	155.1 ± 7.8	126.1 ± 7.3 (81) *
Glutamic acid + Glutamine	2249 ± 156	1571 ± 186 (70)*
Aspartic acid + Asparagine	1816 ± 85	2226 ± 111 (123)*
Proline	112.9 ± 16.6	217.6 ± 2.9 (193)**
Leucine	79.1 ± 15.5	102.5 ± 18.9 (130)
Isoleucine	32.3 ± 5.9	49.7 ± 3.7 (154)*
Methionine	15.7 ± 7.2	22.5 ± 5.8 (143)
Valine	85.0 ± 9.8	156.6 ± 9.2 (184)**
	***cv*. Hemus**	
Soluble protein	149.9 ± 9.4	120.6 ± 4.9 (78)*
Glutamic acid + Glutamine	2084 ± 134	1947 ± 79 (93)
Aspartic acid + Asparagine	1510 ± 121	1959 ± 92 (130)*
Proline	108.6 ± 8.7	272.1 ± 14.2 (251)***
Leucine	84.0 ± 9.3	127.3 ± 11.0 (152)*
Isoleucine	34.8 ± 6.1	63.3 ± 7.9 (182)*
Methionine	17.0 ± 5.6	19.5 ± 8.3 (115)
Valine	126.0 ± 14.1	205.2 ± 15.4 (169)*

*P<0.05 **P<0.01 ***P<0.001

Two reasons for the observed protein and amino acids changes may be proposed: proteolysis or accumulation of specific amino acids. Based on the trends presented by the glutamic and aspartic "polls", we may suggest as more probable the first case. It is known that aspartic acid has a prevailing role in the metabolism of the senescing leaves, while glutamic acid is involved in nitrogen assimilation and transport processes within the plants (Urquhart and Joy, 1981). Thus, the presented trends of both amino acids were well fitted with the general performance of Cd-exposed barley plants, having lower photosynthetic activity and growth rate (Vassilev et al., 1997). Furthermore, they are in line of the Journet et al. (1986) opinion's that the degradation of proteins through amino acid catabolism may act as an adaptation of plant cell to carbohydrates deficiency.

On the other hand, it is also known that plant cells respond to different stress conditions by the so-called osmotic adjustment to maintain water balance (Barcelo et al., 1988). Several amino acids, mainly proline, take part in this process (Fukutoki and Yamada, 1981). In fact, Alia and Saradhi (1991) found that Cd may induce proline accumulation in the tissue, but did not link this effect with osmotic adjustment. Contrarily, Costa and Morel (1994) did not find higher proline level in Cd-exposed lettuce. We found in our study almost two-fold higher proline level in the leaves of Cd-exposed barley. According to Paleg and Aspinall (1981) the critical water potential level (Ψ) when proline accumulation in barley plants begins is around - 0.7 MPa but under drought may reach in order higher values. We have previously reported that Ψ in Cd-exposed barley plants from the same cultivars varied between –0.6 and –0.7 MPa (Vassilev et al., 1997; Vassilev et al., 1998), thus these plants were in the beginning of water stress. Summing up, we consider the observed higher level of both proline as well as the other stress-related free amino acids to be more likely a consequence of Cd-enhanced proteolysis than Cd-imposed osmotic adjustment.

In conclusion, we found in this study that Cd treatment enhanced membrane lipid peroxidation and K^+ leakage from plant tissue as well as decreased chlorophylls content in

barley plants. Cd also diminished the contents of both soluble protein and glutamic acid in leaves as well as increased that of aspartic acid and several sress-related free amino acids. The negative effects were stronger expressed in the less tolerant barley *cv*. Hemus.

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