Plant Growth Regulation **39:** 271–276, 2003.

Accumulation of ammonium ion in cadmium tolerant and sensitive cultivars of *Oryza sativa*

Yi Ting Hsu and Ching Huei Kao*
*Department of Agronomy, National Taiwan University, Taipei, Taiwan, Republic of China; *Author for correspondence (e-mail: kaoch@ccms.ntu.edu.tw; phone: +886-2-23698159; fax: +886-2-23620879)*

Received 11 July 2002; accepted in revised form 18 September 2002

Key words: Ammonium ion, Cadmium, Glutamine synthetase, *Oryza sativa* L

Abstract

Cd-tolerant and Cd-sensitive rice cultivars were used to study the role of NH_4^+ accumulation in Cd-induced toxicity. NH_4^+ accumulation seems to be involved in regulating the toxicity of rice seedlings caused by $CdCl_2$. This conclusion was based on the observations that (a) on treatment with $CdCl_2$, NH_4^+ content increased rapidly in the leaves of the Cd-sensitive cultivar (cv. Taichung Native 1, TN1) but not in the Cd-tolerant cultivar (cv. Tainumg 67, TNG67), (b) pretreatment with abscisic acid (ABA) enhanced Cd tolerance and reduced Cd-induced NH_4^+ accumulation in TN1 seedlings, (c) exogenous application of the ABA biosynthesis inhibitor, fluridone, decreased Cd tolerance and increased NH_4^+ content in leaves of TNG67, (d) exogenous application of phosphinothricin, an inhibitor of glutamine synthetase (GS), which resulted in NH_4^+ accumulation in the leaves, also induced toxicity similar to Cd in TN1 seedlings. Evidence is presented to show that Cd-induced NH_4^+ accumulation in TN1 leaves is attributable to a decrease in GS activity. Since Cd-treated TN1 leaves had higher glutamine and glutamate contents than control leaves, it is unlikely that glutamine (or glutamate) depletion is the mechanism which regulates Cd-induced toxicity.

Abbreviations: ABA – abscisic acid, DW – dry weight, FW – initial fresh weight, GS – glutamine synthetase, PPT – phosphinothricin, TN1 – Taichung Native 1, TNG67 – Tainung 67

Introduction

Cadmium (Cd) is a divalent heavy metal cation and is one of the most toxic heavy metals with no described physiological function. It enters the environment through industrial processes and to a lesser exert from natural weathering (di Toppi and Gabbrieli 1999). Although not essential for plant growth, this metal is readily taken up by roots and translocated into aerial organs where it can accumulate to high levels. It has been shown that Cd interacts with the plant's water balance (Barceló and Poschenrieder 1990; Costa and Morel 1994), inhibits stomatal opening (Barceló and Poschenrieder 1990), lowers chlorophyll content (Larsson et al. 1998), reduces growth (Chen and Kao 1995), damages the photosynthetic apparatus (Krupa 1988; Sidlecka and Baszynsky 1993), and produces oxidative stress (Chien et al. 2001; Hendry et al. 1992; Somashekaraiah et al. 1992). In spite of the considerable literature on the subject, the mechanisms of Cd toxicity are not known with any certainty.

The effect of Cd stress on nitrogen metabolism has been little investigated (Boussama et al. 1999). Nitrate reductase and glutamine synthetase (GS) activities decrease with Cd stress (Boussama et al. 1999; Chien and Kao 2000; Chugh et al. 1992; Ouariti et al. 1997; Singh et al. 1994; Weigel and Jagar 1980). A decline in activity of GS in leaves caused by excess Cd may result, at least in part, in an accumulation of NH_4^+ in leaves. NH_4^+ is a central intermediate of nitrogen metabolism in plants (Miflin and Lea 1976), but a high content of NH_4^+ is known to have toxic effects on plant cells (Givan 1979). Relatively

little work has been done to study the effect of Cd on NH_4^+ accumulation and we know little about the relationship between NH_4^+ accumulation and Cd-induced toxicity. Thus it is of great interest to examine the role of NH_4^+ in regulating Cd-induced toxicity of plants. The two cultivars of rice seedlings investigated herein differ markedly in Cd tolerance and thus have been chosen as model systems to evaluate whether NH_4^+ accumulation is linked to Cd toxicity in rice seedlings. The seedlings of Tainung 67 (TNG67) cultivar used in our experiment are more tolerant to Cd than those of Taichung Native (TN1).

Material and methods

Plant cultivaion and treatment

Two rice (*Oryza sativa* L.) cultivars, an Indica type cultivar, TN1 and a Japonica cultivar, TNG67 were used in this study. Seeds were sterilised with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. These seeds were then germinated in Petri dishes on wetted filter papers at 37 °C in the dark. After 48 h incubation, uniformly germinated seeds were selected and cultivated in a 250 ml beaker containing half-strength Kimura B solution as described previously (Chu and Lee 1989). The hydroponically cultivated seedlings were grown in a Phytotron with natural light at 30 °C day/25 °C night and 90% relative humidity. Twelve-day-old seedlings with three leaves were used in all experiments.

For Cd, abscisic acid (ABA), fluridone, and phosphinothricin (PPT) treatments, the chemicals were added directly to the culture solution during the experiment.

Cd determination

For determination of Cd, leaves or roots were dried at 65 °C for 48 h and the dried material ashed at 550 °C for 20 h. The ash residue was incubated with 31% HNO_3 and 17.5% H_2O_2 at 72 °C for 2 h, and dissolved in 0.1 N HCl. Cd was then quantified using an atomic absorption spectrophotometer (Model AA-6800, Shimadzu Kyoto, Japan).

Determination of chlorophyll, protein, NH_4^+, and amino acids

Chlorophyll was determined according to Wintermans and De Mots (1965) after extraction in 96% (v/v) ethanol. For protein determination, leaves were homogenised in a 50 mM sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,600 g for 20 min, and the supernatants used for determination by the method of Bradford (1976). NH_4^+ was extracted and its concentration determined as described previously (Chien and Kao 2000). For determination of glutamate, glutamine and total amino acids, leaf samples were extracted with 2% sulfosalicylic acid and the homogenate was centrifuged at 15,000 g for 20 min. The supernatant was used directly for amino acid analysis (amino acid analyzer, Beckmann 6300, Palo Alto, USA).

Glutamine synthetase (GS) activity

For extraction of GS, leaf samples were homogenised with 10 mM Tris-HCl buffer (pH 7.6, containing 1mM $MgCl_2$, 1 mM EDTA and 1 mM 2-mercaptoethanol) using a chilled pestle and mortar. The homogenate was centrifuged at 15,000 g for 30 min and the resulting supernatant was used for determination of GS activity. The whole extraction procedure was carried out at 4 °C. GS was assayed by the method of Oaks et al. (1980). The reaction mixture contained in a final volume of 1 ml was 80 μmol Tris-HCl buffer, 40 μmol L-glutamic acid, 8 μmol ATP, 24 μmol $MgSO_4$, and 16 μmol NH_2OH; the final pH was 8.0. The reaction was started by addition of the enzyme extract and, after incubation for 30 min at 30 °C, was stopped by adding 2 ml 2.5% (w/v) $FeCl_3$ and 5% (w/v) trichloroacetic acid in 1.5 N HCl. After centrifugation the absorbance of the supernatant was read at 540 nm. One unit of GS activity is defined as 1 μmol L-glutamate γ-monohydroxamate formed per min.

Experimental design

Cd content was expressed on the basis of dry weight (DW). Chlorophyll, protein, NH_4^+, amino acid content and GS activity were expressed per g initial fresh weight (FW). Absolute levels of each measurement varied among experiments because of seasonal effects. However, the patterns of responses to $CdCl_2$ were reproducible. For all measurements, each treatment was repeated four times. All experiments de-

scribed here were repeated at least three times. Similar results and identical trends were obtained each time. The data reported here are from a single experiment.

Results and discussion

In plants, the most apparent symptom of Cd toxicity is chlorosis of the leaves. When rice seedlings of TN1 and TNG67 were treated with 0.5 mM $CdCl_2$ for 7 d, leaf chlorosis was observed in TN1 seedlings, but not in TNG67 seedlings (data not shown). In short term (3 d) experiments, chlorosis was first observed in the second leaves of TN1 seedlings. In the present investigation, unless otherwise indicated, Cd toxicity in the second leaves caused by excess Cd was indicated by a decrease in chlorophyll and protein contents. Figure 1 shows the time courses of chlorophyll and protein contents in the second leaves of TN1 and TNG67 seedlings treated with or without 0.5 mM $CdCl_2$. It is clear that the decrease in chlorophyll and protein contents in TN1 leaves is more pronounced than that in TNG67 leaves, indicating that TNG67 seedlings are a Cd-tolerant cultivar, whereas TN1 seedlings are a Cd-sensitive. Figure 1 also shows the changes in Cd content in rice seedlings treated with 0.5 mM $CdCl_2$. Cd content in the second leaves of TNG67 seedlings remained unchanged after Cd treatment. In contrast, a marked increase in Cd content in Cd-treated TN1 leaves was observed. It seems that avoiding the build-up of Cd in the second leaves of TNG67 prevents the toxic effect of Cd.

Changes in NH_4^+ content in the second leaves of rice seedlings treated with or without $CdCl_2$ are shown in Figure 2. Ammonium ion content in the second leaves remained unchanged in TN1 seedlings treated without $CdCl_2$. It is clear that accumulation of NH_4^+ in the second leaves of TN1 induced by $CdCl_2$ was evident at 1 d after $CdCl_2$ treatment. However, no NH_4^+ accumulated in the second leaves of TNG67 throughout the $CdCl_2$ treatment.

GS is the primary enzyme responsible for NH_4^+ assimilation in plants (Miflin and Lea 1976). We observed that GS activity in the second leaves of TN1 treated with $CdCl_2$ decreased compared with that given no $CdCl_2$ treatment (Figure 2). In contrast, $CdCl_2$ had no effect on the GS activity of the second leaves of TNG67 (Figure 2).

The decrease in GS activity by $CdCl_2$ in the second leaves possibly results from the direct effect of

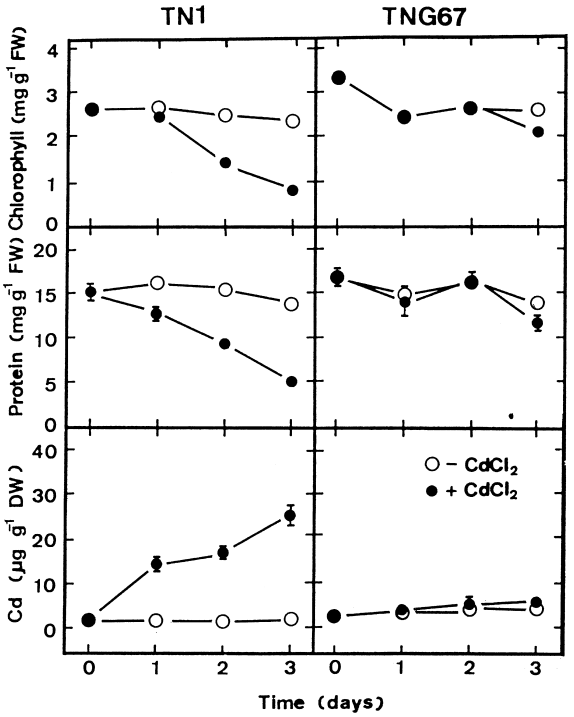

Figure 1. Changes in chlorophyll, protein and Cd contents in the second leaves of rice seedlings either untreated or treated with $CdCl_2$ (0.5 mM). Vertical bars represent standard errors (n = 4)

Cd. This possibility was tested by mixing enzyme extract of the second leaves with various concentrations of $CdCl_2$. GS activity was then assayed 30 min after mixing. It was found that GS activity was directly inhibited by Cd in both TNG67 and TN1 extracts, with the former more inhibited than the latter (Figure 3). Thus, the lack of inhibition of GS activity in the second leaves of TNG67 is mainly due to the lack of increase in Cd content (Figure 1). Current results also suggest that Cd-induced NH_4^+ accumulation in the second leaves of TN1 seedlings is attributable to the decrease in GS activity.

We have observed (unpublished) that ABA plays an important role in Cd tolerance and pretreatment of TN1 seedlings with ABA counteracting Cd-induced toxicity. If accumulation of NH_4^+ is important in regulating toxicity in TN1 seedlings, then pretreatment with ABA is expected to reduce Cd toxicity and NH_4^+ content in TN1 leaves. Since some chlorosis was observed in the second leaves of TN1 treated with ABA for 2 d, Cd toxicity was evaluated by using the third leaves and 1.5 mM $CdCl_2$. As expected, ABA pretreatment reduced Cd toxicity and reduced Cd-induced NH_4^+ accumulation in the third leaves of TN1

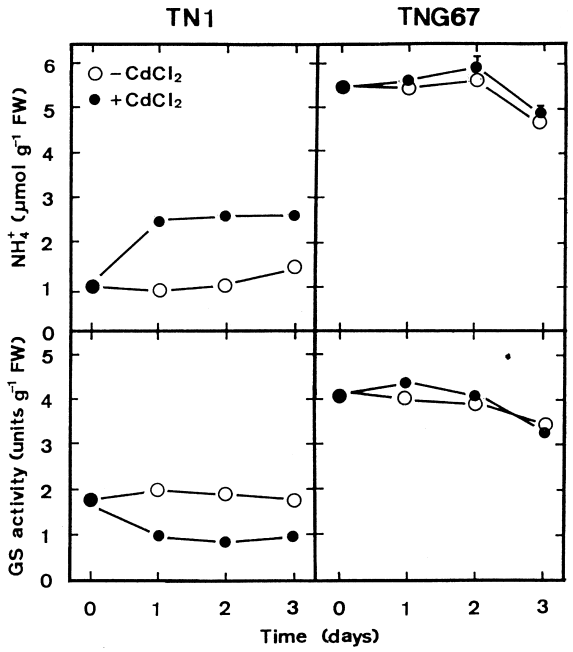

Figure 2. Changes in NH_4^+ and GS activity in the second leaves of rice seedlings either untreated or treated with $CdCl_2$ (0.5 mM). Vertical bars represent standard errors (n = 4)

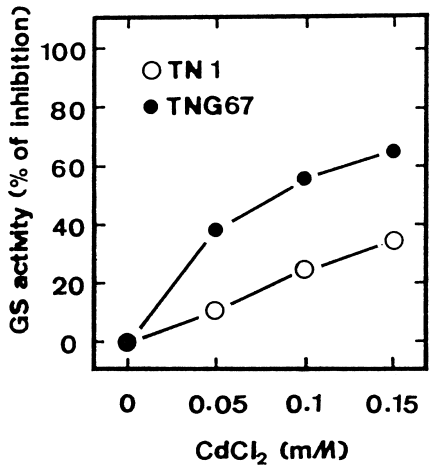

Figure 3. Effect of $CdCl_2$ on the activity of GS extracted from rice leaves. Extracts were prepared from the second leaves of 12-day-old rice seedlings. Assays were conducted 30 min after the addition of various concentrations of $CdCl_2$. Each treatment was repeated four times. The data reported here were from one of three independent experiments

seedlings (Figure 4). Figure 4 also shows that ABA pretreatment reduced the decrease in GS activity in the third leaves of TN1 seedlings.

The results presented in Table 1 show that treatment with fluridone, an inhibitor of ABA biosynthesis, inhibited the increase in ABA content, enhanced

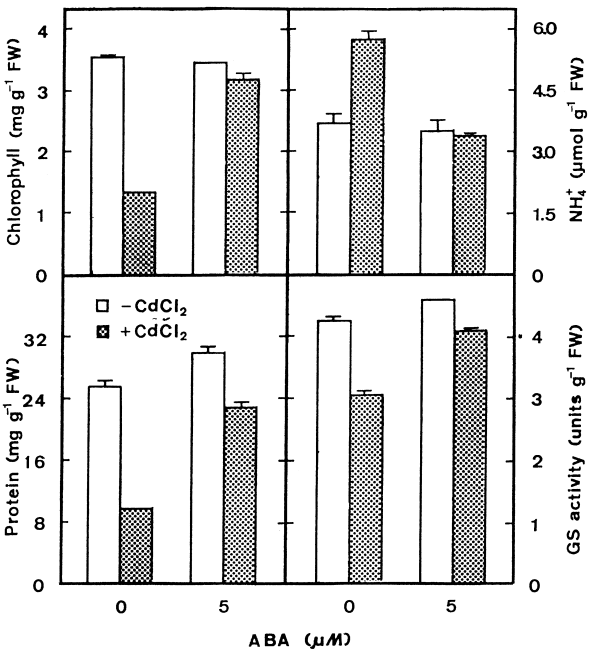

Figure 4. Effect of ABA-pretreatment on the contents of chlorophyll, protein, NH_4^+ and the activity of GS in the third leaves of TN1 rice seedlings. TN1 rice seedlings were pretreated with ABA for 2 d and then either untreated or treated with $CdCl_2$ (1.5 mM) for 2 d. Vertical bars represent standard errors (n = 4)

Cd toxicity, increased NH_4^+ content, and decreased GS activity in the second leaves of TNG67 seedlings.

PPT [2-amino-4-(methylphosphinyl)-butanoic acid, known as glufosinate] is a potent inhibitor of GS (Miflin and Lea 1976). If NH_4^+ accumulation is important in regulating Cd toxicity of TN1 leaves, then PPT treatment is expected to result in a similar toxicity as Cd in TN1 leaves. Table 2 shows that PPT treatment resulted in a decrease in GS activity, an increase in NH_4^+ content, and a decrease in chlorophyll and protein contents.

An inhibition of GS activity in the second leaves of TN1 seedlings may also result in a decrease in glutamine content. It is possible that glutamine depletion could be another reason for the Cd-induced toxicity in the second leaves of TN1 seedlings. To test this possibility, we measured the contents of glutamine, glutamate and total amino acids in the second leaves of rice seedlings either untreated or treated with $CdCl_2$. Contrary to our expectation, Cd-treated second leaves of TN1 rice seedlings had higher contents of glutamine, glutamate and total amino acids than control leaves (Figure 5). Only slightly higher glutamine content in the second leaves of TNG67 seedlings was observed at 2 and 3 d after $CdCl_2$ treat-

Table 1. Effect of fluridone on the contents of ABA, chlorophyll, protein and NH_4^+ and the activity of GS in the second leaves of TNG67 rice seedlings.

Treatment		ABA	Chlorophyll	Protein	NH_4^+	GS activity
CdCl2	Fluridone	(pmol g^{-1} FW)	(mg g^{-1} FW)	(mg g^{-1} FW)	(μmol g^{-1} FW)	(units g^{-1} FW)
–	–	538.6 ± 30.6	3.3 ± 0.46	12.4 ± 1.0	4.7 ± 0.41	3.2 ± 0.24
–	+	252.0 ± 34.9	3.1 ± 0.10	12.6 ± 0.41	4.4 ± 0.40	3.3 ± 0.17
+	–	991.4 ± 117.8	2.8 ± 0.21	12.5 ± 0.31	4.3 ± 0.39	2.8 ± 0.21
+	+	548.8 ± 111.9	2.2 ± 0.07	8.0 ± 0.79	6.1 ± 0.86	2.1 ± 0.02

$CdCl_2$ (0.5 mM) or fluridone (0.2 mM) was added to the culture solution for 2 d. The data represent mean values ± standard errors, n = 4.

Table 2. Effect of phosphinothricin (PPT) on the activity of GS and the contents of NH_4^+, chlorophyll, and protein in the second leaves of TN1 rice seedlings.

Treatment	GS activity (units g^{-1} FW)	NH_4^+ (μmol g^{-1} FW)	Chlorophyll (mg g^{-1} FW)	Protein (mg g^{-1} FW)
– PPT	2.0 ± 0.11	1.6 ± 0.16	3.4 ± 0.09	12.1 ± 1.5
+ PPT	1.5 ± 0.20	4.0 ± 0.49	2.5 ± 0.12	9.0 ± 1.5

PPT (10 μM) was added to the culture solution for 2 d. The data represent mean values ± standard errors, n = 4.

ment (Figure 5). Thus, the conclusion to be drawn from the present work is that NH_4^+ accumulation rather than glutamine depletion is associated with Cd-induced toxicity in TN1 seedlings.

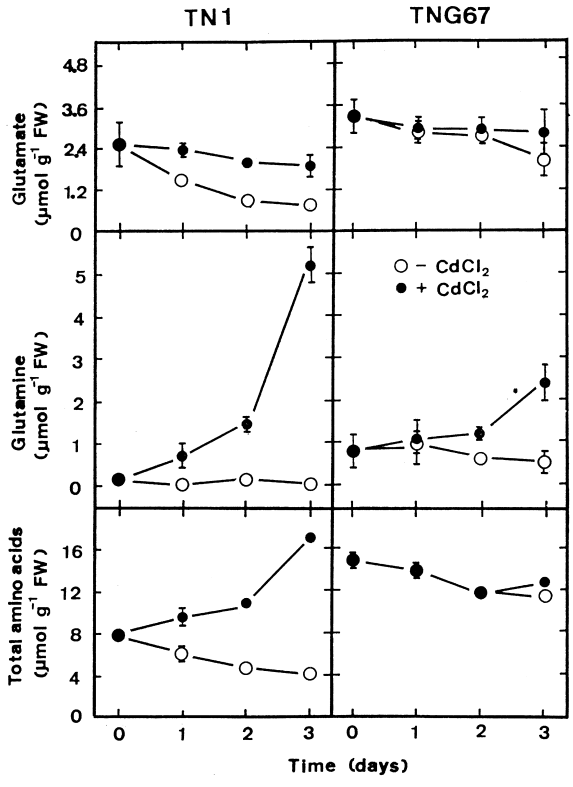

Figure 5. Changes in the contents of glutamate, glutamine, and total amino acids in the second leaves of rice seedlings either untreated or treated with $CdCl_2$ (0.5 mM). Vertical bars represent standard errors (n = 4)

Acknowledgements

This work was supported by the National Science Council of the Republic of China.

References

Barceló J. and Poschenrieder C. 1990. Plant water relations as affected by heavy metal stress: review. J. Plant. Nutr. 13: 1–37.

Boussama N., Ouariti O. and Ghorbal M.H. 1999. Changes in growth and nitrogen assimilation in barley seedlings under cadmium stress. J. Plant. Nutr. 22: 731–752.

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Chen S.L. and Kao C.H. 1995. Cd induced changes in proline level and peroxidase activity in roots of rice seedlings. Plant Growth Regul. 17: 67–71.

Chien H.-F. and Kao C.H. 2000. Accumulation of ammonium in rice leaves in response to excess cadmium. Plant Sci. 156: 111–115.

Chien H.-F., Wang J.-W., Lin C.C. and Kao C.H. 2001. Cadmium toxicity of rice leaves is mediated through lipid peroxidation. Plant Growth Regul. 33: 205–213.

Chu C. and Lee T.M. 1989. The relationship between ethylene biosynthesis and chilling tolerance in seedlings of rice (*Oryza sativa*). Bot. Bull. Acad. Sin. 30: 263–273.

Chugh L.K., Gupta V.K. and Sawhney S.K. 1992. Effect of cadmium on enzymes of nitrogen metabolism in pea seedlings. Phytochemistry 31: 395–400.

Costa G. and Morel J.-L. 1994. Water relations, gas exchange and amino acids content in Cd-treated lettuce. Plant Physiol. Biochem. 32: 561–570.

di Toppi L.S. and Gabbrieli R. 1999. Response to cadmium in higher plants. Environ. Exp. Bot. 41: 105–130.

Givan C.V. 1979. Metabolic detoxification of ammonia in tissues of higher plants. Phytochemistry 18: 375–382.

Hendry G.A.F., Baker A.J.M. and Ewart C.F. 1992. Cadmium tolerance and toxicity, oxygen radical process and molecular damage in cadmium-tolerant and cadmium-sensitive clones of *Holcus lanatus*. Acta Bot. Neerl. 41: 271–281.

Krupa Z. 1988. Cadmium-induced changes in the composition and structure of the light-harvesting complex II in radish cotyledons. Physiol. Plant. 73: 518–524.

Larsson E.H., Bornman J.F. and Asp H. 1998. Influence of UV-B radiation and Cd^{2+} on chlorophyll fluorescence, growth and nutrient content in *Brassica napus*. J. Exp. Bot. 49: 1031–1039.

Miflin B.J. and Lea P.J. 1976. The pathway of nitrogen assimilation in plants. Phytochemistry 15: 873–885.

Oaks A., Stulen J., Jones K., Winspear M.J. and Booesel I.L. 1980. Enzymes of nitrogen assimilation in maize roots. Planta 148: 477–484.

Ouariti O., Gouia H. and Ghorbal M.H. 1997. Responses of bean and tomato plants to cadmium: growth, mineral nutrition, and nitrate reduction. Plant Physiol. Biochem. 87: 199–202.

Sidlecka A. and Baszynsky T. 1993. Inhibition of electron flow around photosystem I in chloroplasts of cadmium-treated maize plants in due to cadmium-induced iron deficiency. Physiol. Plant. 87: 199–202.

Singh R.P., Bharti N. and Kumar G. 1994. Differential toxicity of heavy metals to growth and nitrate reductase activity of *Segamun indicum* seedlings. Phytochemistry 35: 1153–1156.

Somashekaraiah B.V., Padmaja K. and Prasad A.R.K. 1992. Phytotoxicity of cadmium ions on germinating seedlings of mung bean (*Phaseous vulgaris*): involvement of lipid peroxides in chlorophyll degradation. Physiol. Plant. 85: 85–89.

Weigel H.-J. and Jagar H.-J. 1980. Different effect of cadmium in vitro and in vivo on enzyme activities in bean plants (*Phaseolus vulgaris* L. cv. Sankt andreas). Z. Pflanzenphysiol. 97: 103–113.

Wintermans J.F.G.M. and De Mots A. 1965. Spectrophotometric characteristics of chlorophyll a and b and their pheophytins in ethanol. Biochim. Biophys. Acta 109: 448–453.