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Combined toxicity of cadmium and arsenate to wheat seedlings and plant uptake and antioxidative enzyme responses to cadmium and arsenate co-contamination

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Abstract

To demonstrate the combined toxicity of cadmium (Cd) and arsenate (As) to early developmental stages of six wheat varieties, young seedlings were exposed to solutions containing both contaminants and seed germination frequency and seedling biomass, root length and shoot height, Cd and As uptake, amylase activity, superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), soluble protein and malondialdehyde (MDA) concentrations in the seedlings were investigated. Seed germination and seedling biomass and root and shoot elongation decreased significantly ($P < 0.01$) with increasing concentrations of Cd and As and root length appeared to be the most sensitive parameter. Uptake of Cd and As by seedlings increased with increasing Cd and As concentrations in the test solutions and obeyed Michaelis–Menten kinetics. Average total amylolytic, α -amylase and β -amylase activities seemed to decrease with Cd concentrations $> 4 \text{ mg L}^{-1}$ and As $\geq 4 \text{ mg L}^{-1}$. Seedling contents of soluble protein, MDA and POD increased and the activities of SOD and CAT decreased with increasing concentrations of Cd and As following an initial increase. The MDA content was linearly and positively correlated with seed germination frequency, biomass increment, root length and shoot height elongation ($P < 0.01$), suggesting that MDA may be useful as a biological indicator of Cd and As toxicity in wheat. Combined exposure to Cd and As produced greater toxicity to wheat than single exposure to each metal separately, and Cd and As in combination had an additive effect on seed germination frequency and antagonistic effects on seedling biomass and shoot and root elongation.

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Keywords: Antagonistic effects; ID_{50} ; Amylase activity; Antioxidative enzymes; MDA; Soluble protein

1. Introduction

Cadmium (Cd) is a potentially hazardous trace metal which enters the environment mainly through industrial processes, irrigation with wastewater and application of municipal based composts and phosphatic fertilizers (Lepp, 1981). Arsenic is a metalloid that is ubiquitous in the environment and the major anthropogenic sources are metal processing, burning of coal and application of arsenic-based pesticides or herbicides (Kabata-Pendias and Pendias, 1992). Anthropogenically modified geochemical parameters also

induce heavy metal pollution and have received considerable attention.

Studies have shown that Cd and As can affect each other's uptake by plants as well as that of other elements (Das et al., 1997; Carbonell et al., 1995). Therefore interactions may occur between them in plant uptake and toxicity although they utilize completely different uptake mechanisms, with Cd being taken up by some bivalent cation transporters and arsenate by the phosphate uptake system (Clarkson and Lüttge, 1989; Meharg and Macnair, 1990). Numerous studies have investigated the toxicity of Cd or As alone on plant growth. However, coexistence of Cd and As in the environment is a common phenomenon and little information is available on the effects of their

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combined contamination on plant growth and uptake. Assessment of their combined toxicity is more relevant because it reflects more closely actual environment pollution in the field. Due to the complexity involved in combined metal uptake by plants few studies on mixtures of contaminants have been reported. It is unknown whether and how Cd and As interfere with each other in their effects on plant growth and metal uptake.

The toxic symptoms of metals in plants can be recognized by changes in biochemical and physiological processes (Sgherri et al., 2002) or by organ and intact plant responses such as growth inhibition, reduction in yield, or foliar injury (Taylor, 1984) or by changes in plant communities (Folkeson and Andersson-Bringmark, 1988). However, visible injuries and significant changes in growth inhibition and poor yield become apparent only after the plants are exposed to relatively high levels of pollutants or after a certain growth period. If appropriate methods can be established, toxic effects of metals on biochemical processes may be detected much earlier and at relatively low levels of pollution. In comparison, seed germination frequency and the early seedling growth are more sensitive to metal toxicity because some of the plants' defense mechanisms have not yet developed and hence effects at early stages of plant development can be very useful for toxicity assessment. In addition, starch is the main form of storage polysaccharide for energy in seeds. Evidence indicates that in germinating seeds starch degrades predominantly via the amylolytic pathway (Swain and Dekker, 1966a, b) and the participation of α -amylase and β -amylase in the hydrolytic pathway for starch degradation must be considered. It is unknown whether or how the coexistence of Cd and As may affect amylolytic activity.

Cadmium has been found to induce oxidative stress in cells (Sandalio et al., 2001). Depending on its concentration, Cd can either inhibit or stimulate the activity of several antioxidative enzymes before any visible symptoms of toxicity appear (Corrêa et al., 2006; León et al., 2002). Reactive oxygen species (ROS) may be generated through the conversion of arsenate to arsenite. This may result in damage to DNA, proteins and lipid membranes (Mishra and Singhal, 1992). Plants have antioxidant defense systems comprising catalases (CAT), peroxidases (POD), and superoxide dismutases (SOD) which play an important role in scavenging ROS produced under oxidative stress (Alscher and Hess, 1993). Therefore, increased activities of these enzymes may be considered as circumstantial evidence for enhanced production of reactive oxygen radicals such as $O_2^{\bullet-}$, $\bullet OH$, and H_2O_2 (Drazkiewicz et al., 2003). Malondialdehyde (MDA), an indicator of lipid peroxidation and soluble protein contents, has also been used to assess oxidative stress (Polle et al., 1997).

In the present study the effects of co-contamination by Cd and As on seed germination and seedling biomass, root and shoot elongation, amylase activities and Cd and As uptake were studied. Furthermore, to achieve a better understanding of the biochemical mechanisms of defense

against the stress from co-occurring Cd and As, soluble protein, MDA and the contents of antioxidative enzymes (SOD, POD and CAT) in wheat shoots were evaluated.

2. Materials and methods

2.1. Seed germination and seedling biomass and toxicity assay

Effects of different concentrations of Cd (0, 2, 4, 6, 8, and 10 mg L⁻¹) and As (0, 1, 2, 4 and 8 mg L⁻¹) on seed germination frequency were evaluated. The Cd and As solutions were freshly prepared by dissolving $Na_3AsO_4 \cdot 12H_2O$ and $CdCl_2 \cdot 2.5H_2O$ in deionized water and adjusting their pH to 5.8 with 2 mM Mes-Tris buffer.

Six varieties of wheat (*Triticum aestivum* L. cv. DuoKang-1, Jing-9428, Jing-411, Jingdong-8, Zhongmai-8 and Jingdong-11) were obtained from the Chinese Academy of Agricultural Sciences, Beijing, China. Prior to germination the seeds were surface-sterilized in 3% (v/v) H_2O_2 and then rinsed with deionized water. Seed germination was tested on filter papers placed in Petri dishes and moistened with 5.0 mL aqueous mixtures of Cd and As. Controls were obtained by moistening the filter papers with 5 mL deionized water. Thirty seeds of each variety were placed in each dish, covered by the lid, and incubated in the dark at $23 \pm 2^\circ C$ and the proportion of seeds that had germinated after 4 days was counted. Seeds were considered to have germinated when both the plumule and radicle were over 2 mm long. Seedling biomass was also determined and each treatment was set up in triplicate.

After germination for 4 days seedlings were transferred to colored vitreous pots containing 50 mL test mixtures of 0, 1, 2, 4 and 8 mg As L⁻¹ and 0, 2, 4, 6, 8 and 10 mg Cd L⁻¹, and each treatment was set up in triplicate. The pots were placed randomly in a growth cabinet with the temperature maintained at $23 \pm 2^\circ C$. The test solutions were renewed every day to avoid any changes in the concentration and speciation of the two contaminants.

After 2 days of seedling growth, shoot height was measured from the base of the culm to the tip of the longest leaf and root length was measured from the root-shoot junction to the tip of the longest root. Root length and shoot height of seedlings grown in test solutions containing Cd and As were expressed as percentage inhibition (%) of root length and shoot height compared with the controls.

2.2. Determination of Cd and As in seedlings

After 6 days' growth, three uniform seedlings were selected from each pot for the determination of Cd and As concentrations. Seedlings were first rinsed with ice-cold $CaCl_2$ solution and deionized water four times. They were then oven dried at $70^\circ C$ for 48 h, weighed and digested with ultra pure concentrated HNO_3 (5 mL) and 30% w/v H_2O_2 (3 mL) using a microwave digestion protocol (CEM Mars V, Matthews, NC, USA). Arsenic was determined by hydride generation atomic fluorescence spectrometry (HG-AFS, Model AFS-610A atomic fluorescence spectrophotometer, Beijing BRAIC Instrumental Company, Beijing, China) and Cd by inductively coupled plasma-atomic emission spectrometry (ICP-AES, Optima 2000 DV, Perkin-Elmer Co., Wellesley, MA, USA). All measurements were performed in triplicate.

2.3. Determination of amylase

Total amylolytic activity, α -amylase activity and β -amylase activity were measured according to the method of Swain and Dekker (1966a). The method has been described in detail in our previous paper (Liu et al., 2005).

2.4. Determination of antioxidative enzymes

Enzyme solutions were extracted by homogenizing the fresh seedlings with mortar and pestle under liquid nitrogen after 6 days' growth. Fresh

seedlings (1.0 g) were homogenized in 10 mL of an extraction buffer (0.05 M $\text{NaH}_2\text{PO}_4 \cdot \text{Na}_2\text{HPO}_4$ in 1% polyvinylpyrrolidone, pH 7.8). The details were described by Polle et al. (1997). After filtration through four layers of cheesecloth to remove debris, the homogenate was centrifuged at 10,000 g for 10 min. All operations were performed at 4 °C. The supernatant was used for enzyme activity and soluble protein assays.

Superoxide dismutase (SOD) was assayed on the basis of its ability to inhibit the photochemical reduction of nitro blue tetrazolium (Beauchamp and Fridovich, 1971). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM nitro blue tetrazolium, 2 μM riboflavin, 100 nM EDTA, and 0–200 μL of enzyme extract. The riboflavin was added last. The reaction mixture was read at 560 nm. One unit of SOD activity (U) was defined as the amount of enzyme that caused 50% inhibition of the initial rate of the reaction in the absence of enzyme. Total SOD activity was expressed as $\text{U g}^{-1} \text{FW}$.

Catalase (CAT) was measured by the method of Aebi (1984). The principle of the method was based on the hydrolysis of H_2O_2 and decreasing absorbance at 240 nm. A 0.1 reduction of A_{240} in 1 min was considered as one unit of enzyme activity (U) and CAT activity was expressed as $\text{U min}^{-1} \text{g}^{-1} \text{FW}$.

Peroxidase (POD) activity was determined with guaiacol by spectrophotometry (Lagrimini, 1991). In the presence of H_2O_2 , POD catalyzes the transformation of guaiacol to tetraguaiacol. This reaction can be recorded at 470 nm. The reaction mixture contained 100 mM phosphate buffer (pH 6.0), 33 mM guaiacol and 0.3 mM H_2O_2 . Enzyme specific activity was expressed as $\Delta\text{OD}_{470} \text{ min}^{-1} \text{g}^{-1} \text{FW}$ (OD: optical density).

2.5. Determination of lipid peroxidation and soluble protein

Oxidative damage of lipids was measured in terms of the total content of two thio-barbituric acid (TBA) reactive substances and expressed as equivalent of malondialdehyde (MDA) with some modifications (Cakmak and Horst, 1991). MDA was measured by a colorimetric method (Heath and Packer, 1968). The absorbance of the MDA was read at 532 nm and the nonspecific absorption at 600 nm was subtracted. The concentration of MDA was calculated at its extinction coefficient ($155 \text{ mM}^{-1} \text{ cm}^{-1}$).

The method of ultraviolet absorption was used to determine the concentration of soluble protein in wheat shoots (Li et al., 2000). Absorption was recorded at 280 and 260 nm. Soluble protein was expressed as $\text{mg g}^{-1} \text{FW}$.

2.6. Statistical analysis

Statistical analysis was performed using Microsoft Excel, Origin 7.0, SPSS 11.5 and the geochemical speciation model MINTEQAII (Allison et al., 1991). The data represent means calculated from three replicates. The multiple comparison procedure (LSR test) was used to compare the effect of Cd and As interaction on germination, biomass, root length and shoot height and statistical significance was set at $P < 0.05$ (Breslow, 1974). The least significant difference test (LSD test) was employed for comparison of antioxidative enzyme activity changes at $P < 0.05$.

3. Results

3.1. Toxicity of Cd and As co-contamination on wheat early developmental stages

Inhibition of seed germination and seedling biomass, root and shoot elongation increased with increasing concentrations of Cd and As in the test solution as shown in Fig. 1. Significant positive linear relationships ($P < 0.01$) existed between the inhibition rates (%) of seed germination and seedling biomass, root and shoot elongation and the concentrations of Cd in the growth medium when the

concentration of As was maintained at 0, 1, 2, 4, or 8 mg L^{-1} (Table 1).

The slope coefficients of the equations for germination inhibition rates and Cd concentrations were 2.72, 2.74, 2.37, 2.30 and 2.10, respectively, when As concentrations were 0, 1, 2, 4 and 8 mg L^{-1} (Table 1). Although the slope coefficients decreased with increasing As concentration in the growth medium, these differences were not significant except when the As concentration was 8 mg L^{-1} . If Cd and As have interactive effects with each other, either synergistic or antagonistic, the slope coefficients in the equations for the Cd and As combined treatment should be different from the treatment with Cd alone. If the slope coefficients are the same for the two treatments, only an additive effect can be inferred. Therefore we can conclude that co-contamination by Cd and As exerted additive effects on seed germination when As concentrations were 0, 1, 2 and 4 mg L^{-1} and antagonistic effects when the As concentration was 8 mg L^{-1} .

Seedling biomass (average of all 6 varieties) decreased significantly ($P < 0.05$) with increasing concentration of Cd or As (Fig. 1b and Table 1). The slope coefficients in the equations were 3.20, 3.05, 2.50, 2.42 and 2.11 when As was 0, 1, 2, 4 and 8 mg L^{-1} . A greater reduction in biomass was observed for the Cd and As combined treatment than exposure to Cd or As separately. Significant decreases in the slope coefficients ($P < 0.05$) confirmed the combined effects of Cd and As on biomass. However, this occurred only when the As concentration was higher than 1 mg L^{-1} in the growth medium.

Effects of Cd and As on root and shoot elongation are presented in Figs. 1c and d. Significant reductions in root length and shoot height were observed with increasing Cd and As concentrations. The equation slope coefficients (Table 1) were 6.69, 6.25, 6.13, 5.49 and 3.85 for root length, and were 4.36, 3.90, 3.84, 2.90 and 2.75 for shoot height when the As concentration was 0, 1, 2, 4 and 8 mg L^{-1} . Significant differences ($P < 0.05$) in the slope coefficients between the treatments with Cd alone and the mixture of Cd and As indicated interactive effects of Cd and As on root and shoot elongation.

Arsenate in the growth medium produced greater inhibitory effects on biomass and shoot and root elongation than when As was absent ($P < 0.05$). The sum of the individual effects of Cd and As separately on biomass increment, root and shoot elongation was higher than the effect of Cd and As when present together ($P < 0.05$). Significant trends were observed for the reduction in slope coefficients and the increase in the intercept coefficients of the outcome equations with increasing As concentrations for the inhibitory effects on biomass increment, shoot and root elongation. Thus, we can conclude that Cd and As had an antagonistic effect on these three growth parameters and the antagonistic effect increased with increasing Cd and As concentrations in the growth medium.

If we define the inhibitory dose 50% (ID_{50}) as the concentration of Cd or As at which the inhibitory

Fig. 1. Inhibitory effect (%) on seed germination (a) and seedling biomass (b), root length (c) and shoot height (d) of different Cd and As concentrations (0 (■), 1 (○), 2 (△), 4 (▽), 8 (☆) mg L⁻¹) (*n* = 6).

Table 1
Relationships between inhibition rate of seed germination frequency (G), biomass increment (B), root length (R) and shoot height (S) and concentration of added Cd at the same concentration of added As (*n* = 6)

	Equation	<i>R</i> ²
Germination	$G_{Cd+0As} = 2.72x + 1.38$	0.972
	$G_{Cd+1As} = 2.74x + 7.47$	0.957
	$G_{Cd+2As} = 2.37x + 15.37$	0.989
	$G_{Cd+4As} = 2.30x + 20.92$	0.996
	$G_{Cd+8As} = 2.10^{*}x + 30.61$	0.983
Biomass	$B_{Cd+0As} = 3.20x - 0.07$	0.996
	$B_{Cd+1As} = 3.05x + 6.22$	0.999
	$B_{Cd+2As} = 2.50^{*}x + 21.87$	0.967
	$B_{Cd+4As} = 2.42^{*}x + 30.10$	0.979
	$B_{Cd+8As} = 2.11^{*}x + 46.96$	0.992
Root length	$R_{Cd+0As} = 6.69x + 2.28$	0.985
	$R_{Cd+1As} = 6.25x + 6.81$	0.983
	$R_{Cd+2As} = 6.13^{*}x + 15.64$	0.974
	$R_{Cd+4As} = 5.49^{*}x + 27.73$	0.991
	$R_{Cd+8As} = 3.85^{**}x + 52.49$	0.948
Shoot height	$S_{Cd+0As} = 4.36x + 0.52$	0.995
	$S_{Cd+1As} = 3.90x + 5.79$	0.996
	$S_{Cd+2As} = 3.84^{*}x + 9.06$	0.974
	$S_{Cd+4As} = 2.90^{**}x + 27.19$	0.980
	$S_{Cd+8As} = 2.75^{**}x + 36.69$	0.985

The probability level of *P* < 0.01; difference in slope coefficient between the treatments by mixture of Cd and As and by Cd alone at the significant level of *, *P* < 0.05; **, *P* < 0.01.

effect reached 50%, we can calculate the ID_{50} for each parameter. The ID_{50} values for Cd (Table 2) indicate an increase in toxicity with increasing As concentration and the most sensitive parameter for toxicity was root length.

3.2. Accumulation of Cd and As in wheat seedlings

The concentrations of Cd and As in seedlings of the six wheat varieties increased with increasing Cd and As in the growth medium (Fig. 2). Uptake of Cd and As fitted the Michaelis–Menten equation (Fig. 2 and Table 3). With increasing As concentration, the increments of Michaelis constant values (K_m) for Cd over control of 52.2, 112.3, 125.9, and 195.6% were significant ($P < 0.05$), however, the increments of the maximum influx rate (V_{max}) were not significant ($P > 0.05$). The K_m and V_{max} values for As uptake did not change significantly with different levels of added Cd ($P > 0.05$). Therefore, the presence of As decreased Cd uptake by the seedlings but the presence of Cd did not significantly change As uptake.

3.3. Effect of Cd and As on amylases

Average total amylolytic activity and α -amylase and β -amylase activities in germinated seeds with different concentrations of Cd and As treatment are presented in Table 4. The average total amylolytic activities of wheat seeds in $< 4 \text{ mg As L}^{-1}$ or $\leq 4 \text{ mg Cd L}^{-1}$ medium were comparable to the control, but activity was progressively depressed with increasing concentration of Cd ($6\text{--}10 \text{ mg L}^{-1}$) or As ($4\text{--}8 \text{ mg L}^{-1}$). Similar patterns of response were observed for α -amylase and β -amylase activities. The total amylolytic activity of germinated seeds declined by 22.7–53.4%, α -amylase activity by 29.7–64.8%, and β -amylase activity by 20.8–50.4%, respectively, over the controls with 8 mg L^{-1} added As. The Cd and As combined treatment produced a greater reduction in amylase activity than Cd or As alone and the α -amylase activity declined more markedly with increasing Cd or As concentrations than did the total amylolytic activity or β -amylase activity.

Table 2
Half-inhibition dose of Cd with different As concentrations

Added As (mg L^{-1})	0	1.0	2.0	4.0	8.0
G_{ID50}	17.88	15.52	14.61	12.64	9.23
B_{ID50}	15.65	14.35	11.25	8.22	1.44
R_{ID50}	7.13	6.91	5.61	4.06	— ^a
S_{ID50}	11.35	11.34	10.66	7.87	4.84

G_{ID50} , B_{ID50} , R_{ID50} , S_{ID50} represent the Cd concentrations for 50% inhibition of seed germination, biomass, root length and shoot height, respectively.

^aWhen the added concentration of As was 8 mg L^{-1} , the inhibition rate of root elongation was higher than 50% irrespective of the As concentration.

Fig. 2. Concentrations of Cd (a) and As (b) in seedlings with different concentrations of As and Cd (lines represent fitting to the Michaelis–Menten equation).

3.4. Estimation of peroxidation products and soluble protein

The TBA assay can be regarded as a reliable method for evaluating the degree of lipid peroxidation (Luna et al., 1994). MDA contents were analyzed on the basis of fresh weight and the results are shown in Fig. 3a. The MDA concentrations increased with increasing Cd and As concentrations, that is, increased lipid peroxidation occurred with increasing Cd and As concentrations. A higher increment in MDA level was observed with Cd and As combined compared with either Cd or As alone. When both Cd and As were present at 8 mg L^{-1} , the MDA value increased by 105.6% over the control. A similar increase was observed in the soluble protein (Fig. 3b). The results indicate some sensitivity of MDA and soluble protein to Cd and As.

3.5. Effects of Cd and As on antioxidative enzyme activities

To better understand the biochemical basis of resistance in wheat caused by Cd and As contamination, superoxide

Table 3

Kinetic parameters calculated from mean influx of Cd (a) and As (b) into wheat seedlings using the Michaelis–Menten function for different Cd and As concentration treatments ($n = 3$ for each of the six wheat varieties)

(a)				
	R^2	P	K_m (mM)	V_{max} (nmol g ⁻¹ FW h ⁻¹)
As (V) in solution (mg L ⁻¹)				
0	0.997	<0.01	50.37	1257.08
1.0	0.997	<0.01	76.66*	1427.30
2.0	0.997	<0.01	106.94**	1549.95
4.0	0.997	<0.01	113.78**	1464.06
8.0	0.982	<0.01	148.89**	1551.72
(b)				
Cd in solution (mg L ⁻¹)				
0	0.982	<0.01	25.99	58.05
2.0	0.966	<0.01	26.15	58.38
4.0	0.959	<0.01	25.69	51.89
6.0	0.988	<0.01	28.42	51.19
8.0	0.982	<0.01	28.28	48.70
10.0	0.935	<0.01	30.89*	46.63

Significant difference, *, $P < 0.05$; **, $P < 0.01$.

dismutase (SOD) activity and catalase (CAT) and peroxidase (POD) activities in wheat shoots were measured and the results are displayed in Fig. 4. All the treatments led to changes in shoot SOD, CAT and POD activities. SOD activity increased at first and then decreased when the Cd concentration increased from 2 to 8 mg L⁻¹. In the absence of Cd, SOD activity increased with the increasing As concentration, but when the Cd concentration in the growth medium was 2 or 8 mg L⁻¹, SOD activity decreased with increasing As concentration. CAT activity increased with increasing Cd concentration in the absence of As. When As was present in the culture solution, CAT activity first increased and then decreased as the Cd concentration increased. When the Cd concentration was 0 or 2 mg L⁻¹, CAT activity in wheat shoots increased with increasing As concentration but they decreased with increasing As concentration when the Cd concentration was 8 mg L⁻¹. The POD activity in wheat shoots increased with increasing Cd and As concentrations in the growth medium. The increments over the control values were 31.1% when both Cd and As were both at a concentration of 8 mg L⁻¹. Co-occurrence of Cd and As had a significant influence on the three antioxidative enzymes ($P < 0.05$).

4. Discussion

Significant linear relationships ($P < 0.01$) were found between the inhibitory effects (%) on seed germination frequency and seedling biomass, root and shoot elongation and concentrations of Cd and As. Coexistence of Cd and As had an additive effect on seed germination and antagonistic effects on biomass, root and shoot elongation.

Table 4

Amylolytic activity in wheat seeds with different concentrations of Cd and As

As in solution (mg L ⁻¹)	Cd in solution (mg L ⁻¹)	Total amylase	α -amylase	β -amylase
0	0	10.41 ± 0.78	2.19 ± 0.12	8.22 ± 0.30
	2.0	10.51 ± 1.12	2.24 ± 0.28	8.27 ± 0.16
	4.0	10.42 ± 0.41	2.28 ± 0.15	8.14 ± 0.10
	6.0	9.50 ± 0.33	1.92 ± 0.21	7.58 ± 1.43
	8.0	8.49 ± 0.82	1.71 ± 0.19	6.78 ± 0.40
	10.0	7.62 ± 0.56	1.43 ± 0.06	6.19 ± 0.40
1.0	0.0	10.28 ± 1.02	2.21 ± 0.12	8.07 ± 0.55
	2.0	10.13 ± 0.76	2.08 ± 0.31	8.05 ± 1.14
	4.0	9.97 ± 0.88	1.97 ± 0.23	8.00 ± 0.43
	6.0	8.98 ± 0.45	1.82 ± 0.17	7.16 ± 0.59
	8.0	7.95 ± 0.24	1.65 ± 0.25	6.30 ± 0.34
	10.0	7.23 ± 0.38	1.47 ± 0.18	5.76 ± 0.22
2.0	0	10.12 ± 1.33	2.03 ± 0.03	8.09 ± 0.24
	2.0	10.07 ± 0.68	2.04 ± 0.11	8.03 ± 1.02
	4.0	9.35 ± 0.97	1.90 ± 0.17	7.45 ± 0.31
	6.0	8.73 ± 0.38	1.77 ± 0.21	6.96 ± 0.56
	8.0	7.72 ± 0.64	1.53 ± 0.13	6.19 ± 0.28
	10.0	6.51 ± 0.76	1.33 ± 0.12	5.18 ± 0.57
4.0	0	9.43 ± 0.84	1.87 ± 0.11	7.56 ± 0.42
	2.0	8.91 ± 0.62	1.75 ± 0.23	7.16 ± 0.59
	4.0	8.04 ± 0.38	1.66 ± 0.09	6.38 ± 0.26
	6.0	7.47 ± 0.91	1.54 ± 0.26	5.93 ± 0.65
	8.0	6.33 ± 1.02	1.37 ± 0.15	4.96 ± 0.32
	10.0	5.52 ± 0.34	0.98 ± 0.10	4.54 ± 0.33
8.0	0	8.05 ± 0.35	1.54 ± 0.16	6.51 ± 0.71
	2.0	7.76 ± 0.56	1.44 ± 0.12	6.32 ± 0.38
	4.0	7.27 ± 0.42	1.20 ± 0.11	6.07 ± 0.53
	6.0	6.42 ± 0.41	1.02 ± 0.09	5.40 ± 0.55
	8.0	5.56 ± 0.33	0.85 ± 0.05	4.71 ± 0.29
	10.0	4.85 ± 0.46	0.77 ± 0.08	4.08 ± 0.37

The antagonistic effects may be ascribed to reductions in the ion activity in the medium when Cd and As were present together, thereby lowering metal(loid) uptake. The calculated results obtained using MINTEQAII indicate that the co-occurrence of Cd and As decreased the total ion activity in the culture solution with a maximum rate of 22% and this supports our interpretation of the results.

Increases in the concentrations of Cd and As in the growth medium led to more pronounced reduction in root length than the other parameters and the degree of inhibition followed the order: root length > shoot height, biomass > germination frequency. The calculated ID₅₀ dose also showed root length to be the most sensitive indicator among the growth parameters (Table 2). In our previous study on the toxicity of As to wheat (Liu et al., 2005) we observed that root length and shoot height had similar sensitivities to As. However, the present study indicates that when Cd and As are present together root length is more sensitive than shoot height. The differences between the two studies may be due to the presence of Cd. Cadmium can readily penetrate the root

Fig. 3. MDA (a) and soluble protein (b) concentrations in wheat seedlings (different letters indicate significant differences at $P < 0.05$).

cortex (Yang et al., 1998), consequently the roots are likely to experience Cd damage first (di Toppi and Gabbrielli, 1999). Blum (1997) also found root length to be the most sensitive parameter to Cd treatment.

Seed germination relies almost exclusively on seed reserves for the supply of metabolites for respiration as well as other anabolic reactions. Starch is quantitatively the most abundant storage material in seeds and available evidence indicates that in germinating seed starch is degraded predominantly via the amylolytic pathway (Juliano and Varner, 1969). In the present study the average total amylolytic, α -amylase and β -amylase activities were significantly depressed by the higher concentrations of Cd and As tested and α -amylase activity was the most sensitive parameter. α -amylase is the major enzyme involved in the initial degradation of starch into more soluble forms while phosphorylase and β -amylase

Fig. 4. SOD (a), CAT (b) and POD (c) activities in wheat seedlings (different letters indicate significant differences at $P < 0.05$).

assist in the further conversion to free sugars which affords the nutrition of seed germination (Juliano and Varner, 1969). Reduction of amylase activity may therefore be the major factor involved in the depression of seed germination.

Uptake of Cd and As by wheat seedlings in the range of concentrations studied followed Michaelis–Menten kinetics. The biological interpretation of the kinetics parameters is thought to be protein carriers situated in the plasmalemma. The $V_{\max}$ of ion uptake is a function of the carrying capacity of the uptake site and the concentration of the carrier protein present in the plasmalemma. The increased K_m and similar $V_{\max}$ for Cd indicate that Cd uptake decreased with increasing concentration of As (Fig. 2a and Table 3a) and also that the affinity of wheat roots to Cd decreased with increasing As concentration. Similar K_m and $V_{\max}$ for As suggests that there were no significant changes in As uptake by wheat seedlings with increasing Cd concentration (Fig. 2b and Table 3b). The combined presence of Cd and As decreased the ion activity on the root surfaces. The Cd and As equilibrium reaction ($\text{Cd}^{2+} + \text{AsO}_4^{3-} \rightleftharpoons \text{Cd}_3(\text{AsO}_4)_2$) in solution may also have limited the ion activity of Cd. Reduced ion activity may play an important role in the depression of Cd uptake by wheat seedlings in the presence of As.

Increased production of toxic oxygen free radicals was directly or indirectly generated by Cd and As. One of the most damaging effects of these free radicals and their products in cells is the peroxidation of membrane lipids of which MDA is an indicator. In the present study MDA increased with increasing Cd and As concentrations, indicating that the degree of damage to membrane lipids depended on the Cd and As concentrations in the culture medium.

Under certain conditions plants can protect themselves by inhibiting lipid peroxidation due to the effects of antioxidative enzymes. It is generally recognized that once antioxidative enzymes are activated, MDA content will decrease. However, our study showed increasing MDA with increasing concentrations of Cd and As. High levels of Cd and As can cause oxidative damage to plants. The degree of cell damage under heavy metal stress depends on the rate of ROS formation and on the efficiency and capacity of detoxification and repair mechanisms. The antioxidant enzymes indicate important cellular defense system against oxidative stress. For example, SOD converts $\text{O}_2^{\bullet-}$ to H_2O_2 and O_2 (Scandalios, 1993), while CAT and POD are important enzymes in oxidative defense systems by catalyzing H_2O_2 hydrolysis. Efficient destruction of $\text{O}_2^{\bullet-}$ and H_2O_2 requires the action of several antioxidant enzymes acting in synchrony. In our study, with increasing concentrations of Cd and As, POD, SOD and CAT activities did not increase in synchrony and MDA therefore did increase.

In the present study the changes in SOD and POD activities at all concentrations of Cd and As tested suggest that Cd and As toxicity induced superoxide radicals ($\text{O}_2^{\bullet-}$) in the seedlings. The SOD and POD activities increased at low Cd and As concentrations. Higher concentrations of Cd and As resulted in inhibition of SOD and POD activities. This suggests that there was insufficient increase in SOD and POD activities to scavenge

Table 5

Regression equations between MDA contents and inhibition rates of seed germination and seedling biomass increment, root length elongation and shoot height elongation

	Equation	R^2	Significance
Germination	$G = 8.83x - 39.09$	0.731	$P < 0.01$
Biomass	$B = 11.47x - 50.21$	0.582	$P < 0.05$
Root length	$R = 17.96x - 86.78$	0.765	$P < 0.01$
Shoot height	$S = 11.93x - 58.09$	0.736	$P < 0.01$

excess $\text{O}_2^{\bullet-}$ that accumulated in seedlings at high Cd and As concentrations.

Soluble protein content increased with increasing Cd and As concentrations. This might be another response to the toxicity of Cd and As and the start of a detoxification mechanism. For example, plants can produce Cd-binding proteins under Cd stress, which can maintain the normal metabolism of the cells by decreasing the toxicity of Cd, increasing cell penetrability and the quantity of functional proteins (Bartolf et al., 1980).

Relationships between MDA contents and the inhibition rates for each growth parameter fitted the equations shown in Table 5. The relationships between the MDA contents and the seed germination inhibition rates and seedling biomass inhibition rates, root length inhibition and shoot height inhibition were all significant ($P < 0.01$). In other words the MDA contents affect seed germination and seedling biomass increase, root length and shoot height elongation significantly. On the other hand, MDA might be a more sensitive biological indicator to Cd and As co-contamination than the other plant characteristics.

5. Conclusions

Co-contamination of Cd and As had interactive effects on wheat growth, an additive effect on seed germination and antagonistic effects on seedling biomass increment and shoot and root elongation. The combined presence of Cd and As decreased the plant uptake of both contaminants. The sensitivity of the plant parameters to the co-contamination of Cd and As followed the order: root length > shoot height, biomass > germination frequency.

The contents of soluble protein and MDA and SOD, CAT and POD activities changed significantly with increasing concentrations of Cd and As. The MDA contents showed significant relationships with seed germination, biomass increase, root length elongation and shoot height elongation and may perhaps be a useful biological indicator of Cd and As toxicity in wheat.

Hydroponic experiments can be criticized because they represent much simpler conditions than plants experience under field conditions. However, they are useful for elucidating the mechanisms by which plants take up metal(loid)s and can provide useful bioassays that help to devise strategies to reduce the risk of metal pollution, especially when they are combined with field experiments.

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