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Wheat phytotoxicity from arsenic and cadmium separately and together in solution culture and in a calcareous soil

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Abstract

The toxicity effect of two deleterious elements of arsenic (As) and cadmium (Cd) (individually or in combination) on root elongation of wheat seedlings (*Triticum aestivum*, L.) were investigated both in hydroponics and in soils freshly spiked with the toxic elements. Median effective concentration (EC_{50}) and non-observed effect concentration (NOEC) were used to investigate the toxic thresholds and potencies of the two elements. The EC_{50} for As was 0.97 μM in hydroponics and 196 $mg{\cdot}kg^{-1}$ in soil, and 4.32 μM and 449 $mg{\cdot}kg^{-1}$ for Cd, respectively. Toxic unit (TU) and additive index (AI) concepts were introduced to determine the combined outcomes, and different behaviors were obtained: synergism in solution culture (EC_{50mix}= 0.36 TU_{mix} and AI: 1.76) and antagonism in soil experiments (EC_{50mix}= 1.49 TU_{mix} and AI: −0.33). Furthermore, the data of soil bioavailable As and Cd can not explain the discrepancy between the results derived from soil and hydroponics experiments.

Keywords: Arsenic; Cadmium; Phytotoxicity; Root elongation; Median effective concentration; Combined effect; Toxic unit; Additive index

1. Introduction

The adverse effects of toxic chemicals on soil fauna and microbes are of the major foci in soil ecotoxicological assessments. As for the effects to the flora, most tests or standard methodologies have been developed to primarily study the behavior of hydrophytes. Test on terrestrial plants has been recognized as an issue of high priority by many governmental agencies around the world [1-2]. The test methods related with phytotoxicity should be enhanced in assessing the impacts of chemicals on terrestrial ecosystem because vegetation is a functional component of terrestrial ecosystem, and crop also serve as an important pathway for human exposure to toxic elements [3].

Currently, some terrestrial plant tests were conducted [1-2, 4-5] to estimate the potential impacts of chemical on non-target species, in order to investigate the effects of remediation [6] and to develop ecological soil screening levels [7]. There are several kinds of standardized plant toxicity tests, i.e. seed germination, root elongation, and early seedling growth tests. Photosynthesis inhibition test and enzyme content fluctuation are also frequently used as endpoints for phytotoxicity [8]. The present study selects wheat for testing as it is the main staple cereal in the world, especially in northern China. Furthermore, root elongation is selected as a quantitative test endpoint in this study as the root accumulate more toxicants, and is more sensitive, than shoot [2, 9].

Current ecotoxicological assessments or criteria have generally been derived from data involving single toxicant, yet rarely is there only one contaminant present in field soils. Combined effects of chemicals should be taken into consideration in the development of ecotoxicologically relevant soil quality criterion. Arsenic and cadmium are two of the typical hazardous elements, and they are non-essential to plant [10-11]. Arsenic (As) was ranked as the No.1 and cadmium (Cd) as the No.8 of hazardous substances which have significant potential threat to human health due to their known or suspected toxicity [12]. Phytotoxicity of single As or Cd was well documented [13-14], but little is known about their combined effects to plants when presented simultaneously in soil. A study performed by Sneller et al. to Silene *Vulgaris* in hydroponics shown the interaction of As and Cd is concentration-depended, and the element's uptake did not affected by another element [15]. While animal studies reported that As may exacerbate Cd toxicity to mice kidney [16].

The objective of this study is therefore to assess the plant toxic effects of As and Cd individually and in combination, and to determine the direction and extent of their interaction both in solution and in soil. To our knowledge, few studies have been published regarding the mixture toxicity of As and Cd to crop plants. Results from this study will help us realistically understand the phytotoxicity of soil As and Cd on plants.

2. Materials and methods

2.1 Preparation of seedlings

Seeds of wheat (*Triticum aestivum*, L., Zhongmai 9) were purchased from Chinese Academy of Agricultural Sciences ,Beijing, China. Seeds were sterilized with 10% (w: w) hydrogen peroxides for 10 min, followed by thorough washing with deionized water. The seeds were submerged in distilled water and cultured in a 37°C incubator for 24 hours. After the radicel appeared, seeds with uniform appearance were chosen for next procedure, i.e. germinated in moist perlite for hydroponics experiments, or embedded in test soil.

2.2 Root elongation test in solution

Six days after germination and initial growth period in perlite seedbed, the seedlings were gently removed from perlite and washed. After measuring the initial root length, groups of 10 seedlings were transferred to 500 ml PVC pots (7.5 cm in diameter and 15 cm in height), in which contained simple phosphate-free nutrient solution (0.1 mM $MgSO_4$, 0.2 mM $Ca(NO_3)_2$, 0.2 mM KNO_3) and the target exposure concentration levels of toxicants [17]. All the hydroponics experiments were conducted with continuous aeration.

In the experiment with a single toxicant, concentrations of As ($Na_3AsO_4 \cdot 12H_2O$, analytical reagent) or Cd ($CdCl_2 \cdot 2.5H_2O$, analytical reagent) were targeted at the following levels: 0, 0.01, 0.03, 0.1, 0.33, 1.00, 3.33, 10, 33.3, 100 µM. In the mixed-toxicant experiment, the treatments were set up according to the single toxicant data [18] and equitoxic mixture with a range of six concentrations (0, 0.4, 0.8, 1.2, 1.6, 2 ∑TU). The equitoxic mixture approach is more reasonable than equi-concentration mixture one because it is supposed that $0.5n \times EC_{50}$ As and $0.5n \times EC_{50}$ Cd had same toxicity and their mutual toxicity should be $1n \times TU_{mix}$. Three replicates were used for each concentration. The exposure time for all experiments was six days, and the culture solution was changed every three days to maintain the target concentration.

2.3 Root elongation test in spiked soil

A sample of non-contaminated soil was collected from the suburb of Beijing (N39°52.15′, E116°37.39′), air dried and sieved through 2mm griddle. The basic physical and chemical properties of the soil were measured and listed in Table 1. Determination of Soil pH was conducted with the soil:water ratio of 1:2.5 [19]. Organic matter was measured with hot $K_2Cr_2O_4$ oxidization and $FeSO_4$ titration, and cation exchange capacity (CEC) by the NH_4OAC methods [20]. Soil texture was classified according to the contents of clay, silt and sand, which were determined by laser diffraction system (Malvern Master Sizer 2000, Malvern Co., England) after the soil particles diffused in 0.5 M $NaPO_3$ solution. Total contents of soil elements were determined by ICP-AES (Optima 2000 DV, PerkinElmer Co. USA) after the digestion with aqua regia [21].

As(V) and/or Cd solution was spiked to soil to make certain levels of soil element content. Then the spiked soil was air dried, passed the 2mm sieve again and aged for at least 1 week before root elongation test. A mass of 300g soil was transferred to a plastic cup; ten germinated wheat seeds were then embedded just beneath the surface and checked daily to keep the moisture at 20% (v/w). After the seeds grew for six days, the seedlings were collected and the main root lengths measured. The arsenate concentrations in eleven soil-arsenate treatments were 0, 2.5, 5, 10, 20, 40, 60, 90, 135, 200, 300 $mg\cdot kg^{-1}$, while the fourteen cadmium treatments are 0, 2.5, 5, 10, 20, 40, 60, 90, 135, 200, 300, 450, 675, 1000 $mg\cdot kg^{-1}$. The combined test had nine concentrations (0, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, 2, 3.2 ∑TU); three replicates were used for each concentration.

2.4 Growth conditions

The experiments were carried out in a growth chamber set with a 14:10 (hour: hour) light:dark photoperiod (260-350 μmol $m^{-2}s^{-1}$), a day/night temperature of 25/20°C and a relative humidity of 70%.

2.5 Statistical analysis

NOEC was determined by Dunnett program, Version 1.5 (USEPA). If a significant F value of $P<0.05$ was obtained, a Dunnett's multiple comparison versus the control group analysis was conducted [22]. EC_{50} calculation was performed by the regression method using SigmaPlot 9.0 (SPSS Inc., Chicago, IL). Logistic formula (Equation 1) of 3 parameters as following was chosen for its good fitness [23]. Parameter ***y*** is the wheat root length, ***x*** is the concentration, ***a*** is the expected root length of wheat when ***x***=0, $\boldsymbol{x_0}$ is median effective concentration (EC_{50}) and ***b*** is the slope parameter.

$$y = \frac{a}{1 + \left(\frac{x}{x_0} \right)^b} \qquad (1)$$

2.6 Principles for preparing mixture and determining combined effects

The concept of toxic unit was proposed by Sprague and Ramsay [24] to express the effect concentration of a chemical, and later proved useful in mixture toxicity studies Toxic unit is non-dimension value calculated by the ratio of concentration and EC_{50}. One toxic unit means the toxic concentration is nearly EC_{50}. According to the toxic data of single As and Cd, the mixed-toxicant experiments were prepared as Equations 2-3 and with equitoxic ratio hypothesis[18, 25]. The combined effect of mixture (EC_{50mix}) was calculated by regression, and expressed with TU_{mix}, then compared with expected effects from concentration additive (1 TU_{mix}) [26-27]. If the EC_{50mix} would not significantly differs from 1 TU_{mix}, the effect of two toxicants is concentration additive. If the EC_{50mix} would be significantly lower (or higher) than 1 TU_{mix}, then less (or higher) than concentration-additive mode is derived about the toxicants interaction.

$$TU = \frac{Conc.}{EC_{50}} \quad \text{(for single toxicant)} \qquad (2)$$

$$TU_{mix} = \sum TU = TU_{As} + TU_{Cd} \quad (3)$$

Marking and Dawson [28] proposed an original method of additive index (AI), which can quantitatively describes additive toxicity of chemicals. Sum of the toxicity (***S***) of chemical A and B was determined by Equations 4, ***i*** and ***m*** are the EC_{50} of the individual and the mixture. By transforming to additive index (Equations 5), it is easy to judge whether the combined effect is less than or greater than additive just by recognizing the value of AI is negative or positive.

$$S = \frac{A_m}{A_i} + \frac{B_m}{B_i} \quad (4)$$

$$AI = \frac{1}{S} - 1 \quad (S \leq 1) \quad \text{or} \quad AI = S(-1) + 1 \quad (S \geq 1) \quad (5)$$

3. Results and discussion

3.1 Single toxicity in solution culture

Concentration-effect curves (Figs. 1-2) were plotted from the data of single toxicant exposures. From the figures, it can be seen that As and Cd had different curve shapes, the values of slope parameter ***b*** were 2.73 for As and 0.97 for Cd. The two toxicants had the similar toxic threshold of 0.33 μM, but the concentrations with an inhibition rate larger than >95% were 3.3 and 100 μM for As and Cd, respectively, which has a 30-fold difference. The EC_{50} values were 0.97 and 4.32 μM for As and Cd, respectively.

Data on arsenate toxicity from our solution culture experiment are much higher than the results of Liu et al. [29], who exposed six varieties of wheat to arsenate in solution for two days and the median effective concentrations of the root length were about 4-16 mg/L. Similarly, a reported Cd EC_{50} for wheat elongation was 66 mg/L [30], which greatly differs from our solution results. These differences indicate that the early-stage test with simple solution, an approach used in this study, seems to be a more sensitive method to identify toxic substances.

3.2 Combined toxicity in solution culture

In the experiment with mixed toxicants, a set of increasing equitoxic concentrations of toxicants was arranged based on the single exposure data. The solutions with 0.4, 0.8, 1.2, 1.6, 2 TU_{mix} were composed according to Table 2. Using TU_{mix} as the horizontal axis, instead of the real concentration, the combined effect curve (Fig. 3) produced a regression result of EC_{50} at 0.36 TU_{mix}. This means that solution containing 0.17 μM As (0.18 TU_{As}) plus 0.78 μM Cd (0.18 TU_{Cd}) could inhibits 50% root elongation. The value of AI was 1.76 (ranging from 1.17~2.55). It is obvious that mixture exposure of arsenate and cadmium in solution has a significant great than additive toxicity to wheat root.

3.3 Single toxicity in soil

Figures 4-5 showed the concentration-effect curves of wheat in the soils spiked with As or Cd. The curve shape for As was steeper than Cd (***b*** value of 3.32 for As and 0.91 for Cd). Soil As had a toxic threshold of 40 $mg \cdot kg^{-1}$ with a higher than 80% root inhibition rate at 300 $mg \cdot kg^{-1}$; while the threshold for soil Cd was found to be 20 $mg \cdot kg^{-1}$ but even the largest concentration of 1,000 $mg \cdot kg^{-1}$ only had the 67%

inhibition rate for the root. The soil EC_{50} was 196 mg·kg^{-1} for arsenate and 449 mg·kg^{-1} for cadmium. In other words, As(V) toxicity is about 2.3 times of that for Cd in the tested soil.

Toxicity data of soil As are in line with several other studies. Song et al. [31] performed 4-days' barley root growth tests with 16 European soils, and the EC_{50} values for freshly spiked arsenate varied from 26.6 to 458.2 mg·kg^{-1}. The EC_{50} value of soil Cd obtained in this study (449 mg·kg^{-1}) is larger than a similar test (98 mg·kg^{-1}) by An [9], who used the soils with different properties, a higher moisture content of 40% and without undergoing ageing process; all of these factors may contribute to the higher toxicity of the later test.

3.4 Combined toxicity in soil

An equitoxic combined experiment for As and Cd spiked soil was conducted based on the soil single toxicant exposure data. The combined test was composed according to Table 3. The combined effect curve (Fig. 6) used TU_{mix} as the horizontal axis, and the regression result indicated that the EC_{50} was 1.49 TU_{mix}, which means, a soil mixtured of 147 mg·kg^{-1} As (0.75 TU_{As}) and 337 mg·kg^{-1} Cd (0.75 TU_{Cd}) would inhibits 50% root elongation comparing with the control. The value of AI was −0.33 (−0.38~−0.28) , therefore, As and Cd had a less than additive toxicity to wheat root in soil.

3.5 Mode of interaction for As and Cd

It seems that the direction changes for interaction of As and Cd when the two elements present simultaneously in solution or in soil. In order to comprehend the notable difference between solution and soil on their EC_{50} value and combined effects, we used Visual MINTEQ (Version. 2.30, Department of Land and Water Resources Engineering, KTH, Sweden) to calculate solution speciation data [32-33]. Most of the As and Cd added were in active forms or toxic forms (Cd^{2+} and $H_2AsO_4^-$/$HAsO_4^{2-}$) in solutions. Labile soil As and Cd were extracted using 0.1 M $NaNO_3$ with a soil:solution ratio of 1:50 [34] to investigate the proportion of extractable elements. However, the EC_{50} and NOEC values expressed with labile As and Cd showed a reversal of the toxic orders for Cd and As, with Cd 5 times more toxic than As (data not shown). This means Cd/As contents extracted with 0.1 M $NaNO_3$ could not represent toxic forms, or the extraction data cannot fully explain the difference between soil and solution. Since the extraction conditions under which availability is measured always differ greatly from the field condition, and to large extent "availability" is composed of different speciation with different toxicity, caution should be exercised when using extraction data to explain the toxicity.

Combined anions affect the toxicity of Cd according to their types and content. In a soil with high level of chloride, the phytoavailablity of Cd is enhanced by forming stable and uptake-facilitating chloride complexes [35]. On the other hand, soil characteristics (pH, organic matter, adsorption capacity and moisture) and factors (ageing) also affect the availability and subsequently its toxicity in the complicated soil matrix [21, 31, 36]. Many compounds of As or Cd (for example, $Cd_3(AsO_4)_2$)

have very low solubility product (K_{sp}) values so precipitation could be formed to reduce the active concentration.

As and Cd exists in solution as ions of different charge, i.e. negative and positive, they may interact on the root surface and then affect another elements uptake by plants [37]. We hypothesize that positively charged Cd is easy to be absorbed to the surface of biomembranes which always have negative charge, so the total charge of membrane more close to neutral, thus the negative anion of arsenate is easy to absorb and pass through the bio-membrane. This may be part of the reason why As and Cd in solution had a more than additive toxicity, but this needs further investigation.

Hochadel and Waalkes [37] reported a test about the sequence of toxicants applied on rats. If pretreated with a NOEC concentration of As, decreased rat mortality compares well with the group only treated with Cd. However, when the exposure order was reversed, there had no significant effect of Cd to As. Exposure history could have a significant influence on the combined effect. Furthermore, interactions of two toxicants may occur at different levels, among the soil, during the uptake and on the target sites in cell [27]. Both the two toxicants are associated with the antioxidant system (for example metallothionein and glutathione) in cell. Sneller's study on Silene *Vulgaris* shown that As and Cd had additive effects on phytochelatins contents [15], which indicate that the interactions in the physiological level are important topics in need of further research.

When assessing the mixture toxicity, it should not hasten to say the elements act strictly similar or independently, usually their action modes are intervenient of the extremes. We had known that both As and Cd could affect the plant growth by inducing oxidation stress or enzyme content fluctuation and other factors. On the other hand, different shapes of concentration-effect curves in our result show that their interactions were not simple similar action.

In general, metal interactions may potentially occur at lower concentrations, more work needs to be conducted, for example, to use alternative methodology other than the simple similar action mode.

4. Conclusions

Generally speaking, arsenate is more toxic than cadmium to wheat root elongation. At the lower concentration, As may have less toxicity or even exhibit stimulation effect. Our solution culture experiment shows that the simultaneous exposure to As and Cd not only produces a toxicity higher than the single exposure, but also with a magnitude larger than the value predicted with a simple similar active mode or concentration addition. In the complex soil matrix, the active or the more toxic form of metal may dramatically decline from metal-soil interactions, so the combined effects judged from nominal concentration appears to exhibit antagonism. The extractable fraction of metals cannot represent the actual toxic parts of metal in soil. Therefore, simple additive mode should be used with caution to study the interaction of multiple toxicants in soil.

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Table 1 Some physicochemical properties of the soil used in the experiment

Parameters	Value	Parameters	Value
pH (H_2O, 1:2.5 soil/water ratio)	8.05	Total As ($mg \cdot kg^{-1}$)	12.2
CEC (cmol/kg)	17.0	Total Cd ($mg \cdot kg^{-1}$	0.04
Organic matter(g/kg)	20.4	Total Cu ($mg \cdot kg^{-1}$)	31.7
Sand (%)	45.99	Total Zn ($mg \cdot kg^{-1}$)	61.2
Silt (%)	47.75	Total Cr ($mg \cdot kg^{-1}$)	60.3
Clay (%)	6.26	Total Pb ($mg \cdot kg^{-1}$	22.6
Soil texture	Silt loam	Total Mn ($mg \cdot kg^{-1}$)	559
Total P ($mg \cdot kg^{-1}$)	887	Total Ni ($mg \cdot kg^{-1}$)	296

Table 2 Composition of combined test in solution experiment

Treatment	(TU_{mix})	**As conc.(μM)**	(TU_{As})		**Cd conc.(μM)**	(TU_{Cd})
1	(0)	**0**	(0)	**+**	**0**	(0)
2	(0.4)	**0.19**	(0.2)	**+**	**0.86**	(0.2)
3	(0.8)	**0.39**	(0.4)	**+**	**1.73**	(0.4)
4	(1.2)	**0.58**	(0.6)	**+**	**2.58**	(0.6)
5	(1.6)	**0.78**	(0.8)	**+**	**3.46**	(0.8)
6	(2)	**0.97**	(1)	**+**	**4.32**	(1)

Table 3 Composition of combined test in soil experiment

Treatment	(TU_{mix})	As conc.(μM)	(TU_{As})		Cd conc.(μM)	(TU_{Cd})
1	(0)	**0**	(0)	**+**	**0**	(0)
2	(0.1)	**9.8**	(0.05)	**+**	**22.4**	(0.05)
3	(0.2)	**19.6**	(0.1)	**+**	**44.9**	(0.1)
4	(0.4)	**39.2**	(0.2)	**+**	**89.8**	(0.2)
5	(0.8)	**78.4**	(0.4)	**+**	**179.6**	(0.4)
6	(1.2)	**117.6**	(0.6)	**+**	**269.4**	(0.6)
7	(1.6)	**156.8**	(0.8)	**+**	**359.2**	(0.8)
8	(2)	**196**	(1)	**+**	**449**	(1)
9	(3.2)	**313.6**	(1.6)	**+**	**718.4**	(1.6)

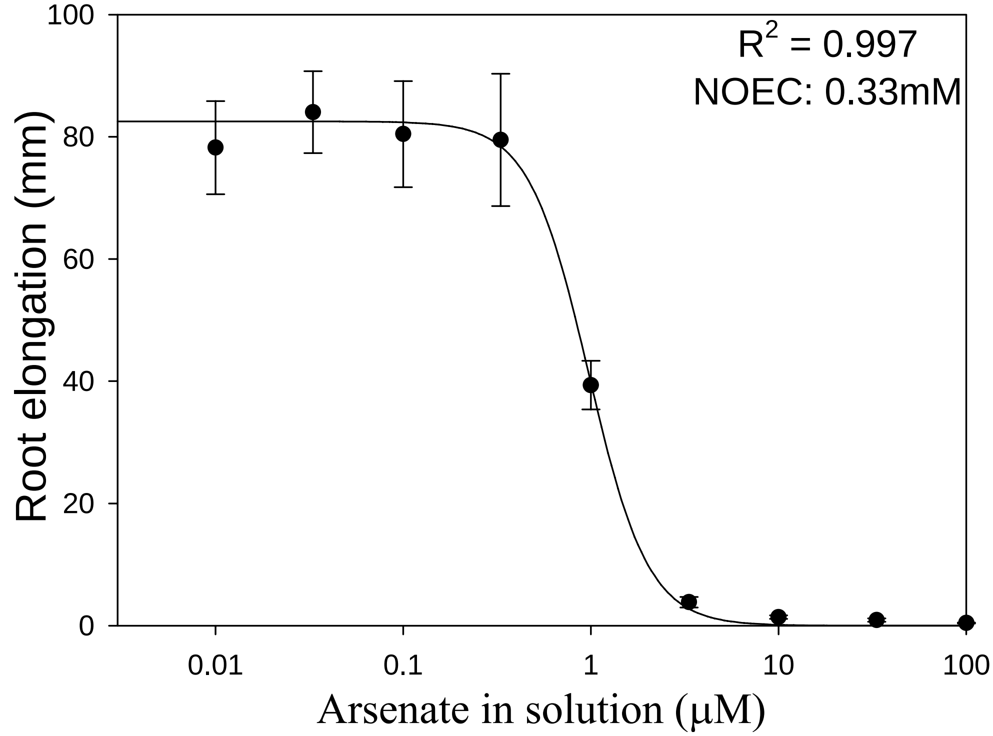

Fig. 1 Observed responses and fitted curve of wheat root to arsenate in solution. NOEC were assessed using Dunnett's procedure (at a 5% significance level) on the data. Error bar stands for standard error.

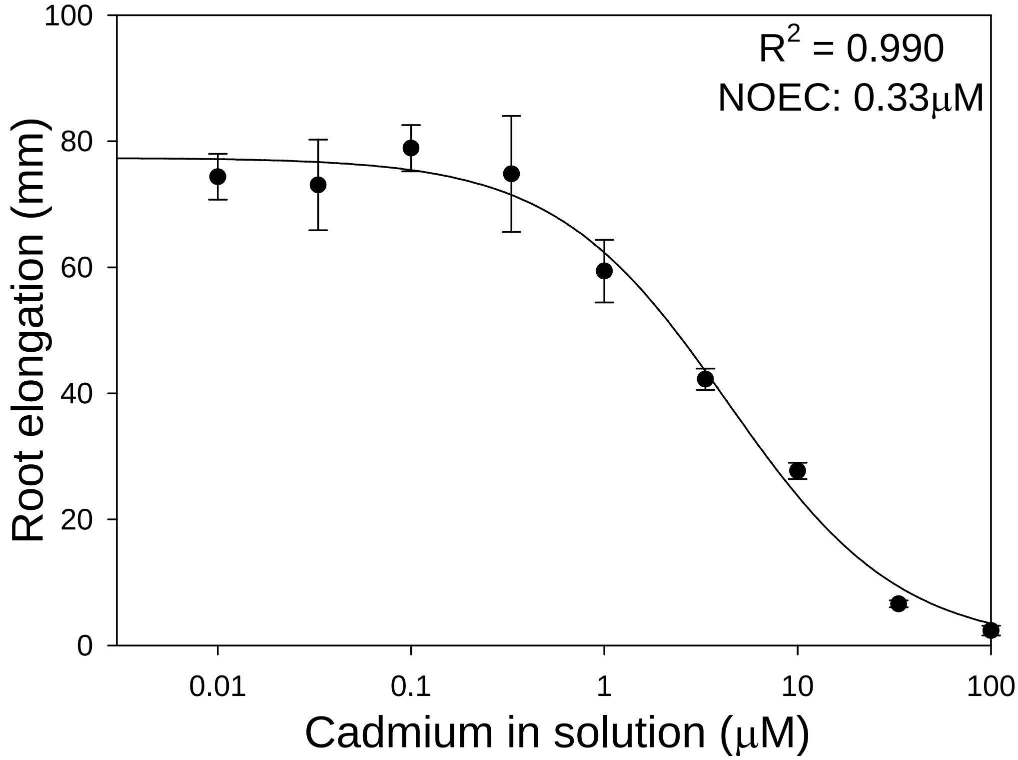

Fig. 2 Observed responses and fitted curves of wheat root to cadmium in solution. NOEC were assessed using Dunnett's procedure (at a 5% significance level) on the data. Error bar stands for standard error.

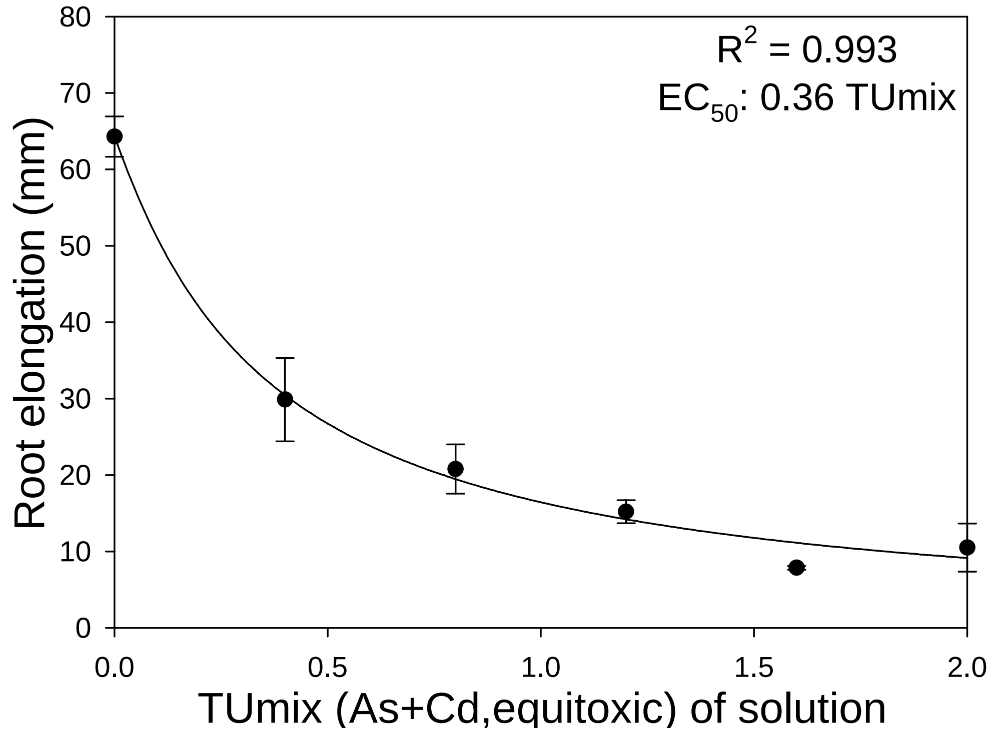

Fig. 3 Responses and fitted curves of wheat root to arsenate and cadmium mixture solution. Error bar stands for standard error.

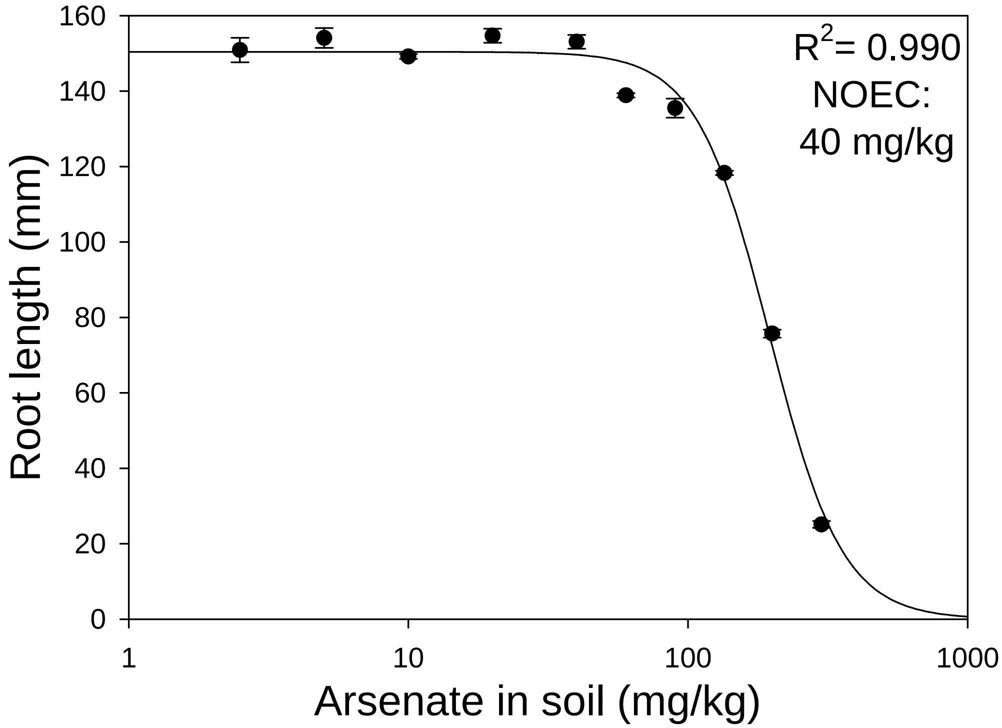

Fig. 4 Observed responses and fitted curves of wheat root to soil arsenate. NOEC were assessed using Dunnett's procedure (at a 5% significance level) on the data. Error bar stands for standard error.

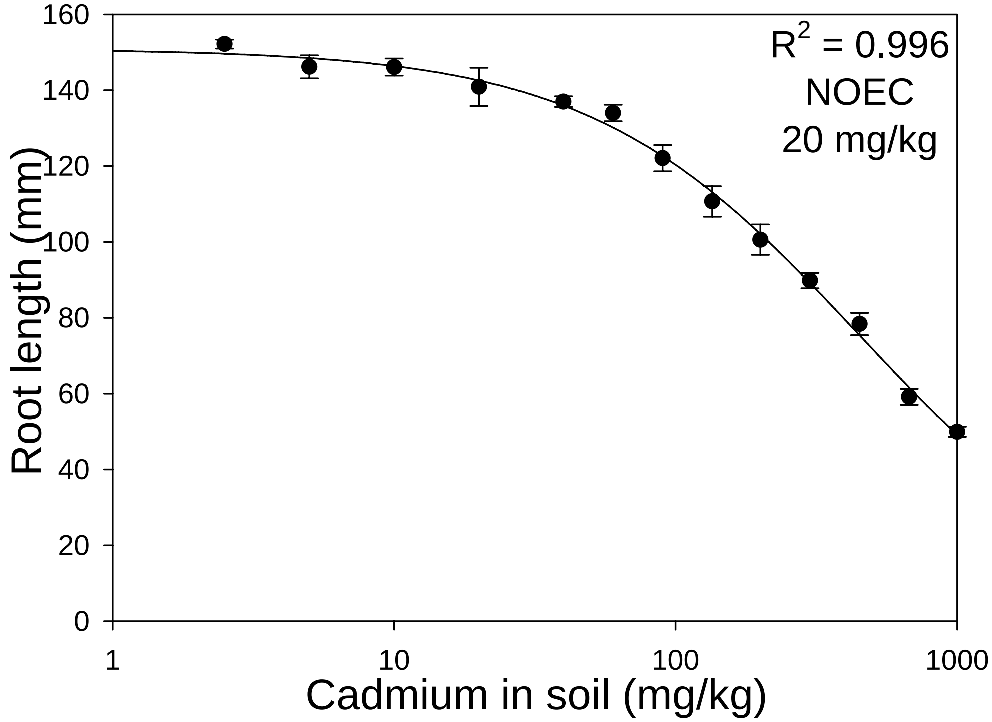

Fig. 5 Observed responses and fitted curves of wheat root to soil cadmium. NOEC were assessed using Dunnett's procedure (at a 5% significance level) on the data. Error bar stands for standard error.

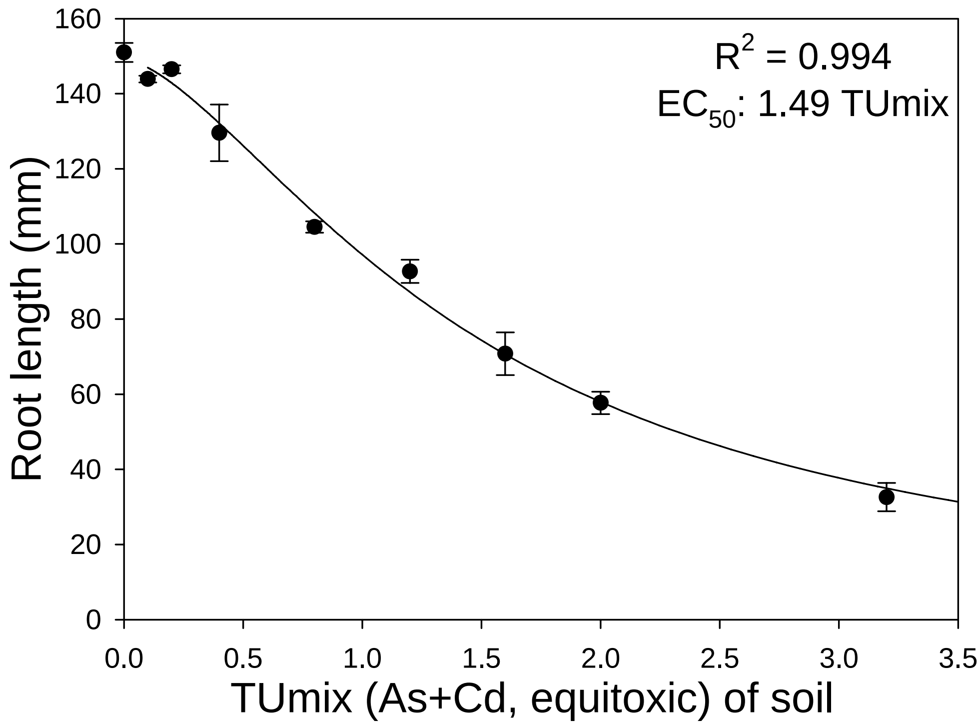

Fig. 6 Responses and fitted curves of wheat root to soil mixed with arsenate and cadmium. Error bar stands for standard error.