# Individual and combined phytotoxic effects of cadmium, lead and arsenic on soybean in Phaeozem

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**ABSTRACT**

Acute laboratory toxicity tests were carried out to assess the individual and combined toxic effects of metals including cadmium (Cd), lead (Pb) and the metalloid arsenic (As) in Phaeozem on the seed germination and seedling growth of soybean, *Glycine max*. Seeds were exposed to varied concentrations of Cd, Pb and As individually and in mixtures including Cd + Pb, Cd + As, Pb + As and Cd + Pb + As. The sum of toxic units (TU) for medium effective concentration of the mixture ($EC_{50mix}$) was calculated based on the dose (TU-based)-response relationship using the Trimmed Spearman-Karber method. Binary metal combinations of Cd + Pb, Cd + As and Pb + As produced additive, synergistic and antagonistic effects, respectively, on shoot growth and synergistic, synergistic and antagonistic effects on root growth. Ternary combination of Cd + Pb + As had a synergistic effect on shoot growth and an additive effect on root growth. Bioaccumulation of metals was observed in soybean and inhibited or enhanced bioaccumulations of individual metals were found in mixtures due to different combinations of metals.

**Keywords:** Phaeozem; cadmium; lead; arsenic; combined toxicity; soybean; toxic unit method

Metal(loid)s are often present in soil in mixtures of three to five, depending on the source of pollution (Spurgeon et al. 1994). Environmental effects of combined heavy metals may be quite different from those of individual pollutants due to interactions between heavy metals (MacFarlane and Burchett 2002, Zhou et al. 2006). Experiments on the toxicity of mixtures of pollutants may reflect the actual toxicity to ecosystems in a more realistic way than experiments in which toxicants are tested individually (Spurgeon et al. 1994), and several studies have investigated the combined effects of heavy metals on certain plant species. Antagonistic effects of cadmium (Cd) and lead (Pb) on Chinese watermelon (Guo and Zhou 2003), additive effects of Cd and arsenic (As) on alfalfa (*Medicago sativa* L.) (Zhou and Gao 1994), and combined effects of copper (Cu), Cd and Pb on cucumber (*Cucumis sativus*) (An et al. 2004) have been reported. Shute and Macfie (2006) studied the accumulation and distribution of Cd and Zn in combination on soybeans (*Glycine max*) and assessed the effect of one metal on the bioavailability of the other metal across the range of concentrations added to the soil. However, there still has been very little published information on combined effects of three toxic elements on soybean in the polluted area.

The Phaeozem region is a major production area of foodstuffs in China and plays an important role in maintenance of food safety. In recent years, with the discharge of industrial wastes, irrigation with polluted water and the application of fertilizers and pesticides, increasing quantities of heavy metals have entered Phaeozem and gradually accumulated (Guo and Zhou 2004), posing the risk of potential toxic effects. Some studies have been reported on Phaeozem pollution with heavy metals. Among all

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the cropland soil samples from the north part of Phaeozem region, 42.9% was polluted by Cd, and 57.1% was polluted by Pb. In the south part, the contents of Cd, Pb and As were relatively higher, and the pollution mainly occurred in the coal mine areas and suburbs (Guo et al. 2005, Cao et al. 2006). Nevertheless, there is little information on the combined toxic effects of heavy metals on crops grown in Phaeozem.

The present study employed Phaeozem to test the individual and combined phytotoxic effects of Cd, Pb and As on soybean (*Glycine max*) as Cd, Pb and As often occur together and soybean is the main crop in the Phaeozem region. The measurement endpoints included seed germination, lengths of roots and shoots. With acute toxicity experiments, using TU (toxic unit) method, the BAF (bioaccumulation factor) was calculated. Based on these results, the individual and combined phytotoxic effects and plant accumulation of Cd, Pb and As were analyzed. Our aim was to provide a theoretical basis for the risk assessment of heavy metal pollution and the maintenance of sustainable agricultural production.

## MATERIAL AND METHODS

**Sources of pollutants and crop species selected.** Salts used in the tests were $CdCl_2$, $PbCl_2$ and $Na_2HAsO_4$ (analytical grade) to supply the metals Cd and Pb and As. Henceforth in this paper we will use the term "metals" to include the two metals and the metalloid. Soybean tested was produced by the seed company of Jilin Agricultural University. Seed germination tests using control soil with no metals added showed that the germination rate was greater than 90%.

**Test soil.** The test soil was a typical Phaeozem (0–20 cm depth) collected from cropland in Northeast China. Physicochemical properties and background concentrations of Cd, Pb and As of the soil are listed in Table 1. The fractions of sand, silt and clay in the soil samples were determined using the hydrometer method (Beverwijk 1967), showing that sand amounted to 7.8%, silt 36.9% and clay 55.3%. Soil pH was measured with a pH meter (Radiometer Model pHM 93, France), and soil organic matter content was determined using the Walkley-Black method (Nelson and Sommers 1996). The cation exchange capacity was analyzed using the neutral $NH_4AC$ method (Lu 1999).

The prepared soil samples were analyzed for their total metal concentrations using an acid digestion method (Li and Thornton 1993). About 0.25 g of dried and homogenized soil samples were weighed and placed into Pyrex test tubes. 4.0 ml concentrated nitric acid and 1.0 ml concentrated perchloric acid were added. The mixtures were heated in a hot plate at 50°C for 3 h, 150°C for 3 h, 190°C for 18 h till complete dryness. After the test tubes were cooled, 5% (0.8M) nitric acid was added and heated at 60°C for 1 h with occasional mixing. Upon cooling, the mixtures were decanted into polyethylene tubes and centrifuged at 3 500 rpm for 10 min. Concentrations of Cd and Pb in solutions were determined using atomic absorption spectrophotometer (GBC-906, Australia). Another 0.5 g of soil samples were digested with concentrated nitric acid, concentrated perchloric acid and hydrochloric acid, and As concentrations were measured using an atomic absorption spectrometer coupled with a FIAS-400-hydride system (Perkin-Elmer 3100, USA). Measurements were made in triplicates and quality control included the analysis of a standard reference soil from the National Institute of Standards and Technology (NIST SRM 2709). Recoveries of metals ranged from 93.7 to 106.8%.

**Acute toxicity tests.** In the individual metal experiment, based on the results of preliminary experiments (An 2004), 7 treatment groups of Cd, Pb and As concentrations were added at 0, 50, 100, 200, 400, 800 and 1600 mg/kg. An additional treatment of 3200 mg/kg was prepared for Pb. The combined concentrations were prepared by

Table 1. Physicochemical properties and background concentrations of Cd, Pb and As of the test soil

| Parameter | Mean value ± SD[1] |
|---|---|
| pH | 6.76 ± 0.03 |
| OM (g/kg) | 27.8 ± 0.7 |
| CEC (cmol/kg) | 28.56 ± 1.1 |
| Cd (mg/kg) | ND[2] |
| Pb (mg/kg) | 17.95 ± 2.3 |
| As (mg/kg) | 10.62 ± 1.1 |
| Sand (1.00–0.05 mm) (%) | 7.8 |
| Silt (0.05–0.002 mm) (%) | 36.9 |
| Clay (< 0.002 mm) (%) | 55.3 |

OM – organic matter; CEC – cation exchange capacity; [1]means and standard deviations of triplicate samples; [2]not detected at detection limit of 0.030 mg/kg adding the same concentration of each metal as in the individual experiments. Combined experiments included three binary mixtures (Cd + Pb, Cd + As and Pb + As), and one ternary mixture (Cd + Pb + As). Different quantities of $CdCl_2$, $PbCl_2$, $Na_2HAsO_4$ according to the 7 treatments were dissolved in deionized water and the solutions were mixed thoroughly into 100 g of dry Phaeozem sieved through a clean 1-mm stainless steel mesh. Then 10 seeds were planted below the surface of the soil. All treatments were replicated three times. The samples were placed in a culture box (LRH-250-A; made in Guangdong) at a constant temperature of 27 ± 2°C for 7 days. Then, all the plants were washed with deionized water, after that, the lengths of the roots and shoots were measured and the seed germination frequency was calculated. The plants were then oven dried at 70°C for 48 h, and 0.10 g of sample was placed in a test tube with nitric acid (65%, 5 ml), allowed to sit at room temperature overnight, then heated to 95–100°C to complete the digestion. Digested samples were filtered through VWR Grade 413 qualitative filter paper and topped to 25 ml with deionized water. Concentrations of Cd, Pb and As in the roots and shoots were determined using the same instruments as used in the analysis of the test soil.

**Data analysis.** According to the Trimmed Spearman-Karber method (An 2004), $EC_{50}$ for root and shoot growth were calculated by expressing each treatment as a percentage of the control treatment. The CEAM (USEPA Center for Exposure Assessment Modeling) model was employed to estimate $EC_{50}$ values of individual metals. Then the $EC_{50}$ values determined for individual metals were used to calculate the $EC_{50}$ for mixtures ($EC_{50mix}$).

The Toxic Unit (TU) method was employed to test the response addition model for the pollutant mixtures since this approach was found to be an effective model to estimate the combined effects of metals in plant systems (Pape-Lindstrom and Lydy 1997, Faust et al. 2003, An et al. 2004). In the TU model, TU values were calculated as the following equation:

$$\sum TU_i = \sum_{i=1}^{n} \frac{c_i}{ECx_i}$$

where: $n$ is the number of mixture components; $c_i$ is the concentration of an individual component in the mixture; $ECx_i$ is the concentration of the $i^{th}$ mixture component that causes x% effect

The quotient ($c_i/ECx_i$) is generally termed as the TU of that component (Faust et al. 2003).

The concentrations of individual metal tests were converted into TUs by dividing the concentrations by the single metal $EC_{50}$ (TU = concentrations/$EC_{50}$). Based on the resulting dose (TU-based)-response relationships, $EC_{50mix}$ and sum of toxic unit (ΣTU) at 50% inhibition for the mixture were calculated. The combined effect was defined as being concentration additive ($EC_{50mix}$ = 1 TU), or as being greater or less than additive ($EC_{50mix}$ > 1 TU or < 1 TU) (Van der Geest et al. 2000) so that combined effects could be additive, synergistic or antagonistic.

All the statistical procedures were carried out using the SPSS software package version 10.0, Microsoft Excel 2003, and Origin 7.5.

## RESULTS AND DISCUSSION

### Effects on seed germination

#### *Inhibitory effects of individual metals*

Seed germination was expressed as a percentage of the mean of the control treatment, and the results are shown in Figure 1. Percent germination of soybean decreased from 100 mg/kg to 1600 mg/kg in three metals treatments, and decreased greatly at 1600 mg/kg. There was no general trend showing that the severity of the germination response increased with increasing concentrations of metals. The statistical analysis showed that there were no significant differences in percent germination of soybean exposed to the seven experimental concentrations of Cd and Pb. By contrast, As had a significantly ($P < 0.01$) inhibitory effect on soybean germination under the experimental conditions. Soybean is sensitive to toxicological effects of As, because the inhibitory rate of seed germination reached 100% when the concentration of As in soil was 1600 mg/kg.

#### *Inhibitory effects of combined metals*

The inhibitory effects of binary combinations of Cd + Pb, Cd + As, and Pb + As were similar. Percent germination significantly decreased at 100 mg/kg for each combination, and at higher concentrations (> 200 mg/kg) it decreased with increasing combined concentrations of metals.

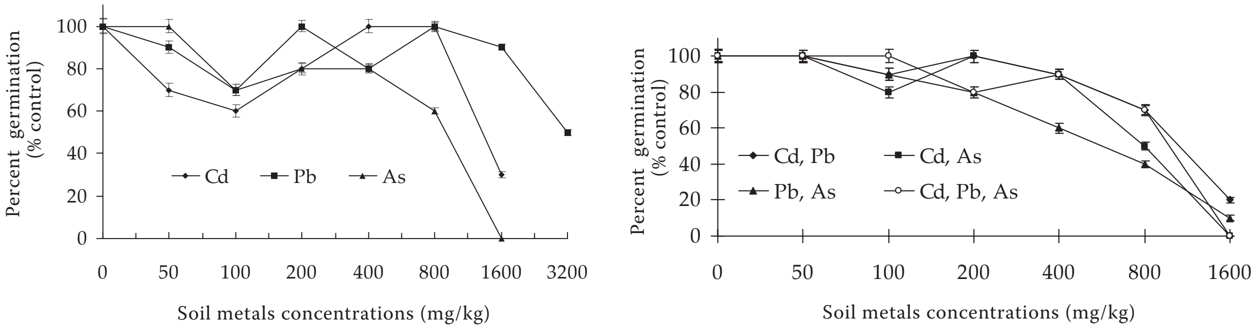


Figure 1. Percent germination of soybean seeds exposed to different metals (Cd, Pb and As) concentrations in the soil

The Pb + As combination had a more inhibitory effect on seed germination. In the case of the ternary combination of Cd + Pb + As, percent germination decreased markedly when the concentrations were greater than 400 mg/kg. There were significant relationships between the percent germination and the concentrations of all three metal combinations ($P < 0.01$).

Seed germination was significantly influenced only when concentrations of Cd, Pb and As in the soil were relatively high (> 800 mg/kg) indicating some resistance of seed germination to toxicity of Cd, Pb and As in most of the experimental treatments. Seed germination was therefore not a sensitive indicator of the phytotoxic effects of Cd, Pb and As in the soil. Similar results were found in previous studies in which seed germination of sorghum, cucumber, wheat and sweet corn were all insensitive to soil Cd concentrations (An 2004).

## Effects on soybean growth

### *Phytotoxic effects of individual metals*

Root and shoot growth of the soybean exposed to Cd, Pb and As in the soil were expressed as percentage of the growth compared to the control treatment (Figure 2). All three metals addition had adverse effects on root and shoot growth. Compared with the control treatment, percent growth of the roots and shoots decreased with the increased concentrations of Cd, Pb and As in the soil and the rate of decrease exposed to As was the highest. The results demonstrated that the toxicity of Cd, Pb and As increased with increasing concentrations of individual metals in the soil. Furthermore, the effect of As on the morphology of the soybean was more pronounced than that of Cd or Pb (Figure 3).

$EC_{50}$ values calculated using the CEAM model are shown in Table 2. The addition of individual Cd, Pb and As resulted in different effects on soybean growth. Among the three metals tested, As was the most toxic, followed by Cd, and Pb was the least toxic. This phenomenon appeared to be associated with the pH of the test soil. Arsenate [$As^{(V)}$] and arsenite [$As^{(III)}$] are the primary forms of inorganic As in soils, and $As^{(III)}$ is the most toxic, soluble, and mobile species found in the environment (Masscheleyn et al. 1991, Smith et al. 1999). The investigations showed that As sorption was largely dependent on soil pH, and there was a general decrease in $As^{(V)}$ sorption as pH

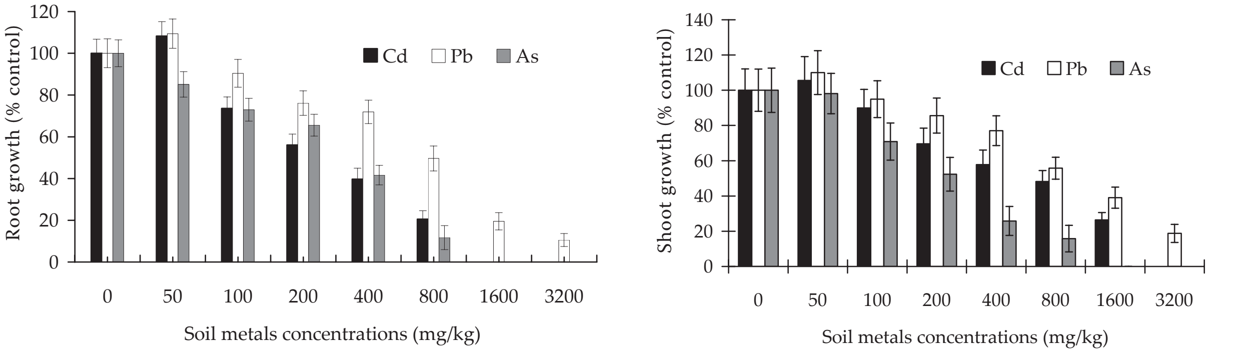


Figure 2. Percentage growth of the roots and shoots exposed to different metals (Cd, Pb and As) concentrations in the soil


Figure 3. Morphology of soybean in the soil with high Cd, Pb and As concentrations

increase, while the proportion of $As^{(III)}$ sorbed by soil increased with increasing pH (Smith et al. 1999). Smith et al. (1999) observed that sorption by the soil ranged from approximately 0.80 of added $As^{(III)}$ at low pH, to approximately 0.95 of added $As^{(III)}$ at pH 6 to 7. As stated previously, $As^{(III)}$ sorbed by the soil in this study was relatively higher at pH 6.76. Therefore, the phytotoxic effect of excessive As under the experimental conditions would be expected.

Table 2. $EC_{50}$ values for growth of roots and shoots exposed to individual Cd, Pb and As in the soil

| | Cd | Pb | As |
|---|---|---|---|
| Roots | 213 (187–241)* | 583 (450–621) | 195 (162–224) |
| Shoots | 377 (287–500) | 829 (692–992) | 299 (253–354) |

*95% confidence levels is in parenthesis

### *Phytotoxic effects of combined metals*

The effects of combined pollution have been classified into three types, namely, additive, synergistic, and antagonistic effects (Khalil et al. 1996). The combined effects of the binary mixtures tested produced all three types of interactions, and the results are shown in Table 3. In terms of root growth, the $EC_{50mix}$ values exposed to combinations of Cd + Pb, Cd + As and Pb + As were 0.68 (0.62–0.79), 0.85 (0.60–1.18), and 1.21 (1.12–1.29), respectively. The $EC_{50mix}$ value for the Pb + As combination was greater than 1, thus the interaction of Pb and As was antagonistic. Synergistic responses to the growth of the roots occurred in Cd + Pb and Cd + As combinations since both $EC_{50mix}$ values were less than 1. In the case of shoot growth, $EC_{50mix}$ values from binary combinations of Cd + Pb, Cd + As and Pb + As were 1.09 (0.91–1.30), 0.51 (0.44–0.58) and 1.27 (1.23–1.28), respectively. The interactions between Cd + Pb, Cd + As and Pb + As were additive, synergism and antagonism with different $EC_{50mix}$ values. The ternary combination of Cd + As + Pb resulted in an additive effect on root growth and a synergistic effect on shoot growth, with $EC_{50mix}$ values of 0.98 (0.47–1.95) and 0.63 (0.62–0.67), respectively.

As for the growth of roots and shoots, the interactions among Cd, Pb and As in the shoots were not always consistent with those in the roots, an

Table 3. $EC_{50mix}$ values and combined effects on root and shoot growth of soybean

| Pollutants | Roots | Shoots |
|---|---|---|
| Cd + Pb | 0.68 (0.62–0.79) synergism | 1.09 (0.91–1.30) additive |
| Cd + As | 0.85 (0.60–1.18) synergism | 0.51 (0.44–0.58) synergism |
| Pb + As | 1.21 (1.12–1.29) antagonism | 1.27 (1.23–1.28) antagonism |
| Cd + Pb + As | 0.98 (0.47–1.95) additive | 0.63 (0.62–0.67) synergism |

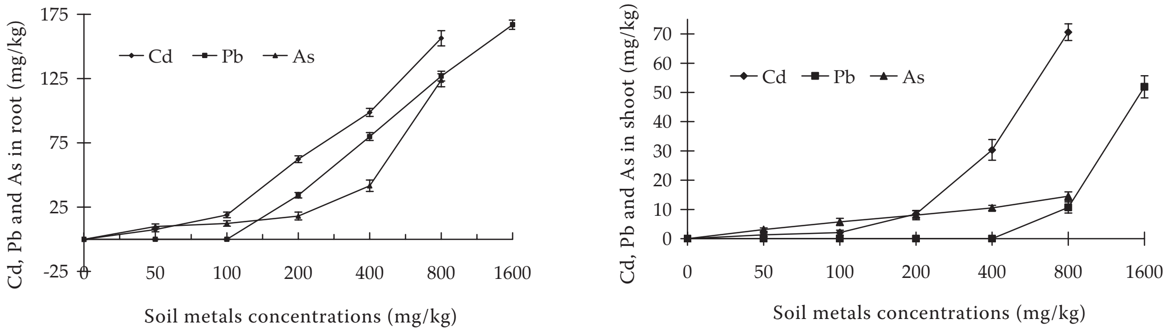


Figure 4. Concentrations of Cd, Pb and As in soybean exposed to different metals (Cd, Pb and As) concentrations in the soil

observation possibly related to differences in both the translocation of metals from roots to shoots and the characteristic of the different plant tissue. Furthermore, according to previous studies, toxicity may exist for some species but not others (Burton et al. 2001). Under our experimental conditions, the interaction between Cd and As was synergistic to the growth of both roots and shoots. The cationic form of Cd can combine with the anionic form of As to form a compound with higher toxicity than that of Cd or As, resulting in an additive phytotoxic effect, thus the combined toxicity of Cd and As was very strong. Consistent with our results, Zhou (1995) found that Cd and As in combination showed synergistic toxic effects to both seed germination and plant growth especially when the two metals occurred together at higher concentrations.

## Plant uptake and translocation of metals

### *Plant uptake and translocation of individual metals*

The plant concentrations of Cd, Pb and As are shown in Figure 4. Concentrations of Cd, Pb and As in the roots and shoots increased significantly

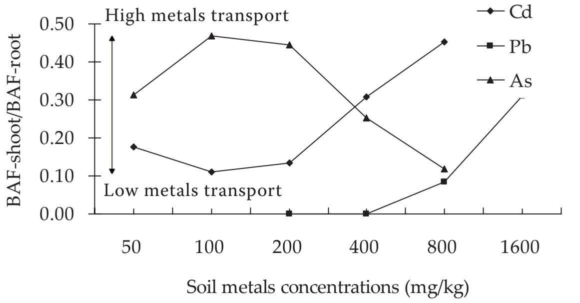


Figure 5. Ratios of BAF-shoot to BAF-root exposed to different metals (Cd, Pb and As) concentrations in the soil

with increasing concentrations of metals in the soil ($P < 0.05$). Concentrations of Cd, Pb and As were greater in the roots than those in the shoots. In the roots, concentrations of Cd were higher than those of Pb and As, and when the Cd concentration in the soil reached 800 mg/kg, root and shoot concentrations were as high as 156 and 71 mg/kg, respectively. Pb concentrations in the roots and shoots were close to 0 mg/kg when the soil concentration was less than 100 mg/kg, but increased significantly with increasing Pb concentration in the soil above 100 mg/kg indicating that low concentrations of Pb in the soil had low mobility throughout the soil-plant system. The results in this study showed that the mobility of Cd was the highest, while the mobility of Pb was the lowest.

To analyze the translocation of Cd, Pb and As in the soil-plant system, the bioaccumulation factor (BAF = metal concentration in the crop/metal concentration in the soil) was calculated, and the ratios of BAF for the shoots to the roots as a function of metal concentrations in the soil are shown in Figure 5. The ratios of BAF for the shoots to the roots were far below 1, which is in agreement with previous studies (Cataldo et al. 1981, Wang and Shen 2001). This confirms that Cd, Pb and As accumulated mainly in the soybean roots, with only small amount of metals translocated to the shoots. Moreover, there were significant differences among the three metals in the amount translocated to the shoots as the metals concentrations in the soil increased. More Pb was translocated as the Pb concentration in the soil increased, and more Pb accumulated in the roots. At 100 mg/kg in the soil, BAF-shoot/BAF-root would be the lowest for Cd and the highest for As, then the ratio increased for Cd and decreased for As with the increase of metals concentrations in the soil. Thus translocation of Cd to the shoots would enhance

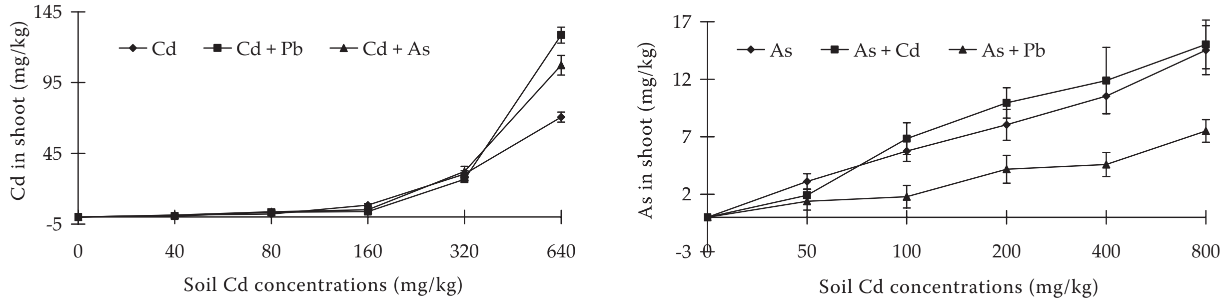


Figure 6. Combined effects on shoots exposed to binary mixtures of Cd, Pb and As

as Cd concentrations in the soil increased, which was contrary to the translocation of As.

### *Plant uptake and translocation of combined metals*

Bioaccumulation of a single metal was influenced by the presence of other metals, which resulted in inhibited or enhanced bioaccumulation of one metal in the mixture (Peralta-Videa et al. 2002, An et al. 2004). The combined effects of binary combinations on the shoots in this study are shown in Figure 6. For exposure to the Cd + As combination, the content of Cd in shoots increased by As addition (Figure 6), and the content of As in shoots was also enhanced in the presence of Cd (Figure 6), which may be the reason for the synergistic effects of Cd and As on shoot growth ($EC_{50mix}$ = 0.51). For the Pb + As combination, As concentration in shoots decreased in the presence of Pb, which indicated that the response of the Pb + As combination on shoot growth was antagonistic ($EC_{50mix}$ = 1.27). However, the accumulation effect of the Cd + Pb combination was not coincident with the soybean growth response. Pb addition had no distinct influence on shoot Cd accumulation (Figure 6), and as a result, for the Cd + Pb combination the Cd-Pb uptake to the shoots had no significant relationship with the combined response in terms of soybean growth.

Under the experimental conditions, the soybean root could not grow normally at the higher metals concentrations; therefore, only parts of samples were used to analyze the combined effects on the root growth. Under the binary combination of Cd + Pb and Cd + As, accumulation concentrations of Cd in roots increased with presence of Pb and As (Figure 7). Due to the presence of Cd, As accumulated in roots increased, too (Figure 7), which was coincident with the result of combined effects calculated using the TU model ($EC_{50mix}$ = 0.85). This phenomenon was the reason of the synergistic effects of Cd and As on the root growth. For the combination of Pb + As, accumulation concentrations of As decreased with the presence of Pb, which indicates that the combined effect of Pb and As on the root growth was antagonistic ($EC_{50mix}$ = 1.21).

This study has demonstrated that for the combination of three metals, the concentrations of each metal in shoot were enhanced (Figure 8), which resulted in an increased toxic effect of Cd, Pb and As on shoot growth. This agrees with the result that the effect of the Cd + Pb + As combination was synergistic ($EC_{50mix}$ = 0.63). No analysis was carried out on root growth because root samples

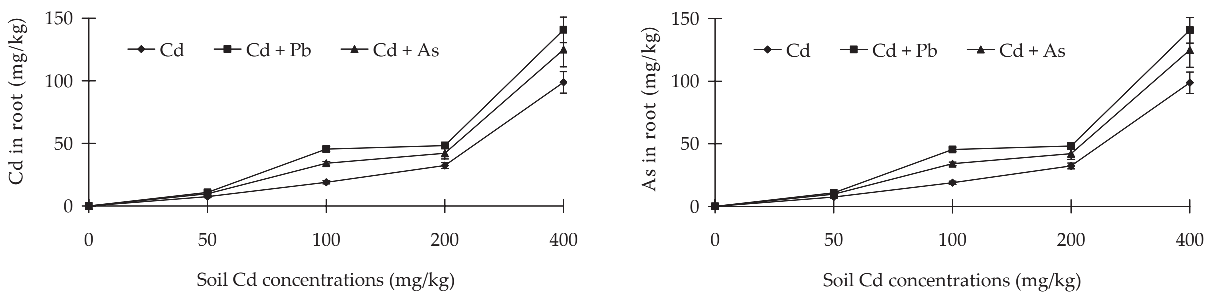


Figure 7. Combined effects on roots exposed to binary mixture of Cd, Pb and As

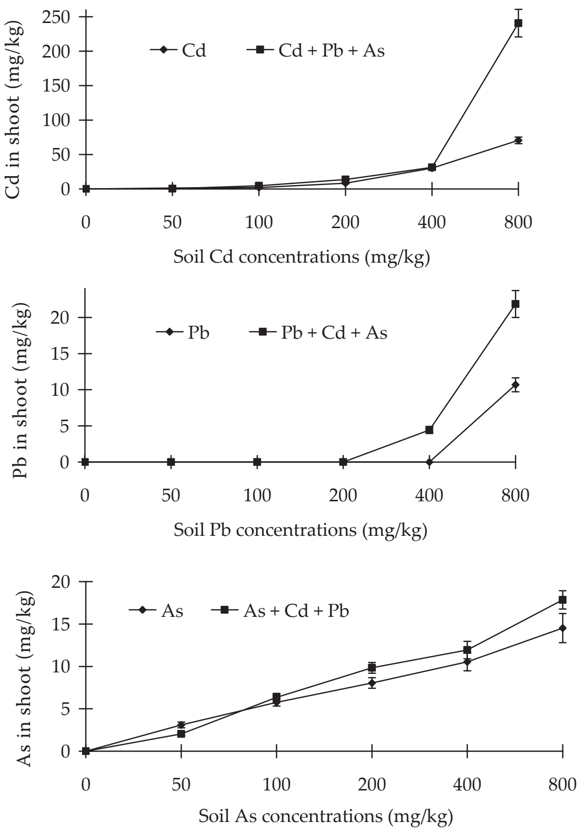


Figure 8. Combined effects on shoots exposed to ternary mixture of Cd, Pb and As

could not be obtained from most treatments subjected to the test concentrations.

The mixture toxicity of Cd, Pb, and As to the soybean was investigated in soil microcosm studies. Individual Cd, Pb and As in the soil had adverse effects on the seedling growth of the soybean, and As was more toxic than Cd and Pb to the soybean in this study. The phytotoxicity of metals depended on the mobility of these metals from the soil to the plant, as well as on the tissue (root, shoot) of plant. The binary mixtures of Cd + Pb, Cd + As and Pb + As produced all three types of interactions (antagonistic, additive, and synergistic responses) depending on the different combinations. Ternary combination resulted in an increased toxicity, and the combined effects were additive and synergistic on root and shoot growths, respectively. The combined effects of metals on the roots were stronger than those on the shoots. Inhibited or enhanced bioaccumulation of individual metals was observed in mixtures, and the mixture toxicity was more dependent on different metals combinations.

The results of this study indicate that combined effects take place in contaminated Phaeozem and should be taken into account when assessing the risk of metal-contaminated field, which also could provide primary information for remediation and prevention. As a part of the research on heavy metals pollution of Phaeozem, this study focuses merely on the combined phytotoxic effects of Cd, Pb and As on the soybean. The changes in phytotoxicity and uptake of individual and combined elements on physiological and biochemical base, as well as the evaluation of elements mobility in combination are essential in the research of combined phytotoxicity, and further research will be conducted.

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