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Effect of arsenic-phosphorus interaction on arsenic-induced oxidative stress in chickpea plants

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ABSTRACT

Arsenic-induced oxidative stress in chickpea was investigated under glasshouse conditions in response to application of arsenic and phosphorus. Three levels of arsenic (0, 30 and 60 mg kg^{-1}) and four levels of P (50, 100, 200, and 400 mg kg^{-1}) were applied to soil-grown plants. Increasing levels of both arsenic and P significantly increased arsenic concentrations in the plants. Shoot growth was reduced with increased arsenic supply regardless of applied P levels. Applied arsenic induced oxidative stress in the plants, and the concentrations of H_2O_2 and lipid peroxidation were increased. Activity of superoxide dismutase (SOD) and concentrations of non-enyzmatic antioxidants decreased in these plants, but activities of catalase (CAT) and ascorbate peroxidase (APX) were significantly increased under arsenic phytotoxicity. Increased supply of P decreased activities of CAT and APX, and decreased concentrations of non-enzymatic antioxidants, but the high-P plants had lowered lipid peroxidation. It can be concluded that P increased uptake of arsenic from the soil, probably by making it more available, but although plant growth was

inhibited by arsenic the P may have partially protected the membranes from arsenic-induced oxidative stress.

Abbreviations: AA - Non enzymatic antioxidant activity, **APX** - Ascorbate peroxidase, **CAT** - Catalase, **MDA** - Malondialdehyde, **NBT** - Nitroblue tetrazolium, **PEDXRF**- Polarized energy dispersive X-ray fluorescence, **ROS** - Reactive oxygen species, **SOD** - Superoxide dismutase, **XRF** - X-ray fluorescence,

Introduction

Arsenic, a non-essential element for plants and animals, occurs naturally in the environment through geological activities. The element is most commonly found in the oxidized states arsenite (As(III)) and arsenate (As(V)). Arsenic contamination is widespread due to anthropogenic activities such as smelting operations, fossil fuel combustion (Nriagu and Pacyna 1988; Ochiai 1995) and arsenic-based agrochemicals, fertilizers and disposal of municipal and industrial wastes (Requejo and Tena 2006). Arsenic-enriched soils are considered major sources of contamination in the food chain and water supplies, and this is of great environmental concern because arsenic is known to be a carcinogen and mutagen (Fayiga and Ma 2006).

Plants take up arsenic mainly as arsenate. Exposure to arsenate causes considerable stress in plants, including inhibition of growth and finally death (Stoeva and Bineva 2003) and causes physiological disorders (Stoeva et al. 2005). However, the biochemical responses of plants to

arsenic stress are insufficiently studied (Hartley-Whitaker et al. 2001). Cytoplasmic arsenate interferes with metabolic processes involving phosphate, giving it the potential to be toxic to plants, but it is probably reduced in the cytoplasm to arsenite (Meharg and Hartley-Whitaker 2002; Stoeva and Bineva 2003). Arsenite reacts with sulfhydryl groups (-SH) of enzymes and tissue proteins, inhibiting cellular function and causing death (Ullrich-Eberius et al. 1989). Even though arsenic is not a redox metal, there is significant evidence that exposure of plants to inorganic arsenic results in the generation of reactive oxygen species (ROS), which are connected with the valence changes that the element readily undergoes from arsenate to arsenite in plants (Meharg and Hartley-Whitaker 2002). The ROS, such as superoxide radicals ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet -}$) and hydrogen peroxide (H_2O_2), are strong oxidizing agents that cause oxidative damage to biomolecules such as lipids and proteins and eventually cause cell death (Molassiotis et al. 2006; Gunes et al. 2007). ROS produced as a result of various abiotic stresses need to be scavenged for maintenance of normal growth. Plants have evolved mechanisms to protect cells and subcellular systems from the effects of ROS by using enzymatic antioxidants such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and ascorbate peroxidase (APX; EC 1.11.1.11), and non-enzymatic antioxidants such as ascorbate, glutathione and α-tocopherol (Sairam et al. 2005; Gunes et al. 2007). Altered activities of these antioxidant enzymes and antioxidants have been commonly reported, and are used frequently as indicators of oxidative stress in plants. Although changes in their activities have been reported under various abiotic stresses, little information is available on the effects of arsenic on antioxidant enzymes. Furthermore, the effects of arsenic and P interactions on oxidative stress and antioxidant metabolism of plants have not been previously investigated.

Studies on arsenic toxicity have focused mainly on arsenate because it is the dominant form in aerobic soils. Arsenic is analogous to phosphorus; both have similar electron configurations and chemical properties and compete for the same uptake carriers in the root plasmalemma (Ullrich-Eberius, et al. 1989; Meharg and MacNair 1992; Meharg and Hartley-Whitaker 2002). In soil, arsenate and phosphate compete with each other for soil sorption sites, and this competition results in a reduction in their sorption by soil and an increase in solution concentrations (Gao and Mucci 2001; Smith et al. 2002). Knowledge of arsenate and phosphate interactions is important for a better understanding of their uptake and accumulation by plants due to the similarities in chemical behaviors of the two ions. Tu and Ma (2003) have reported competitive effects between arsenate and phosphate in Chinese brake fern. They observed that phosphate substantially increased plant biomass and arsenate accumulation by alleviating arsenate phytotoxicity at high arsenate levels. They also reported high doses of arsenic decreased phosphate concentrations of the plants. However, phosphate has also been shown to increase arsenic availability in soil leading to increased plant uptake (Peryea 1991; Fayiga and Ma 2006).

Previous studies have mainly expanded on the toxicology and uptake of arsenic in arsenic hyperaccumulator plants such as ferns (Tu et al. 2004; Tu and Ma 2003; 2004; Srivastava et al. 2005); less is known about arsenic toxicity mechanisms in relation to interactions with phosphorus in field crops. This study was, therefore, designed to investigate the effect of arsenic and P interactions on chickpea growth, concentrations of arsenic and P, and arsenic-induced oxidative stress and oxidative stress response mechanisms in chickpea.

Materials and Methods

Growth conditions and treatments

Chickpea (*Cicer arietinum* L. cv. Uzunlu 99) plants were grown from May 08, 2007 to July 02, 2007 in a naturally lighted glasshouse (approximately 50% relative humidity, and 32-34/18-20^{o}C day/night temperature) at the Faculty of Agriculture, Ankara University (39^{o} 57 44.51 N; 32^{o} 51 46.95 E). Some characteristics of the soil were as follows: field capacity 24 %, texture clay loam, $CaCO_3$ 57.3 g kg^{-1}, pH (1:2.5 water) 7.98, EC 0.44 dS m^{-1}, organic matter 7.60 g kg^{-1}, total N 0.92 g kg^{-1}. The concentrations of NH_4OAc-extractable K, Ca and Na were as follows (mg kg^{-1}); 470, 2566 and 64, respectively. The $NaHCO_3$-available P was 8.54 mg kg^{-1}, and DTPA-extractable Zn, Fe, Cu and Mn were as follows (mg kg^{-1}); 0.72, 6.36, 1.01 and 24 respectively, and water soluble As was 2.14 mg kg^{-1}. PVC pots lined with polyethylene were filled with 3 kg of air-dried soil. Treatments, with three replicates, consisted of four levels of phosphorus 50, 100, 200 and 400 mg P kg^{-1} soil (as KH_2PO_4), and three arsenic rates 0, 30 and 60 mg As kg^{-1} (as $NaAsO_2$). All these treatments were applied and incorporated into the soil before seed sowing. Potassium concentrations were balanced with K_2SO_4. Chickpea seeds were sown at the rate of 8 seeds to each pot. After a good stand of plants developed, they were thinned to 4 plants per pot. For the basal N fertilization, 150 mg kg^{-1} of N was applied as NH_4NO_3 before sowing. During the experiment, soil was kept at approximately 70% of field capacity by watering with tap water.

Sampling and harvest procedure

For fresh matter used for assays, samples were taken from leaflets from fully matured leaves chosen at random. All the measurements with fresh matter were carried out from June 25 - 29, 2007. At the end of the experiment, plants were harvested. Shoots were washed once with tap water and twice in deionized water. They were then dried in a forced-air oven at 60 ^{o}C until constant mass was reached and they were ground (40 mesh sieve) for elemental analysis.

Enzyme extraction and assay

Enzyme extraction procedures were carried out at 0-4 °C. Samples (0.5 g) were taken from the youngest leaflets from fully matured leaves, and were homogenized by a Heidolph, Diax 900 homogenizer in 5 ml 100 *mM* potassium phosphate buffer (pH 7.6) containing 1 *mM* EDTA-Na_2. Because APX is labile in the absence of ascorbate, 0.5 *mM* ascorbate was included for the extraction of this enzyme with the above procedure. The homogenized samples were centrifuged at 10,000 *g* for 5 minutes. The supernatant was used as a crude enzyme extract in SOD, CAT and APX enzyme analyses. All colorimetric measurements (including enzyme activities) were made at 20 ^{o}C in a Shimadzu UV/VIS 1201 spectrophotometer.

Superoxide dismutase (SOD) activity was assayed by the nitroblue tetrazolium (NBT) method (Giannopolitis and Ries 1977). The reaction mixture (3 ml) contained 50 *mM* Na-phosphate buffer, pH 7.3, 13 *mM* methionine, 75 *μM* NBT, 0.1 *mM* EDTA, 4 *μM* riboflavin and enzyme extract (0.2 ml). The reaction was started by the addition of riboflavin, and the glass test tubes were shaken and placed under fluorescent lamps (60 μmol m^{-2} s^{-1}). The reaction was allowed to proceed for 5 minutes and was then stopped by switching off the light. The absorbance was measured at 560 nm. Blanks and controls were run in the same manner but without illumination

and enzyme, respectively. One unit of SOD was defined as the amount of enzyme that produced 50% inhibition of NBT reduction under assay conditions.

Ascorbate peroxidase (APX) activity was determined by following the decrease of ascorbate and measuring the change in absorbance at 290 nm for 1 minute in 2 ml of a reaction mixture containing 50 *mM* potassium phosphate buffer (pH 7.0), 1 *mM* EDTA-Na_2, 0.5 *mM* ascorbic acid, 0.1 *mM* H_2O_2 and 50 µL of crude enzyme extract at 25 °C (Nakano and Asada 1981). The activity was calculated from the extinction coefficient (2.8 mM^{-1} cm^{-1}) for ascorbate.

Catalase (CAT) activity was determined as a decrease in absorbance at 240 nm for 1 minute following the decomposition of H_2O_2, as modified by Cakmak et al. (1993). The reaction mixture (3 ml) contained 50 *mM* phosphate buffer (pH 7.0), 15 *mM* H_2O_2 and 50 µL of crude enzyme extract at 25 °C. The activity was calculated from the extinction coefficient (40 mM^{-1} cm^{-1}) for H_2O_2.

Determination of membrane damage and non-enzymatic antioxidants

Lipid peroxidation (MDA) in fully matured leaves was measured to assess the membrane damage. For the measurement of lipid peroxidation, the thiobarbituric acid (TBA) test which determines MDA as an end product of lipid peroxidation was used (Hodges et al. 1999). For this, sub-samples (500 mg) were homogenized in 4.0 ml of 1% TCA (trichloroacetic acid) solution and centrifuged at 10,000g for 10 minutes. The supernatant was added to 1 ml 0.5% (w:v) TBA in 20% TCA. The mixture was incubated in boiling water for 30 minutes and the reaction was

stopped by placing the tubes in an ice bath. The samples were centrifuged at 10,000 *g* for 5 minutes, and the absorbance of the supernatant was read at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA-TBA complex (pink pigment) was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1}.

The non-enzymatic total antioxidant activity was estimated by the method of Prieto et al. (1999). The assay is based on the reduction of Mo(VI) to Mo(V) and subsequent formation of a green phosphate/Mo(V) complex at acidic pH. 0.5 g of shoot samples was homogenized in 10 ml ethanol and centrifuged at 10,000 *g* for 5 minutes. 0.1 ml ethanolic extract was combined with 3 ml of reagent solution (0.6 *M* sulphuric acid, 28 *mM* sodium phosphate and 4 *mM* ammonium molybdate). The tubes were incubated at 95 ^{o}C for 90 minutes. After the mixture had cooled to room temperature, the absorbance was measured at 695 nm. The antioxidant activity was expressed as the number of equivalents of ascorbic acid on a dry weight basis.

Determination of H_2O_2 concentration

The H_2O_2 content of fully matured leaves was colorimetrically measured as described by Mukherjee and Choudhuri (1983). To determine H_2O_2 levels, leaf samples were extracted with cold acetone. An aliquot (3 ml) of the extracted solution was mixed with 1 ml of 0.1% titanium dioxide in 20% (v:v) H_2SO_4 and the mixture was then centrifuged at 6,000 *g* for 15 minutes. The intensity of yellow colour of the supernatant was measured at 415 nm. The concentration of H_2O_2 was calculated from a standard curve plotted within the range of 100 to 1000 *nM* H_2O_2.

Elemental Analysis

After determination of dry weights, all samples were ground into fine powder in an agate mortar. They were then passed through a 200 μm sieve. Sieved sub-samples were pressed into thick pellets of 32 mm diameter using wax as a binder. USGS Standards, Leaf Standards, GBW 7109 and GBW-7309 sediment as reference standard materials were also pressed into pellets in a similar manner as the samples, and used for quality assurance.

Arsenic and P concentrations were determined by polarized energy dispersive XRF. The spectrometer used in this study was a Spectro XLAB 2000 PEDXRF spectrometer which was equipped with a Rh anode X-ray tube, 0.5 mm Be side window. The detector of the spectrometer is Si (Li) cooled by liquid N_2 with a resolution of < 150eV at Mn Kα, 5000 cps. The spectrometer configures d as source beam, scattered beam and fluorescent beam all at mutually orthogonal angles (Stephens and Calder 2004).

The sample measurements by PEDXRF were mainly done by three types of targets. The first target (Barkla target) is suitable for the light elements with $Z > 22$. The second target (Bragg target) is an oriented crystal target suitable for light elements up to $Z = 22$. The third target is a pure metal target suitable for specific elements or small groups of adjacent elements. Additionally this target is useful for generating Compton scatter peaks which can be used for matrix correction (Ivanova et al. 1999; Chuparina and Gunicheva 2003).

Statistical Analysis

The experiments were set up in a completely randomised design with three replicate pots. Analysis of variance was performed on the data, and significant differences among treatment means were compared by descriptive statistics (± SE) and by Duncan's multiple range tests.

Results

Plant growth, arsenic and phosphorus concentrations

The interaction between P and arsenic, and the effect of P treatment, on the fresh and dry weight of chickpea shoots were not significant, while increasing levels of arsenic significantly reduced dry weights of the shoots (Figure 1). Arsenic concentration and content of chickpea shoots were significantly increased by increased levels of applied arsenic and P (Figure 2). Phosphorus concentration of the shoots was also increased by increasing levels of applied P regardless of applied arsenic levels. However, in all applied P levels increasing levels of applied As significantly reduced P content of the shoots, but not P concentration, because of the lowered biomass (Figure 3).

Lipid peroxidation and antioxidative responses

Hydrogen peroxide (H_2O_2) concentration, enzymatic (SOD, CAT and APX) and non enzymatic total antioxidant (AA) activity are presented in Table 1. The interaction between P x arsenic treatments had a significant effect on lipid peroxidation (MDA concentration) in chickpea leaves. Application of arsenic caused increased MDA concentrations regardless of P levels, although application of P reduced MDA concentrations above 100 mg P kg^{-1}. Plants supplied with 200

and 400 mg kg^{-1} P and 30 mg kg^{-1} As had lower MDA than plants grown with 100 mg kg^{-1} at the same rate of As supply, and plants supplied with 200 mg kg^{-1} P and 60 mg kg^{-1} As had lower MDA than plants supplied with 50 or 100 mg kg^{-1} P at this high rate of As supply.

Phosphorus x arsenic interaction had no significant effect on H_2O_2 concentration in the chickpea leaves. However, the concentration of H_2O_2 was significantly increased by increasing levels of arsenic application. The concentration of H_2O_2 was significantly lower at the highest P treatment (400 mg kg^{-1}) than with application of P at 100 mg kg^{-1}.

Phosphorus treatments had no clear effect on SOD activity, although application of P at 200 mg kg^{-1}gave the lowest values, but it was significantly reduced by arsenic treatments. There was a progressive increase in CAT activity with increasing supply of As, but the three highest rates of P supply gave lower CAT activity than the lowest P rate for both rates of As supply. Phosphorus x arsenic interaction on APX activity was significant. The activity of APX was increased by arsenic treatment, but the two highest levels of P supply gave lower APX activities than the two lowest rates of P supply. With the 30 mg kg^{-1} As treatment the 200 mg kg^{-1} P treatment gave lower APX activity than the two lowest rates of P supply, and the 400 mg kg^{-1} P treatment had lower APX activity than the 100 mg kg^{-1} P treatment. With the 60 mg kg^{-1} As treatment the three highest P treatments all gave lower APX activities than the lowest P treatment. Non-enzymatic antioxidant activities of the chickpea leaves were significantly reduced by both P and arsenic treatments (Table 1).

Discussion

Arsenate and phosphate are chemical analogues and are taken up by plants by the same uptake system. However, according to previous reports, the effects of each ion on the other can be either positive or negative depending on growing conditions (Tu and Ma 2004). In hydroponic systems with arsenic hyperaccumulator plants, phosphate has long been reported to suppress plant arsenic uptake (Tu and Ma 2003). In soil, however, the influence of phosphate on arsenic phytotoxicity is variable, because soil properties affect the availability of phosphate and arsenate (Tu and Ma 2003). Such arsenic-P interactions in soil-grown crops need further investigation, and according to our knowledge this is the first report which comprehensively describes the effects of arsenic-P interactions on oxidative stress mechanisms and ionic imbalances in chickpea grown in calcareous soil. The effects on oxidative stress mechanisms are particularly important in shoots, because of the generation of reactive radicals in the light reactions of photosynthesis, and as the plants were grown in soil it would have been impossible to accurately recover the fine roots, so measurements were made on shoots only.

We used polarized energy dispersive X-ray fluorescence (PEDXRF) spectroscopy to measure arsenic and P in the plant samples. It has been used previously as an analytical tool for plant analysis (Ivanova et al. 1999; Hodson and Sangster 2002; Anjos et al. 2002; Chuparina and Gunicheva 2003), and it is normally used to determine the concentrations of different elements in a sample. It has the advantages of good sensitivity, non-destructiveness and simple relations to the fundamental physics of atom-radiation interaction (Anjos et al. 2002). X-ray techniques have been used to characterize arsenic uptake within plants by other researchers (Pickering et al. 2000; Castillo-Michel et al. 2007; Smith et al. 2008).

In the present study, increasing levels of applied arsenic significantly reduced chickpea shoot growth regardless of applied P levels. Phytotoxicity of arsenic has been previously reported for arsenic hyperaccumulating ferns (Tu and Ma 2003; 2004) and also bean (Stoeva et al. 2005), turnip (Carbonell-Barrachina et al. 1999) and maize (Requejo and Tena 2006). Even though applied P levels had no significant effect on arsenic phytotoxicity, arsenic concentration of chickpea shoots was significantly increased by P application. This result is the opposite of the finding of Meharg and Macnair (1994) that as phosphate and arsenate compete for the high-affinity phosphate transporter, and phosphate binds to it more efficiently than arsenate, high phosphate concentration in the soil favours uptake of phosphate rather than arsenate. However, the result is in good agreement with the finding of Tu and Ma (2003), who reported increasing soluble arsenic in soil solution in response to increasing P additions to the soil. These authors suggested that applying more phosphorus fertilizers enhanced total arsenic accumulation by Chinese brake fern due to the displacement of sorbed arsenate from the soil by phosphate. In the present study, we used calcareous soil of a high pH, therefore having a high P fixation capacity. However, chickpea is a plant with a good ability to release inorganic P from soils, so the plants should not have been P-deficient in the absence of additional P supply, and it is noticeable that there was no increase in shoot growth with supply of additional P. For the plants where As was supplied it seems likely that increasing levels of applied P increased the soluble arsenic concentration in the soil solution and consequently arsenic concentration in the shoots. Other studies on arsenate-P interaction in soils have also shown changes in concentrations in soil solution through competition for sorption sites (Gao and Mucci 2001; Smith et al. 2002).

Although P supply increased the internal As concentrations, phosphorus concentration of the chickpea shoots was unaffected by the increased arsenic levels. This reinforces the suggestion

that P supply made As more available from the soil. It certainly shows that there was not competition for uptake between arsenate and phosphate, as under those circumstances it would be expected that high arsenic concentrations in the tissues would be matched by lower P concentrations. Enhanced uptake of phosphate would have been expected to have given less uptake of arsenate through competition for the phosphate transporter, or at the very least the high internal P status would have been expected to down regulate the transporter, so preventing further uptake of arsenate (Meharg and Hartley-Whitaker 2002).

There have been a number of reports testing the effect of P on arsenic phytotoxicity (Meharg et al. 1994; Tu and Ma 2003; 2004) and several reports on biochemical responses of plants to arsenic stress (Stoeva et al. 2005; Stoeva and Bineva 2003; Srivastava et al. 2005), but the effects of arsenic-P interaction on arsenic-induced oxidative stress mechanisms are insufficiently studied.

Biochemical responses of plants to toxic metals are complex and several defence strategies have been suggested. These include complexation of ions, reduced influx, and enhanced production of antioxidants that detoxify ROS that are produced in response to the metals (Meharg 1994). This is a process that readily occurs in plants (Stoeva and Bineva 2003). Although arsenic is not a redox metal, there is significant evidence that exposure of plants to inorganic arsenic results in the generation of ROS, which is connected with arsenic valence change. Mittler (2002) reported that membrane damage might be caused by high levels of H_2O_2 resulting in $OH^{\cdot}$ formation and consequently lipid peroxidation. It is well known that ROS-induced lipid peroxidation of membranes is a reflection of stress-induced damage at the cellular level (Jain et al. 2001). Therefore, the level of MDA, a decomposition product of polyunsaturated fatty acids produced

during peroxidation of membrane lipids, is often used as an indicator of oxidative damage (Mittler 2002). In the present study, MDA content was utilized as a biomarker for lipid peroxidation and application of arsenic increased MDA concentration in the plants. The implication here is that the application of arsenic caused both increased MDA concentration and lower shoot growth, with MDA concentration increasing 34% with supply of As and shoot dry mass decreasing 40%. If the scavenging system of a plant does not cope well with the formation of free radicals or ROS, it leads to uncontrolled oxidation and radical chain reactions, which result in oxidative stress to the plant (Srivastava et al. 2005), and with application of arsenic such oxidative stress appears to have occurred. Increased levels of MDA have also been observed in bean plants (Stoeva et al. 2005) and hyperaccumulator and sensitive fern species (Srivastava et al. 2005) exposed to arsenic toxicity.

As plants have antioxidant enzymes and antioxidants to combat oxidative stress, altered activities of these systems are also frequently used as indicators of oxidative stress in plants (Mittler 2002). Increased SOD activity in response to arsenic toxicity has been observed in arsenic hyperaccumulator and sensitive fern species (Srivastava et al. 2005), in maize (Mylona et al. 1998) and in the grass *Holcus lanatus* (Hartley-Whitaker et al. 2001). However, our results apparently contradict these as in the present study the activity of SOD was reduced by increasing levels of applied arsenic. SOD catalyses the conversion of highly reactive superoxide to hydrogen peroxide, which itself is a highly reactive oxidizing agent. Hydrogen peroxide is scavenged by CAT and APX, two enzymes that themselves are used as markers of oxidative stress. The CAT and APX activities of the chickpea plants were significantly increased by arsenic, although these plants also contained increased concentrations of H_2O_2. It seems to be the case that treatment of chickpea plants with arsenic causes peroxidation of membrane lipids through accumulation of

H_2O_2, despite the fact that enzymes for the removal of H_2O_2 (CAT and APX) increase in activity and an enzyme that catalyses the formation of H_2O_2 (SOD) shows decreased activity. The increased activities of CAT and APX are presumably insufficient to remove the additional H_2O_2 formed, and the lower activity of SOD implies that the arsenic treatment does not give rise to increased concentrations of superoxide, and the H_2O_2 comes from another source. Similar induction of CAT and APX activities with exposure to arsenic has previously been reported in arsenic hyperaccumulator and sensitive fern species (Srivastava et al. 2005), and increased CAT activity has been seen in maize (Mylona et al. 1998). Decreased SOD activities for different types of oxidative stress have also been reported previously (Gong et al. 2005; Mishra and Choudhuri 1999). In the study on *Holcus lanatus*, two clones resistant to arsenic had higher activity of SOD than sensitive clones, but in one of the sensitive clones SOD activity was only slightly higher than the background rate at rates of supply of arsenate that inhibited growth of the plants (Hartley-Whitaker et al. 2001). Indeed, in all of the clones the highest rate of arsenate supply gave lower rates of SOD activity than some of the lower rates of arsenate supply, so it may be that increase in SOD activity in plants is a response to mild arsenic toxicity, but the activity of the enzyme is depressed by higher levels of toxicity. This was certainly shown to be the case in maize, where expression of different *sod* genes in the leaves was increased at low rates of supply of arsenate and arsenite, but was decreased at higher rates of arsenic supply (Mylona et al. 1998).

As the supply of P significantly increased the concentrations of arsenic in the chickpea shoots it might be expected that the responses of the antioxidant enzymes to P supply would be the same as their responses to arsenic. However, application of P at high rates of supply limited the increase in CAT activity seen with supply of As, and gave rise to lower rates of APX activity in

plants supplied As than application of P at the lowest rate of supply. MDA increased with supply of As, but there was some tendency for As-supplied plants grown in the two highest P levels to have lower MDA concentrations than the plants grown with P supplied at 100 mg kg^{-1}. Indeed, the plants supplied with 200 mg kg^{-1} P had lower MDA values at 60 mg kg^{-1} supply of As than the plants grown in 50 and 100 mg kg^{-1} P despite the higher internal As concentrations. The plants supplied with high P accumulated more phosphate in the shoots, but although this might have had a protective effect against oxidative damage it was not to a sufficient extent to prevent lowered shoot growth. Possibly the accumulated phosphate influenced cellular pH, and limited the conversion of arsenate to arsenite and so lowered the formation of ROS. At the highest rate of P supply (400 mg kg^{-1}) any possible protective effect was counteracted by the increased availability of As, and the value for MDA in the plants grown with 60 mg kg^{-1} As was not significantly different from MDA in the plants grown in high As with the other three rates of P supply.

Concentrations of non-enzymatic antioxidants, which react not only with H_2O_2, but also with superoxide ($O_2\bullet^-$) and hydroxyl ($OH\bullet^-$) radicals, were decreased by both increased arsenic and P levels. In the case of the arsenic treatment this is presumably because non-enzymatic antioxidants are used in counteracting the effects of the ROS. Thus, for example, ascorbate would be used by the increased activity of APX. Similar decreases in concentration of non-enzymatic antioxidants have been seen previously, with lowered concentrations of reduced glutathione occurring in sensitive clones of *Holcus lanatus* exposed to low concentrations of arsenic (Hartley-Whitaker at al. 2001). However, the effect of P application on concentration of non-enzymatic antioxidant concentration is difficult to explain given that P treatment decreased APX activity. This response

would seem to be more likely linked to the increased concentrations of arsenic in the high P treatments.

In conclusion, applied P significantly increased arsenic concentration of chickpea shoots, probably through making arsenic in the soil more available. Exposure to arsenic resulted in oxidative stress and membrane deterioration in chickpea shoots, resulting in lowered growth. Even though use of P increased arsenic concentration, it had some effect on decreasing membrane damage (lipid peroxidation) in arsenic-stressed plants despite giving lower activity of some antioxidant enzymes. The decrease in membrane damage was not sufficient to give a protective effect of P on chickpea growth, and given the effect of P on increasing availability of arsenic the use of phosphorus fertilizers in arsenic-contaminated soils should be carefully considered, unless plants are being grown specifically to remove arsenic from the soil.

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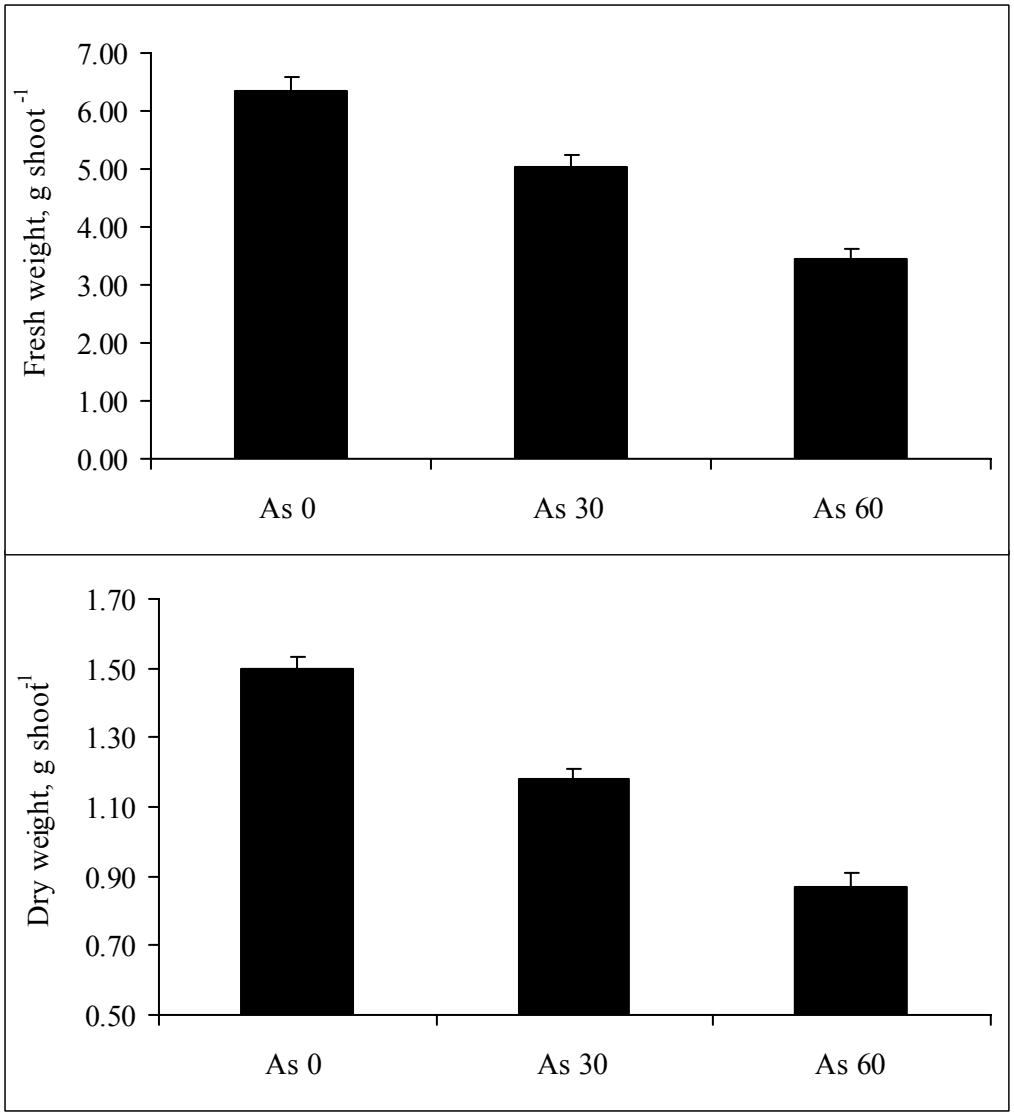

Figure 1. Fresh and dry weights of chickpea shoots. The values are means of 3 replicates ± standard error (SE). F values: the PxAs interaction, P treatment and As treatment are 1.97^{ns}, 1.55^{ns} and 65.37** for fresh weight and 1.61^{ns}, 1.72^{ns} and 58.32** for dry weight, respectively. Significance of ANOVA: **: $P<0.01$; ns: non significant

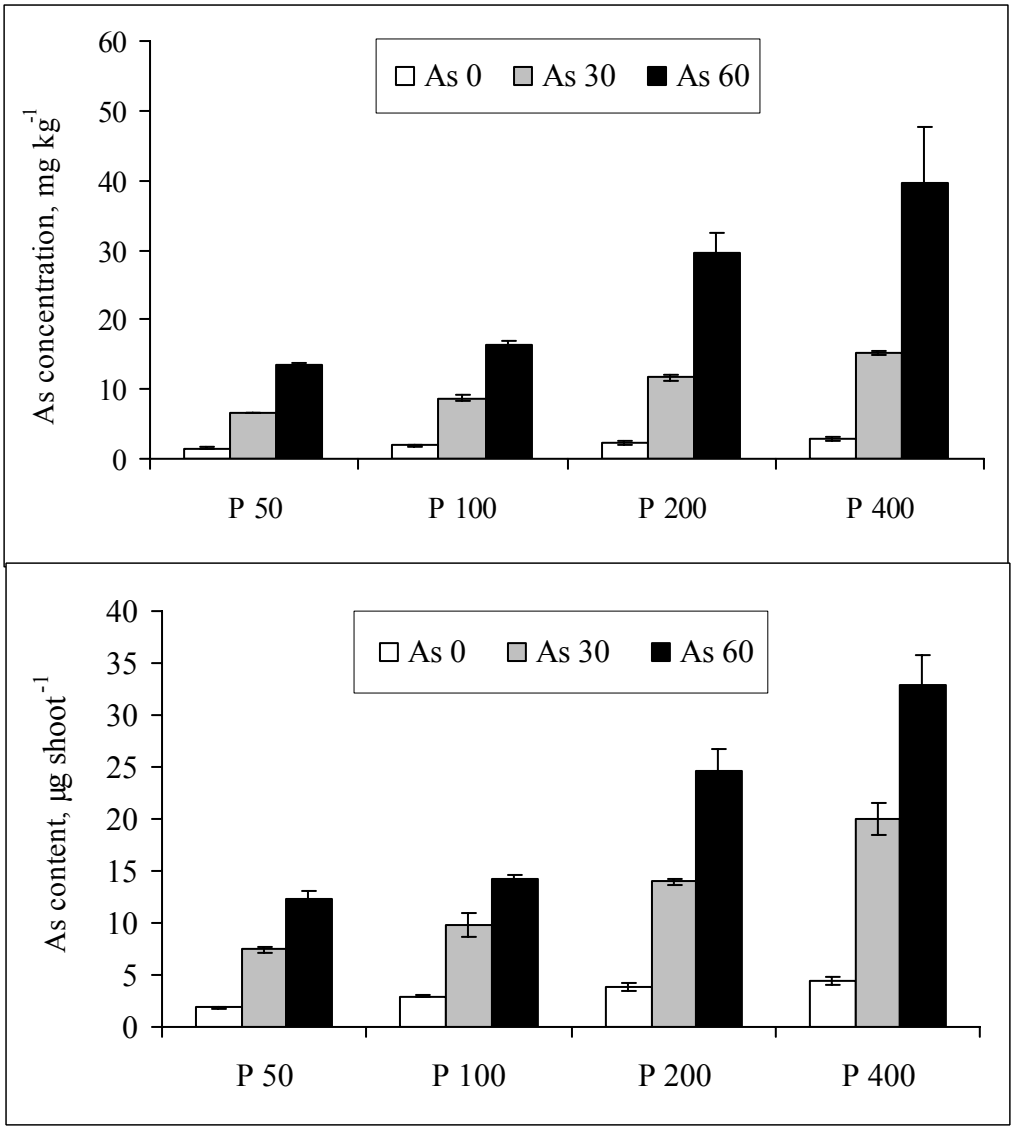

Figure 2. Arsenic concentration and content of chickpea shoots. The values are means of 3 replicates ± standard error (SE). F values: the PxAs interaction, P treatment and As treatment are 5.89**, 14.80** and 85.55** for As concentration and 11.98**, 56.35** and 203.9** for As content, respectively. Significance of ANOVA: **: $P<0.01$;

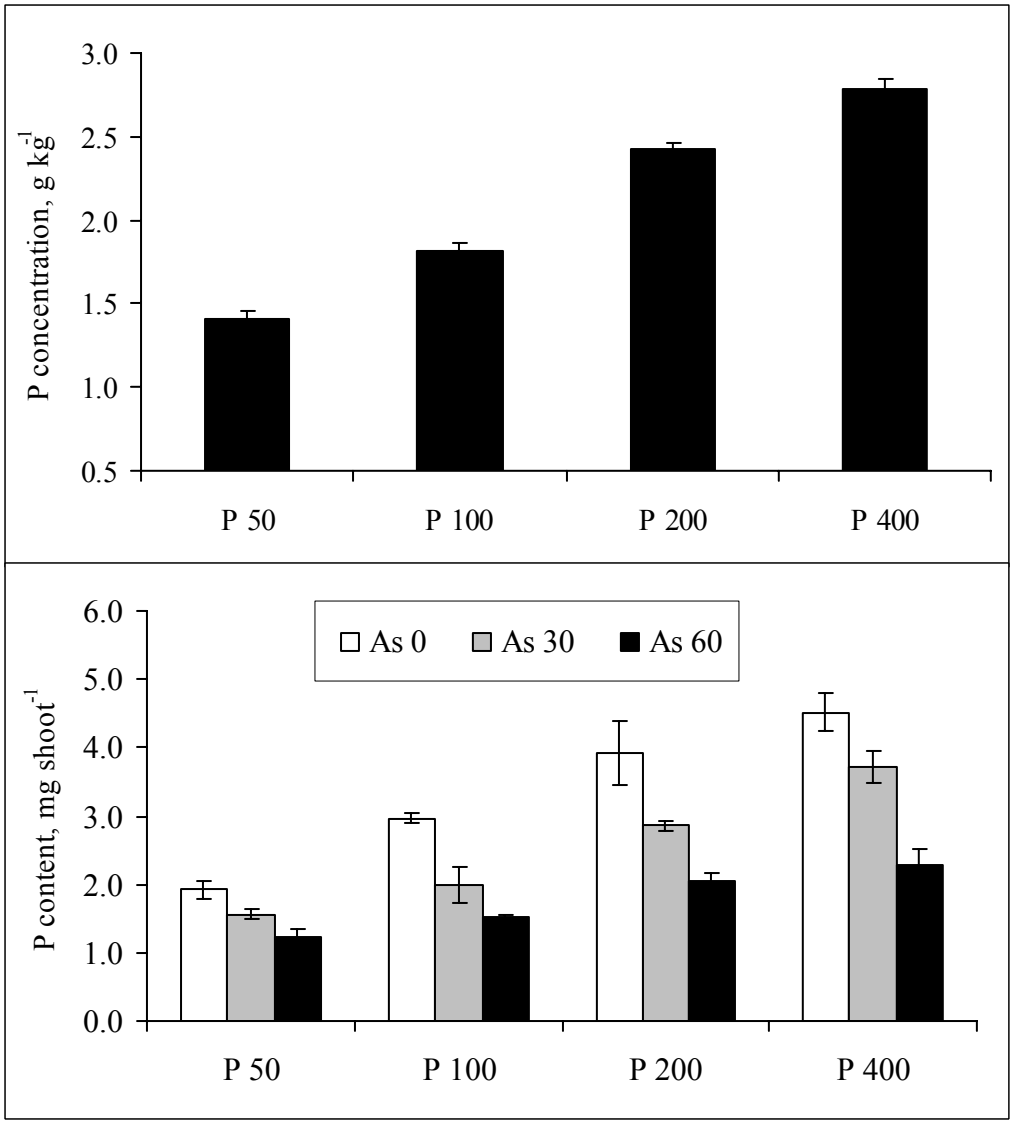

Figure 3. Phosphorus concentration and content of chickpea shoots. The values are means of 3 replicates ± standard error (SE). F values: the PxAs interaction, P treatment and As treatment are 0.84^{ns}, 201.13** and 3.39^{ns} for P concentration and 2.80*, 48.75** and 54.14** for P content, respectively. Significance of ANOVA: *: $P<0.05$; **: $P<0.01$; ns: non significant

Table 1. Interactive effect of soil-applied P and arsenic on MDA and H_2O_2 concentration, and superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) enzyme and non-enzymatic antioxidant activities (AA) of chickpea. The values are means of 3 replicates. Means for P treatments and arsenic (As) treatments are shown under the PxAs interaction means. F values for the PxAs interaction, P treatment and As treatment are shown. Different letters in each column for PxAs interaction, P treatment and As treatment separately represent significant difference at the $P < 0.05$ level based on Duncan's multiple range tests.

Phosphorus treatments mg kg^{-1}	Arsenic treatments mg kg^{-1}	MDA nmol g^{-1}, FW	H_2O_2 µmol g^{-1}, FW	SOD U mg^{-1}, FW	CAT µmol g^{-1}, DW	APX µmol g^{-1}, DW	AA µmol g^{-1}, DW
50	0	15.8 cde	8.9	0.16	0.10 g	1.25 d	86.6
	30	17.5 abcd	13.1	0.13	0.25 ab	1.54 bc	84.3
	60	17.7 abc	17.1	0.09	0.28 a	1.93 a	74.7
100	0	12.5 fg	11.8	0.14	0.12 fg	1.41 cd	93.2
	30	20.0 a	14.1	0.12	0.18 de	1.70 b	69.3
	60	18.8 ab	18.3	0.09	0.19 cd	1.55 bc	67.4
200	0	11.4 g	9.6	0.12	0.13 fg	0.90 e	70.9
	30	15.8 cde	13.3	0.11	0.14 efg	1.20 d	53.5
	60	14.6 ef	17.3	0.07	0.22 bc	1.56 bc	48.5
400	0	12.6 fg	6.7	0.16	0.14 efg	0.97 e	73.5
	30	14.8 def	13.6	0.14	0.15 def	1.32 cd	46.8
	60	17.0 bcde	13.3	0.10	0.16 def	1.42 cd	46.0
Lsd, p<0.05		*2.53*	-	-	*0.037*	*0.22*	-
50		17.0 a	13.0 ab	0.13 ab	0.21 a	1.57 a	81.8 a
100		17.1 a	14.7 a	0.12 b	0.16 b	1.55 a	76.6 a
200		14.0 b	13.4 ab	0.10 c	0.16 b	1.22 b	57.6 b
400		14.8 b	11.1 b	0.14 a	0.15 b	1.24 b	55.4 b
Lsd, p<0.05		*1.46*	*2.34*	*0.013*	*2.13*	*0.127*	*9.03*
	0	13.1 b	9.2 c	0.15 a	0.12 c	1.13 c	81.1 a
	30	17.0 a	13.5 b	0.13 b	0.18 b	1.44 b	63.5 b
	60	17.0 a	16.5 a	0.09 c	0.21 a	1.61 a	59.1 b
Lsd, p<0.05		*1.26*	*2.03*	*0.013*	*0.018*	*0.110*	*7.82*
F values	*PxAs Interaction*	*2.90**	*0.82^{ns}*	*1.29^{ns}*	*10.8***	*3.62***	*1.07^{ns}*
	P treatment	*9.97***	*3.40**	*3.57***	*11.1***	*19.4***	*18.5***
	As treatment	*27.8***	*27.9***	*5.99***	*51.8***	*41.5***	*18.7***

Significance of ANOVA: *: $P<0.05$; **: $P<0.01$; ns: non significant