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Antioxidant responses of peanut roots exposed to realistic groundwater doses of arsenate: identification of glutathione S-transferase as a suitable biomarker for metalloid toxicity

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Abstract

Arsenic (As)-polluted groundwater constitutes a serious problem for peanut plants, as roots can accumulate the metalloid in their edible parts. Characterization of stress responses to As may help to detect potential risks and identify mechanisms of tolerance, being the induction of oxidative stress a key feature. Fifteen-day old peanut plants were treated with arsenate in order to characterize the oxidative stress indexes and antioxidant response of the legume under realistic groundwater doses of the metalloid. Superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) histochemical staining along with the activities of NADPH oxidase, superoxide dismutase (SOD), catalase (CAT) and thiol (glutathione and thioredoxins) metabolism were determined in roots. Result showed that at 20 µM $H_2AsO_4^-$, peanut growth was reduced and the root architecture was altered. $O_2^{\bullet-}$ and H_2O_2 accumulated at the root epidermis, while lipid peroxidation, NADPH oxidase, SOD, CAT and glutathione S-transferase (GST) activities augmented. These variables increased with increasing As concentration (100 µM) while glutathione reductase (GR) and glutathione peroxidase/peroxiredoxin (GPX/PRX) were significantly decreased. These findings demonstrated that the metalloid induced physiological and biochemical alterations, being the NADPH oxidase enzyme implicated in the oxidative burst. Additionally, the strong induction of GST activity, even at the lowest $H_2AsO_4^-$ doses studied, can be exploited as suitable biomarker of As toxicity in peanut plants, which may help to detect risks of As accumulation and select tolerant cultivars.

Keywords: arsenic; glutathione S-transferase; oxidative stress; peanut

Abbreviations:

1-chloro-2, 4-dinitrobenzene (CDNB)

3,3-diaminobenzidine (DAB)

Arsenic (As)

Catalase (CAT)

Glutathione (GSH)

Glutathione peroxidase/peroxiredoxin (GPX/PRX)

Glutathione reductase (GR)

Glutathione S-transferase (GST)

Hydrogen peroxide (H_2O_2)

Nitroblue tetrazolium (NBT)

Potassium iodide (KI)

Superoxide anion ($O_2^{\bullet -}$)

Superoxide dismutase (SOD)

Thiobarbituric-reactive substances (TBARS)

1. Introduction

Arsenic (As) is an ubiquitous metalloid present in many ecosystems. The average concentration of As in the lithosphere ranges between 20 and 30 µM, occupying the position number 52 in the list of the most abundant elements in nature (Adriano 2001). Arsenate ($H_2AsO_4^-$, As(V)) and arsenite ($H_2AsO_3^-$, As(III)) are the prevalent inorganic chemical species, being more toxic than the organic forms (Mandal and Suzuki 2002; Zhao et al. 2010). These contaminants are receiving much attention due to human population chronic poisoning caused by drinking water polluted in several regions of Asia and South America (Zhao et al. 2010). In addition, crop plants can accumulate large amounts of As in edible parts when irrigated with As-containing water; acting as the first stage of As distribution in the trophic chain (Smedley and Kinniburgh, 2002). In Argentina, particularly in Córdoba province, As has become a public health problem due to numerous cases of human poisoning. Francisca et al. (2006) found that 90% of this region is affected by groundwater As concentrations that exceeds the maximum allowed level of the metalloid in drinking water (0.1 µM) (FAO, OMS-OPS, 2007), reaching in some areas up to 20 µM (Cabrera et al. 2005). Peanut is a very important crop in Argentina, specifically in Córdoba, as it represents approximately 90% of the Argentinean production (Ministry of Agroindustry, Argentina, 2006). The fruit of this legume is a pod with one to five seeds that develops underground, in putative intimate contact with soil contaminants. One of the major constraints that Córdoba province has, given the water scarcity in the area, is that crops require artificial irrigation by sprinkler systems using groundwater rich in As (Bustingorri and Lavado, 2012; 2014) representing a potential risk for crops. This is aggravated by the deep penetration of plant roots in soil strata, which is a typical response of plants suffering from drought stress (Sharp and LeNoble 2002).

Arsenate form ($H_2AsO_4^-$ As(V)) is an analog of phosphate ($H_2PO_4^-$) and it can be easily absorbed by plant by $H_2PO_4^-$ transporters (Tripathi et al. 2007; Verbruggen et al. 2009). Once inside the cell, most of $H_2AsO_4^-$ (As(V)) is reduced to $H_2AsO_3^-$ (As(III)), by different $H_2AsO_4^-$

reductases (Dhankher et al. 2006; Duan et al. 2007; Sánchez-Bermejo et al. 2014). Therefore, As is present predominantly in roots and shoots as $H_2AsO_3^-$, which binds to sulfhydryl groups of thiol-rich peptides such as glutathione (GSH) and phytochelatins (PCs) (Pickering et al. 2000). Although the precise mechanisms of toxicity are not completely understood, plants exposed to both As species showed substantial redox homeostasis imbalance, with accumulation of reactive oxygen species (ROS) (Requejo and Tena 2005; Talukdar 2013), and changes in antioxidant responses (Mylona et al. 1998). It is feasible that the As high affinity for sulfhydryl groups causes GSH depletion and changes in enzymatic activity that leads to oxidative stress (Hernández et al. 2015). Biomolecules such as lipids, proteins and nucleic acids are principal targets of ROS, which cause the characteristic oxidative damage symptoms (Verbruggen et al. 2009). Another source of ROS is constituted by the plasma membrane-associated NADPH oxidase family that has a low redox potential able to reduce O_2 to superoxide anion ($O_2^{\bullet-}$) (Mitler et al. 2011). Moreover, under metal(loid)s stress these enzymes are activated increasing ROS accumulation (Hernández et al. 2015).

Plants possess an effective antioxidant defense system that scavenges ROS accumulated by aerobic metabolism, and is comprised of antioxidant enzymes and metabolites (Noctor et al. 2012). Superoxide dismutase (SOD) constitutes the first line of defense dismutating $O_2^{\bullet-}$ into hydrogen peroxide (H_2O_2), which is then reduced to water and oxygen by ascorbate peroxidase (APX) and catalase (CAT) (Mishra et al. 2006). These enzymes constitutes the major ROS scavenging mechanisms of plants (Mittler 2012) and their induction under different metal(loid) treatments has been demonstrated in several species (Sharma and Dietz, 2009). One of the most studied antioxidant metabolite essential to cope with the oxidative stress induced by toxic elements such as Cd, Hg and As is the tripeptide GSH (Li et al. 2006; Bianucci et al. 2012; Sobrino-Plata et al. 2013). GSH transits from its reduced form (thiol) to its oxidized form (GSSG, disulfide), keeping a strong reducing environment in the cell which plays an important role in

preventing excessive oxidation of cellular biomolecules under different stressing conditions (Jozefczak et al. 2012). Besides, a series of antioxidant enzymes related to GSH metabolism are involved in the detoxification of free radicals (Foyer and Noctor 2011). Glutathione reductase (GR) regenerates GSH from GSSG at the expense of NADPH to maintain a high GSH/GSSG ratio (Gill et al. 2013). Glutathione-S-transferases (GSTs) are a large and diverse group of enzymes involved in the detoxification of dangerous xenobiotics catalyzing their conjugation with GSH. Moreover, GSTs can bind GSH with organic peroxides and radicals generated by ROS attack, which can amplify in a chain reaction the oxidative damage caused by toxic elements, preventing their reaction with many important biomolecules (Gill and Tuteja 2010). The enzyme named misleadingly glutathione peroxidase (GPX), a peroxidase able to use GSH and thioredoxin (TRX), constitutes a special case (Herbette et al., 2002). The particularity is that the TRX is known to be a more efficient reducing substrate than GSH in certain plant species (Iqbal et al., 2006). Thus, these enzymes should be considered as peroxiredoxins (PRX) rather than GPX (Navrot et al., 2006) and are named accordingly in our work as GPX/PRX.

Arsenate and arsenite are highly soluble and stable in water under a wide range of pH and redox potential conditions. However, under oxidizing environments as occur in many areas of Córdoba province, the prevalent arsenic form is arsenate (Blarasin et al. 2014). Thus, our experiments were conducted to characterize oxidative stress indexes and the antioxidant response of peanut (*Arachis hypogaea* L.) plants exposed to environmentally realistic As(V) levels, considering the economic relevance of this legume crop in Córdoba province. This study will provide information about the mechanisms of toxicity evoked by As(V), which can be used as bioindexes of putative risks to consumers. Most sensitive stress indexes could be used to select tolerant cultivars with lower toxic symptoms and able to exclude As from edible parts of the plants.

2. Material and Methods

2.1. Plant material and experimental design

Peanut seeds, kindly supplied by "El Carmen S.A." Córdoba, Argentina, were surface sterilized following the method previously described by Vincent (1970). Then, they were germinated at 28ºC in Petri dishes on a layer of Whatman Nº1 filter paper and moistened cotton, until the radicle reached 3-5 cm. Seedlings were transferred to an hydroponic system with Hoagland´s nutrient solution (Hoagland and Arnon 1950) devoid of As (control) or containing different concentrations of $Na_2HAsO_4 \cdot 7H_2O$. The metalloid doses used are equivalent to those found in groundwater in Córdoba province (6 and 20 µM) (Cabrera et al., 2005) and a concentration above those mentioned (100 µM).

Plants were grown in a greenhouse under controlled environment (light intensity of 200 µmol m^{-2} s^{-1}, 16/8 h day/night cycle, constant temperature of 28ºC and relative humidity of 50%) for 15 days. At harvest, plant length was measured and shoots and roots were used for different analysis.

2.2. Root structure and histochemical detection of H_2O_2 and $O_2^{\bullet -}$

For anatomical and histological studies, the main root of a fresh peanut plant was cut into 5 mm length portions, at 1 cm from the root tip as described in Bianucci et al. (2012). The tissues were cut with a rotary microtome and the samples were stained as described by Johansen (1940) and O'Brien and Mc Cully (1981). The photomicrographs were taken using an Axiophot Carl Zeiss microscope (Germany).

In situ localization of superoxide anion ($O_2^{\bullet -}$) was done incubating freshly roots segments in 1 mM nitroblue tetrazolium (NBT), prepared in 10 mM sodium citrate buffer pH 6, for 8 hours, following the procedure described by Frahry and Schopfer (2001). H_2O_2 was visually detected incubating freshly roots segments in 1 mg mL^{-1} 3,3-diaminobenzidine (DAB) as substrate for 8 hours (Orozco-Cárdenas and Ryan 1999). The roots were observed and photographed under a

stereoscopic microscope Stemi SV6, Carl Zeiss (Germany), with a digital camera Canon (China).

2.3. Plant arsenic concentration

Peanut leaves and roots were dried at 50 ºC for 72 h, homogenized with a mortar and pestle, and digested under acidic conditions in pressurized chromatographic vials (4 ml capacity) sealed with polytetrafluoroethylene stoppers. A 0.1 g aliquot of the dried powder was mixed with 1 ml digestion reagent (H_2O:HNO_3:H_2O_2 (5:3:2)) in closed glass vials, using an autoclave (Presoclave-75 Selecta, Barcelona, Spain) at 120^{o}C and 1.5 atm for 30 min (Sobrino-Plata et al. 2009). The digests were filtered through a PVDF filter and diluted in milli-RO water to 5 ml. As concentration was measured using inductively coupled plasma mass spectroscopy (ICP-MS) NexION 300 (Perkin-Elmer Sciex, San Jose, CA, USA).

2.4. Stress indexes

NADPH oxidase activity was determined spectrophotometrically by NBT reduction at 560 nm (Sagi and Fluhr 2001). Roots were homogenized according to Sobrino-Plata et al. (2009) procedure. The activity was measured in 1 ml reaction buffer containing: 0.02 mg protein extract, 0.5 mg ml^{-1} NBT, 0.2 mM NADPH, 4 mM $CaCl_2$ and 0.2 mM $MgCl_2$. One unit of NADPH oxidase was defined as the quantity of enzyme needed to reduced 1 µmol NADPH min^{-1}.

Hydrogen peroxide (H_2O_2) content was measured spectrophotometrically after reaction of root extracts with potassium iodure (KI) following the procedure described by Alexieva et al. (2001). The amount of H_2O_2 was calculated using a standard calibration curve prepared with known H_2O_2 concentrations.

Lipid peroxidation was analyzed in peanut roots by determining the concentration of thiobarbituric-reactive substances (TBARS) as described by Heath and Packer (1968). TBARS

were quantified by measuring absorbance at 535 nm, corrected for non-specific turbidity by subtracting the absorbance at 600 nm, using a UV-visible light spectrophotometer (Spectronic® Genesys 2, USA).

2.5. Enzymatic assay

The superoxide dismutase (SOD, EC 1.15.1.1) and catalse (CAT, EC 1.11.1.6) root samples were obtained by homogenization of roots with an extraction buffer containing 50 mM potassium phosphate, pH 7.8, 0.5 mM EDTA, insoluble polyvinylpyrrolidone, and 0.5% (v/v) Triton X-100 after centrifugation at 10,000 g for 10 minutes at 4℃. GPX/PRX (EC 1.11.1.9), GR (EC 1.6.4.2) and GST (EC 2.5.1.18) root samples were obtained by homogenization of roots with an extraction buffer containing 100 mM potassium phosphate, pH 7.4, 1 mM EDTA after centrifugation at 10,000 *g* for 10 minutes at 4℃. The supernatants were used to determine enzymatic activities. Total protein content was assessed according to Bradford (1976) using bovine serum albumin as standard.

SOD activity was determined according to Beauchamp and Fridovich (1973) by measuring the ability of the enzyme extract to inhibit the photochemical reduction of NBT in the presence of riboflavin. SOD specific activity was determined spectrophotometrically at 560 nm and expressed as units mg^{-1} protein. One unit of SOD activity was defined as the amount of enzyme required to inhibit in a 50% the reduction of NBT. CAT activity was measured using the method described by Aebi (1984). The assay mixture contained 50 mM phosphate buffer at pH 7.4, 12.5 mM H_2O_2 and 100 µg protein extract. The reaction was measured by the H_2O_2 decomposition at 240 nm. One unit of CAT was defined as the quantity of enzyme needed to degrade 1 mmol of H_2O_2. GPX/PRX activity was determined as described by Flohé and Günzler (1984). One ml of the assay mixture contained 50 ml of protein extract, 0.1 M phosphate buffer at pH 7.4, 0.24U GR and 10mM GSH. Then, 1.5 mM NADPH and 1.5 mM H_2O_2 were added. The reaction was

measured following NADPH oxidation at 340 nm. One unit of GPX/PRX was defined as the quantity of enzyme needed to produce 1 mmol $NADP^+$ min^{-1}. This assay likely describes glutathione or glutaredoxin-dependent thiol peroxidase activity since plants do not contain Se-dependent GPX. Thus, plant GPX functions as a TRX-linked thiol peroxidase (Navrot et al. 2006). GR activity of root extracts was determined as described by Sheadle and Bassham (1977): the reduction of GSSG was followed by the NADPH oxidation at 340 nm. One unit of GR was defined as the quantity of enzyme needed to reduced 1 µmol NADPH min^{-1}. Finally, GST activity was assayed according to Habig et al. (1974), by measuring the conjugation of GSH with 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate, which was measured at 340 nm. One unit of GST was defined as the quantity of enzyme needed to produce 1 µmol conjugated CDNB-GSH min^{-1}.

2.6. Analysis of glutathione content

Peanut roots were homogenized in 5 % (w/v) 5-sulfosalicylic acid and centrifuged at 10,000 *g* for 10 min at 4℃ to remove cell debris. The supernatan t was used for measuring the total GSH equivalents content by the method of Anderson (1985) using baker's yeast GR. The reaction was followed at 412 nm and total GSH-Eq was calculated from a standard curve using GSH (0-30 µM). The intracellular GSH-Eq content was expressed as nmol GSH g^{-1} fresh weight (FW).

2.7. Statistical analysis

Experiments were conducted in a completely randomised design and repeated three times. The data were analyzed using ANOVA and Duncan's test at $P<0.05$. Prior to the test of significance, the normality and homogeneity of variance were verified using the modified Shapiro-Wilk and Levene tests, respectively.

3. Results

3.1. Arsenate-induced peanut growth inhibition and root architecture alteration

Arsenic addition caused plant growth alterations as revealed by some morphological features as the diminution of root volume and altered architecture (Fig. 1). Exposure to 20 µM As(V) led to limited proliferation of secondary roots (Fig. 1c), while at 100 µM As(V) root growth was severely inhibited and suffered an evident darkening possibly associated with an induction of oxidative stress (Fig. 1d). These apparent changes were confirmed by measuring of growth variables. Peanut root dry weight (DW) was significantly decreased at 6 µM $H_2AsO_4^-$ however, shoot DW only showed a reduction at the higher arsenate dose evaluated (100 µM) compared to control. At 20 µM $H_2AsO_4^-$ root and shoot length was significantly decreased (Table 1). Roots accumulated the largest proportion of As(V) in plants, reaching values of two to three orders of magnitude in compare to shoots (Table 2). At 100 µM As(V), the amount of metalloid in root had no significant difference with those plants exposed to 20 µM As(V), however, a significant increase in As concentration of shoot was observed at the higher As concentration tested (Table 2). The histological cuts of roots showed three well-developed tissue systems at the epidermis, the cortex and the central cylinder (Fig. 2), where we could observe small deposits of unknown amorphous materials on the cell walls of epidermal cells of plants exposed to 6 µM $H_2AsO_4^-$ (Fig. 2b). These deposits were more numerous in roots of plants treated with higher As(V) doses (20 and 100 µM), where thicker cell walls were also found (Fig. 2c,d). In addition, there were several epidermal and parenchymal cells of the cortex that suffered disintegration and rupture at the highest dose indicating acute cellular damage (Fig. 2d).

3.2. ROS production and oxidative damage caused by arsenate

Oxidative stress parameters were determined in peanut roots, the organ that accumulated the largest proportion of As. Supply of 20 µM $H_2AsO_4^-$ led to the subsequent induction of NADPH

oxidase activity as the metalloid dose was increased, with the maximum activity at 100 µM (Fig. 3a). The same response was observed in SOD activity (Fig. 3b). In parallel, H_2O_2 concentration rose at doses over 20 µM $H_2AsO_4^-$, reaching levels approximately 2-fold higher than in control roots (Fig. 3d). H_2O_2 content significantly enhanced CAT activity (Fig. 3c). Finally, ROS production induced to lipid peroxidation (Fig. 3e), which reflects the onset of oxidative damage caused by the exposure of peanut roots to $H_2AsO_4^-$. The accumulation of H_2O_2 observed in roots (Fig. 3b) is consistent with the histochemical detection of H_2O_2 production on peanut roots (Fig. 4). Generation of H_2O_2 *in situ* was detected by DAB reaction, and there was a noticeable accumulation of H_2O_2 at the epidermis and cortex of plants treated with 20 µM $H_2AsO_4^-$ (see arrows in Fig. 4). DAB staining was much stronger when $H_2AsO_4^-$ concentration augmented to 100 µM with deposits only in the epidermis cells, probably the thickening of the outer tangential wall did not allow the colorant entrance to the cell (Fig. 4). A similar pattern was observed with the histochemical staining of superoxide ($O_2^{\bullet-}$), which accumulated remarkably at the epidermis of plants exposed to 6 µM $H_2AsO_4^-$ (Fig. 5). Interestingly, $O_2^{\bullet-}$ was also found in cortex cells of roots under 20 µM dose, while only epidermis was heavily stained when $H_2AsO_4^-$ treatment rose to 100 µM As, possibly as observed in H_2O_2 detection, at this concentration the epidermis cells were thick and did not allow the colorant entrance consequently, blue precipitates were not detected (Fig. 5).

3.3. Arsenate alters thiol metabolism

The concentration of GSH increased significantly only in peanut plants exposed to 100 µM $H_2AsO_4^-$ (Fig. 6a). On the other hand, at this As(V) concentration GPX/PRX and GR enzymatic activities decreased by almost 50% of control plants (Figs. 6b,c). In contrast, GST activity augmented in a dose-dependent manner from 6 µM $H_2AsO_4^-$, reaching values of almost 10-times bigger at the highest dose of $H_2AsO_4^-$ (Fig. 6d).

4. Discussion

Peanut is an annual herbaceous legume plant with a remarkable importance as crop for food uses. Argentina is one of the major peanut producers in the world and 90% of this production is located in Córdoba province. In this region, there is a serious health problem since approximately 90% of the groundwater is naturally contaminated with As (Francisca et al., 2006). This water is used as artificial irrigation of crops or can be directly absorbed by roots. Therefore, contamination of crops with heavy metals and metalloids constitute not only an agronomic problem but also a human health issue due the transference along the chain food. The experiments performed in this manuscript describe the oxidative stress parameters of peanut plants exposed to As(V) as well as the antioxidant response evoked by the metalloid. The doses used are equivalent to those found in contaminated groundwater in Córdoba province (Cabrera et al., 2005) and a concentration above those mentioned. Arsenic is a non-essential element that is highly toxic even at relatively low concentrations and causes growth inhibition (Stoeva and Bineva 2003; Sobkowiak and Dekert 2003; Alaoui-Sossé et al. 2004; Finnegan and Chen 2012). In our experiments, the strongest growth inhibitory effect was observed in roots, where dry weight and length were significantly decreased above 6 and 20 µM $H_2AsO_4^-$, respectively. Reduction in plant growth was also observed in *Phaseolus vulgaris*, *Pisum sativum***,** *Lupinus albus* L. exposed to large doses of As(V) (Liu et al. 2005; Vázquez Reina et al. 2005; Bundschuh et al. 2012; Talukdar, 2013). Interestingly, at realistic environmental doses of

$H_2AsO_4^-$ (above 3 µM) there was also a significant reduction in root growth of pea seedlings, which matched with inhibition of cell mitosis and cytokinesis (Dho et al. 2010). In fact, As has potent genotoxic effects that causes aberrant chromosomal arrangement and cell cycle arrest, resulting in poor root meristematic activity (Wu et al. 2010). Moreover, root growth inhibition caused by As(V) was associated with the onset of oxidative stress in rice seedlings (Shri et al. 2009). This can explain the darkening of the peanut root system as arsenic doses increased, in the same manner as there was this blackish response in white lupin roots treated with at 100 µM As (Vázquez et al. 2008). The evolution of alterations observed in peanut plant is in agreement with As accumulation trend in roots and shoots. The metalloid content in roots was higher than in shoots, showing that more than 97% of the As taken up by peanut plants was retained in the root system. This is a common response of non-hyperaccumulator plants where roots accumulate the major metalloid concentration (Dhankher et al. 2006; Carbonell-Barrachina et al. 2009; Sobrino-Plata et al. 2013). Probably, this toxic level of contaminant inhibited peanut growth, metabolism and transport allowing the passage of the metalloid to shoots disrupting the root protective “barrier” against As(V). It is feasible that the retention of As in roots occurs mainly at the cell walls of epidermal cells by the formation of an iron plaque as a product of the oxidation of the ferrous iron to ferric iron, that is precipitated as iron (Fe) onto the root surface, developing the plaque (Armstrong 1967). This plaque could acts as a barrier to the uptake of some potentially harmful metals or metalloids (Hansel et al 2002; Liu et al 2004). At the root endodermis As can be accumulated in vacuoles bound probably with GSH-derived ligands (Moore et al. 2011). The alterations of root anatomy, morphology and structure observed in peanut plants also occurred in plants grown with toxic metals like Cd (Vitória et al. 2001; Rodríguez-Serrano et al. 2006; Groppa et al. 2008; Gallego et al. 2012; Bianucci et al. 2012, 2013). Furthermore, in this work, plants growing with As showed a deposit of unknown material over the epidermis and cortex cells. Considering that lateral root emergency begins at pericycle level, the amount of these unknown deposits in roots, that augmented as $H_2AsO_4^-$ concentration

increased, could be limiting lateral root development, maintaining an invariable root:shoot ratio at all $H_2AsO_4^-$ dose tested, but modifying root architecture. This depends not only on metalloid toxicity but in probable changes in phytohormone balances, as may occur with cytokinins (Mohan et al., 2016). Similar deposits were detected in peanut roots under Cd stress (Bianucci et al. 2012; 2013). Thus, the deposition of a material on peanut epidermis induced by As could be the response of the dermal tissue preventing $H_2AsO_4^-$ entrance in the plant.

Previous research demonstrated that As induces generation of ROS leading to oxidative stress in plants (Hartley-Whitaker et al. 2001; Mascher et al. 2002; Molina et al. 2008; Shri et al. 2009; Finnegan and Chen 2012). ROS overproduction is the most harmful process that contributes to cellular damage however, the mechanisms by which As(V) causes the ROS burst are yet unknown (Sharma 2012). Among different putative sources of ROS production in plants, plasma membrane NADPH oxidase enzymatic activity constitutes an important one. This plasma membrane enzyme is involved on $O_2^{\cdot-}$ production which is rapidly disrupted to H_2O_2, spontaneously or *via* enzymatic reactions (Torres and Dangl 2005), and is known to be activated under metal stress (Hernández et al. 2015). The enzymatic reaction by which $O_2^{\cdot-}$ is disrupted to H_2O_2 is catalyzed by SOD, therefore is considered the first enzyme to cope with oxidative stress. Then, the conversion of H_2O_2 to H_2O is accomplished in peroxisomes by CAT enzyme (Noctor and Foyer 1998). Several studies demonstrated that SOD and CAT activities are enhanced by arsenate (Srivastava et al. 2005; 2010; Geng et al. 2006; Singh et al. 2007; Duman et al. 2010; Rai et al. 2011) as observed in this work.

Our findings revealed that NADPH oxidase was significantly enhanced by metalloid addition moreover, $O_2^{\bullet-}$ was found in cortex cells of peanut roots under 20 µM As(V), while epidermis was heavily stained when $H_2AsO_4^-$ treatment rose to 100 µM. The epidermis comprises cells that are readily exposed to toxic metals and metalloids, and are thought to be the first to respond to the induced stress (Hernández et al. 2015). Some metal trigger a quick accumulation of ROS and cell oxidative stress, that can be observed mainly at epidermal cells even after few minutes

of treatment with Hg and Cd (Ortega-Villasante et al. 2007). All these findings allowed to confirm that As induced oxidative stress in young peanut plants and not only reduced growth but also modified the architecture of the root system. Finally, the accumulation of ROS also causes the induction of lipid peroxidation (Cho and Park 2000; Hartley-Whitaker et al. 2001), which explains the strong level observed in peanut roots at the highest dose of $H_2AsO_4^-$.

GSH is the most abundant intracellular non-protein thiol in all living cells and acts as an important antioxidant to ameliorate excessive ROS generated by different stresses (Millar et al. 2003; Foyer and Noctor 2005; Shao et al. 2008). This thiol is also involved in several mechanisms of As detoxification in cells, and has a special interest due its ability to detoxify xenobiotics and heavy metals by conjugation (Jozefczak et al. 2012). Phytochelatins are the major ligand of metabolized $H_2AsO_4^-$, which forms an array of different As-PCs complexes in different plant species (Moore et al. 2011), which are synthesized by the condensation of GSH (Sobrino-Plata et al. 2014). This imposes a demand of GSH to generate sufficient PCs to limit the amount of toxic As in plant cells, leading to transient GSH accumulation (Hernández et al. 2015). Therefore, this mechanism may explain the significant increase in GSH concentration detected in peanut roots exposed to the highest dose of $H_2AsO_4^-$.

One of the hypothesized mechanisms of ROS accumulation under metal stress is the inhibition of antioxidant enzymatic activity (Sharma and Dietz 2009), particularly when stress symptoms are strong enough (Gratão et al. 2005). In this sense, we found a significant inhibition of GPX/PRX and GR activities only in plants treated with the higher arsenate dose. As-sensitive *B. juncea* genotype TM-4 suffered also from GPX/PRX and GR activity inhibition, upon exposure to acute toxic conditions (Srivastava et al. 2010). However, contradictory responses were observed in roots of *P. sativum* and *Oryza sativa* L. exposed to Cd and As, respectively, where the GPX/PRX activity was decreased and GR activity was significantly augmented (Dixit et al. 2001; Shri et al. 2009; Kumar et al. 2014); implying alterations specific to toxic metal(loid)s. On the opposite, GST activity rose remarkably at increasing concentrations of $H_2AsO_4^-$ in peanut-

treated plants. Similar induction of GST activity occurred in *Oryza sativa* and *Prosopis* sp. plants exposed to As (Mokgalaka-Matlala et al. 2009; Tripathi et al. 2012; Singh et al. 2016). GST comprise a large family of isoenzymes involved in the detoxification of organic peroxides, radicals and xenobiotics at the expense of GSH, and are induced by Cd and $H_2AsO_4^-$ (Schröder et al. 2009). GST deplete GSH cytosolic content directly to reduce H_2O_2 and to detoxify oxidation subproducts, promoting oxidizing conditions (Anjum et al. 2012). It is feasible that the pool of GSH will cover the demand imposed for GST activity and allow peanut plant to grow up to 20 µM H_2AsO4^- with minor observable symptoms of toxicity.

Taken together, the results presented in this manuscript revealed that 20 µM As(V) was the maximum concentration that allowed growth even when induced an overproduction of ROS in peanut plants with a relative tolerable As accumulation in shoots. Furthermore, root NADPH oxidase activity and lipid peroxidation contents increased after this As(V) supply, meanwhile GST enzyme activity was activated at 6 µM H_2AsO4^-. This phenomenon shows the ability of peanut GST enzyme to be induced and stimulate free metal binding before any ROS overproduction or oxidative damage to macromolecules can be detected. In this sense, it is important to highlight that 20 µM of arsenate supply resembles to As(V) levels found in contaminated Córdoba groundwater zones. Therefore, this finding contribute to understand how low doses of As could generate contamination of crops, affecting not only economically the peanut production but also generating a contamination of the food chain.

5. Conclusion and perspectives

This is the first report of As(V) impact on peanut plant which demonstrates that physiological and biochemical alterations are induced by the metalloid being NADPH oxidase enzyme implicated in the oxidative burst. Furthermore, the most sensitive stress parameter was the GSH related enzyme GST, which could be used in biotechnological approaches to select cultivars with lower

403 toxic symptoms, more tolerant and able to exclude As(V) from edible parts of the plants in order
404 to reduce As contamination.
405
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Fig. 1. Alteration of plant architecture of peanut plants treated with different concentrations of arsenate (µM): (a) 0 (control), (b) 6, (c) 20 and (d) 100.

Fig. 2. Cross section of peanut root treated with (a) 0 (Control), (b) 6, (c) 20, and (d) 100 µM arsenate. c: cortex, e: epidermis, en: endodermis, ma: unknown material

Fig. 3. Effects of arsenate exposure on root (a) NADPH oxidase activity, (b) SOD activity, (c) CAT activity, (d) hydrogen peroxide concentration, (e) lipid peroxidation. Data represent the mean ± SE (n =10). Different letters denote significant statistical differences ($P < 0.05$) according to Duncan´s test.

Fig. 4. Distribution of hydrogen peroxide in peanut root sections treated with 0 (control), 6, 20 and 100 µM arsenate. The arrows indicate the dark deposits resulting from the reaction of H_2O_2 with DAB.

Fig. 5. Distribution of superoxide radical in peanut root sections treated with 0 (control), 6, 20 and 100 µM arsenate. The arrows indicate reduced formazan blue deposits caused by the reduction of NBT with $O_2^{\bullet-}$.

Fig. 6. Changes in GSH content and thiol-related enzyme activities in peanut roots in response to 0 (control), 6, 20 and 100 µM arsenate**.** (a) Total GHS-Eq concentration, (b) GPX/PRX activity, (c) GR activity, and (d) GST activity. Data represent the mean ± SE (n =10). Different letters indicate significant statistical differences ($P < 0.05$) according to Duncan´s test.

Table 1. Penaut plant growth treated with different doses of arsenate.

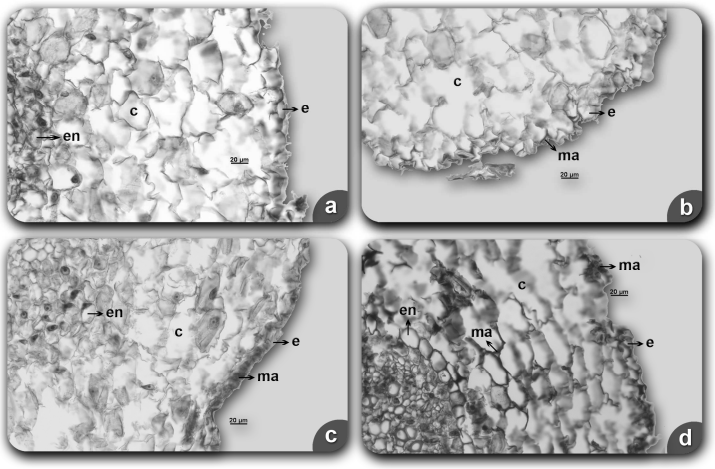
As addition (µM)	Shoot Dry weight (g)	Root Dry weight (g)	Shoot Length (cm)	Root Length (cm)
0	0.38 ± 0.02^{B}	0.14 ± 0.01^{C}	9.46 ± 0.20^{C}	13.44 ± 0.57^{C}
6	0.41 ± 0.01^{B}	0.11 ± 0.00^{B}	9.65 ± 0.28^{C}	12.13 ± 0.34^{C}
20	0.37 ± 0.01^{B}	0.09 ± 0.01^{B}	8.38 ± 0.26^{B}	10.20 ± 0.28^{B}
100	0.14 ± 0.01^{A}	0.05 ± 0.01^{A}	5.04 ± 0.32^{A}	6.15 ± 0.36^{A}

Data represent the mean ± SE (n =10). Different superscript letters indicate significant statistical di□erences between treatments (P <0.05) according to the Duncan's test.

Table 2. Concentration of As in peanut plants treated with different doses of arsenate.

As treatments (µM)	As concentration (µg g^{-1} dry weight)	
	Shoot	Root
6	0.28 ± 0.05^{B}	149.40 ± 25.87^{B}
20	0.66 ± 0.12^{B}	520.20 ± 40.45^{A}
100	7.80 ± 0.62^{A}	538.60 ± 84.79^{A}

Data represent the mean ± SE (n =10)**.** Different superscript letters indicate significant statistical di□erences between treatments (P <0.05) according to the Duncan's test.



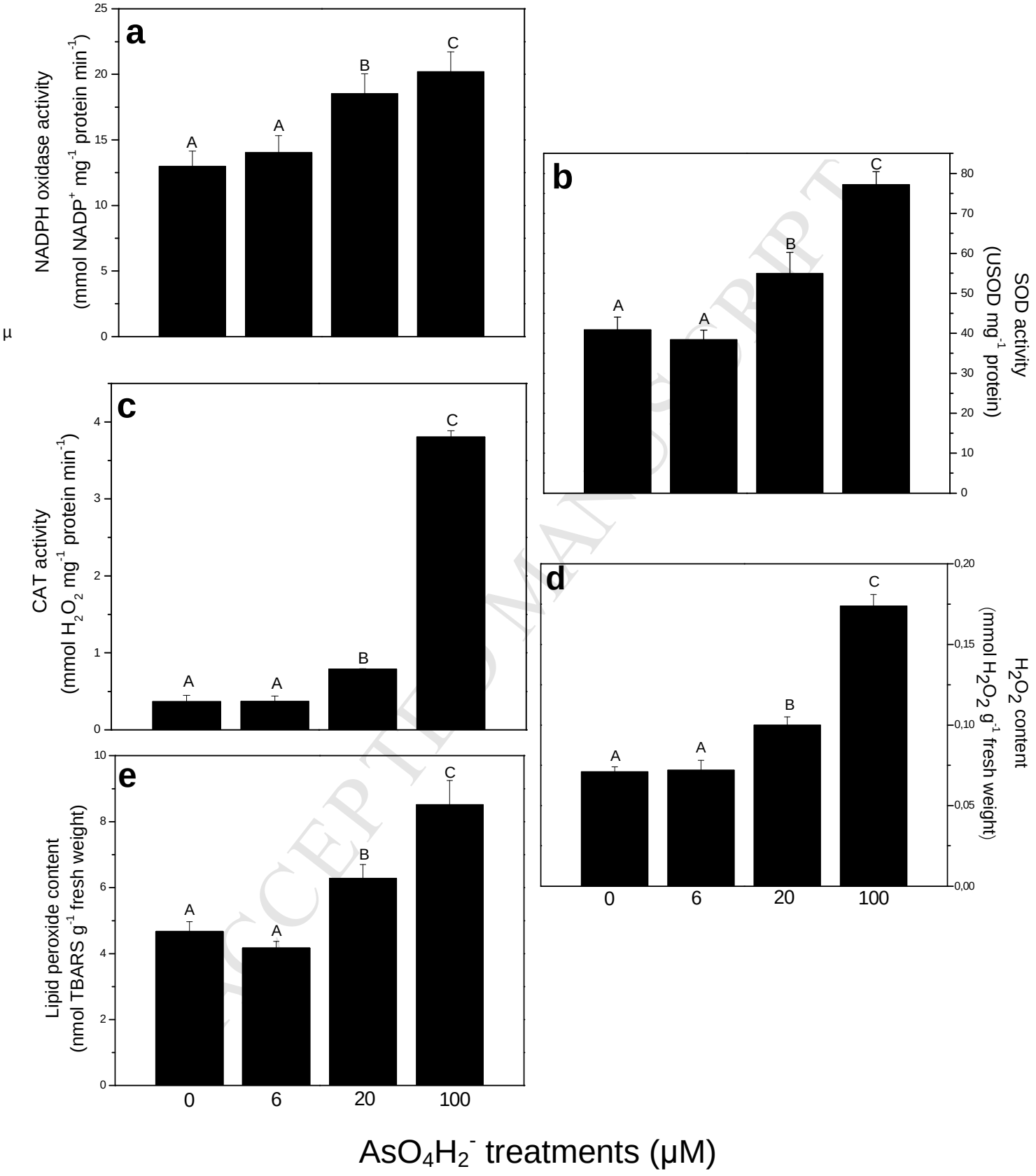
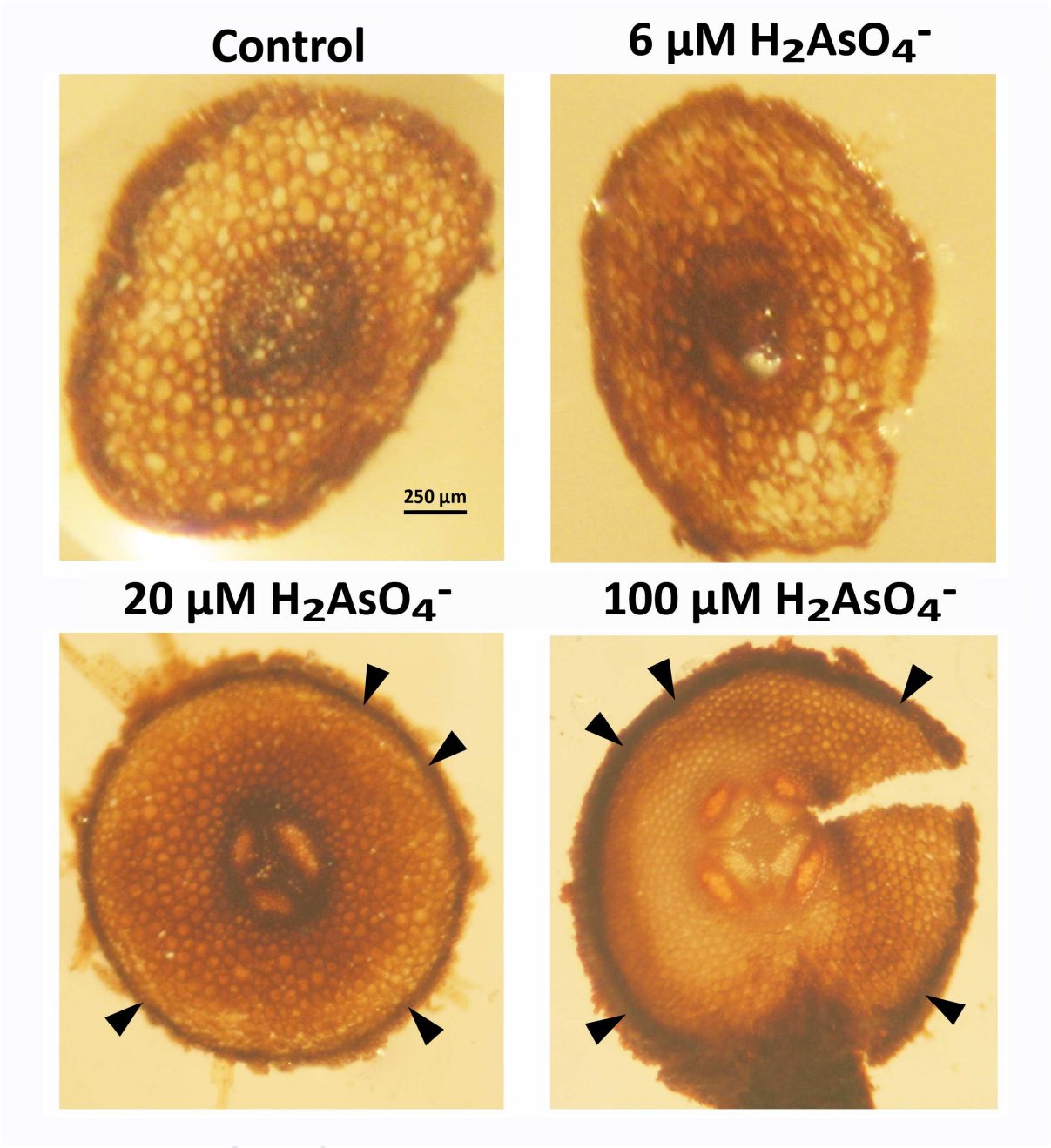
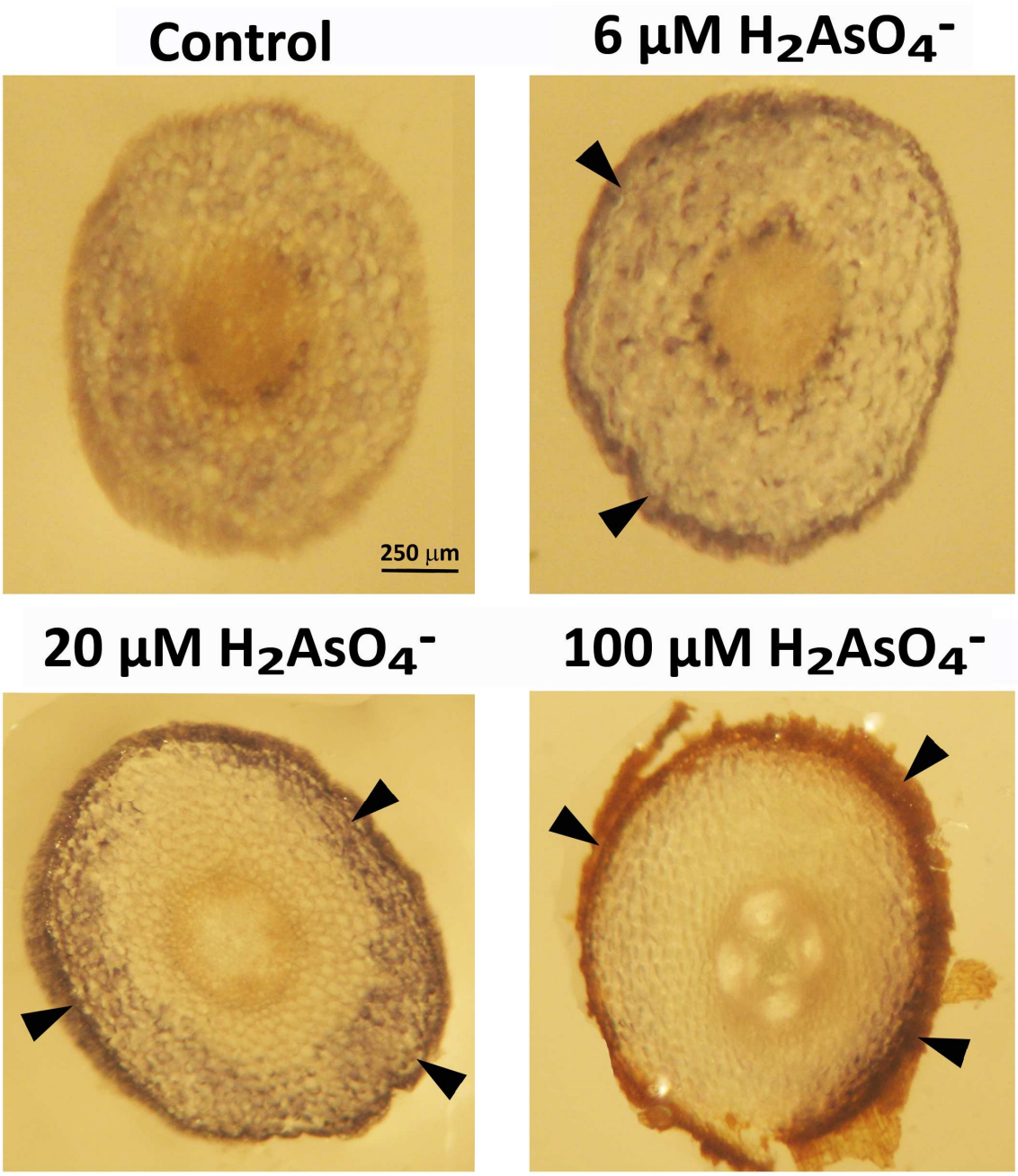
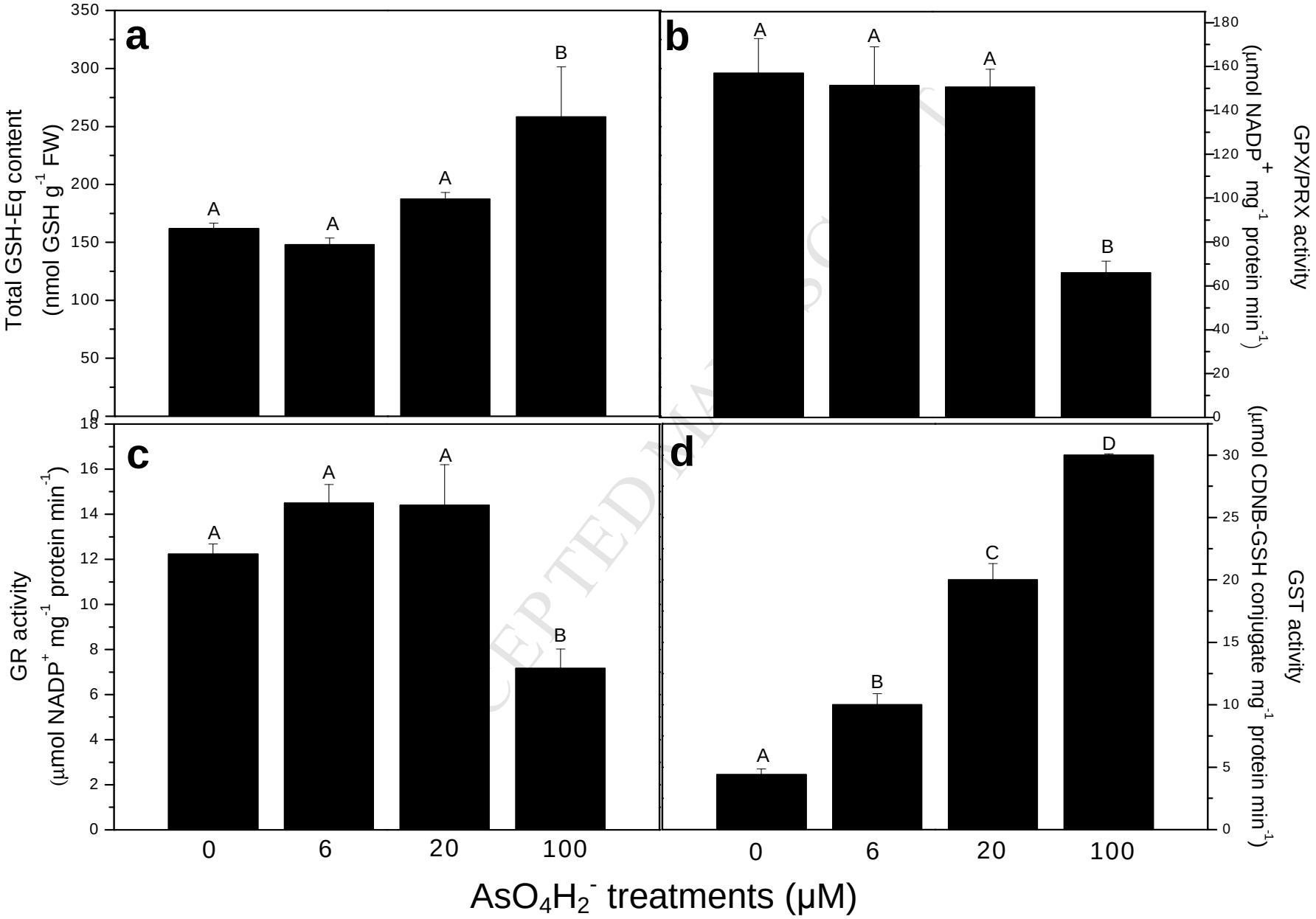

a
NADPH oxidase activity
(mmol NADP+ mg-1 protein min-1)
b
SOD activity
(USOD mg-1 protein)
c
CAT activity
(mmol H2O2 mg-1 protein min-1)
d
H2O2 content
(mmol H2O2 g-1 fresh weight)
e
Lipid peroxide content
(nmol TBARS g-1 fresh weight)
A
A
B
C
0
6
20
100
AsO4H2- treatments (µM)

Fig 3.






a
b
c
d
Total GSH-Eq content (nmol GSH g-1 FW)
GPX/PRX activity (µmol NADP+ mg-1 protein min-1)
GR activity (µmol NADP+ mg-1 protein min-1)
GST activity (µmol CDNB-GSH conjugate mg-1 protein min-1)
A
A
A
B
A
A
A
B
A
A
A
B
A
B
C
D
0
6
20
100
AsO4H2- treatments (µM)

Highlights

This is the first report showing the effect of arsenic on peanut plant in hydroponics

Root histological structure modification and damage to macromolecules are induced by As

Glutathione S-Transferase exhibits a remarkable increase upon exposure to As

GST activity is proposed as a suitable biomarker of peanut arsenic contamination