PERALTA Juan Manuel (Orcid ID: 0000-0003-3220-1221)
Bianucci Eliana (Orcid ID: 0000-0002-0697-7870)
Romero-Puertas Maria (Orcid ID: 0000-0002-4854-896X)

Targeting redox metabolism of the maize-*Azospirillum brasilense* interaction exposed to arsenic-affected groundwater

Juan Manuel Peralta[1,2], Eliana Bianucci[1,*], María C. Romero-Puertas[2], Ana Furlan[1], Stella Castro[1], Claudia Travaglia[1]

[1] Instituto de Investigaciones Agrobiotecnológicas – Consejo Nacional de Investigaciones Científicas y Técnicas (INIAB-CONICET), Departamento de Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto (UNRC). Ruta 36, Km 601, X5800 Río Cuarto, Córdoba, Argentina.

[2] Departamento de Bioquímica, Biología Celular y Molecular de Plantas, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, E-18008 Granada, Spain.

Correspondence

* Corresponding Author

e-mail: ebianucci@exa.unrc.edu.ar

Arsenic in groundwater constitutes an agronomic problem due to its potential accumulation in the food chain. Among the agro-sustainable tools to reduce metal(oid)s toxicity, the use of Plant Growth-Promoting Bacteria (PGPB) becomes important. For that, and based on previous results in which significant differences of As translocation were observed when inoculating maize plants with Az39 or CD *Azospirillum* strains, we decided to decipher the redox metabolism changes and the antioxidant system response of maize plants inoculated when exposed to a realistic arsenate (As^V) dose. Results showed that As^V caused morphological changes in the root exodermis. Photosynthetic pigments decreased only in CD inoculated plants, while oxidative stress evidence was detected throughout the plant, regardless of the assayed strain. The antioxidant response was strain-differential since only CD inoculated plants showed an increase in superoxide dismutase, glutathione S-transferase (GST) and glutathione reductase (GR) activities while other enzymes showed the same behaviour irrespective of the inoculated strain. Gene expression assays reported that only *GST23* transcript level was upregulated by arsenate, regardless of the inoculated strain. As^V diminished the glutathione (GSH) content of roots inoculated with the Az39 strain, and CD inoculated plants showed a decrease of oxidized GSH(GSSG) levels. We suggest a model in which the antioxidant response of the maize-diazotrophs system is modulated by the strain and that GSH plays a central role acting mainly as a substrate for GST. These findings generate knowledge for a suitable PGPB selection, and its scaling to an effective bioinoculant formulation for maize crops exposed to adverse environmental conditions.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ppl.13514

1. Introduction

Maize (*Zea mays* L.) is one of the most relevant crops grown worldwide due to its production and sown area (Nuss and Tanumihardjo 2010, FAOSTAT 2012). Besides grain production, maize plants are used for other purposes such as fermentation and industrial products (Requejo and Tena 2014). Maize is the most important cereal in Argentina, this crop represents a source of essential nutrients as well as economic incomes from export for the agricultural sector. Moreover, half of the production takes place in Córdoba´s province (SINAVIMO 2019). This *Poacea* interacts with soil microorganisms, commonly those of the genus *Azospirillum* giving rise to an associative rhizocenosis, since no specialized structures are formed in the plant root. Among the main strains of *Azospirillum brasilense*, the commercial strain Az39 has been widely used as inoculant in maize, wheat and barley crops due to its ability to increase plant root development and also yield. In addition, it has been shown that Az39 improved maize and wheat growing under unfavorable environments such as drought and salinity stress. The strain CD, a reference strain commonly used at laboratory and field studies also improved maize and wheat crops (Perrig et al., 2007). These strains have been described as a model of Plant Growth-Promoting Bacteria (PGPB) and field traceability and a great amount of information regarding the physiology of its growth and development has been published (Coniglio et al., 2019; Cassán et al., 2020). These free-living diazotrophs, play a fundamental role in biological nitrogen fixation and represent a model system to study auxin biosynthesis and its effects on roots (Pedraza et al., 2010; Molina et al., 2018). However, different abiotic stresses, such as metal(loid)s in the environment, could negatively affect this interaction. A metalloid that disturbs natural environments worldwide is arsenic (As; Smedley and Kinniburgh 2002; Bianucci et al., 2020). This element is naturally present in soil and water, where groundwater is the main source of human poisoning. Particularly arsenic implies a severe problem in some maize producing areas in Córdoba, where groundwater contains high metalloid concentrations coming from natural geological events (Blarasin et al., 2014). Although there is a maximum allowed level of arsenic in drinking water (0.1 µM according to FAOSTAT 2016), Córdoba´s groundwater reaches values up to 24 µM (Cabrera et al., 2005). Consequently, the use of this water for artificial crop irrigation, or the direct absorption by roots, could lead to significant grain contamination. In this way, the metalloid could enter the food chain, constituting not only an agronomic problem due to yield losses, but also a risk for human health (Bustingorri and Lavado 2014). The inorganic form arsenate (As^V) is one of the most toxic and its predominant in aerobic environments (Zhao et al., 2010) such as those found in groundwater from Córdoba (Blarasin et al., 2014). In plants, As^V absorption takes place through phosphate (PO_4^{2-}) transporters and its impact on plant cell metabolism has been widely described. (Catarecha et al., 2007; Wang et al., 2018; Bianucci et al., 2020).

One of the main consequences of inorganic arsenic exposure in plants is the oxidative burst due to an overproduction of reactive oxygen species (ROS). Among them, superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH; Mittler 2017; Sies et al., 2017). In addition, the NADPH oxidase complex significantly contributes to this oxidative response (Mittler et al., 2011). Therefore, oxidative damage can occur on essential biomolecules like lipids, proteins and nucleic acids, leading to oxidative stress that is translated into

a plethora of morphological, physiological, biochemical and molecular alterations (Begum et al., 2016; Singh et al., 2017a, b; Shahid et al., 2017; Bianucci et al., 2017, 2018, 2019, 2020; Peralta et al., 2019, 2020).

The use of PGPB constitutes a very important biotechnological tool to reduce metal(oid)s toxicity. It has been taken into consideration due to PGPBs activity on the metal(oid)s chemical transformation, chelation, or precipitation and sorption (Alka et al., 2020; Marwa et al., 2020). Combined with the metal(oid)s resistance mechanisms of microorganisms (Yang and Rosen 2016; Fagorzi et al., 2018), plants can activate an antioxidant system that scavenges accumulated ROS, being comprised of enzymes and metabolites with antioxidant properties (Noctor et al., 2012; You and Chan 2015). The enzymatic antioxidative defense enzymes are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase/peroxirredoxin (GPX/PRX; Iqbal et al., 2006), glutathione S-transferase (GST) that constitutes a large and diverse group of enzymes involved in the arsenic detoxification (Gill and Tuteja 2010), and the enzymes of the Foyer–Halliwell–Asada Cycle [ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR; Foyer and Noctor 2015)]. The non-enzymatic components include among others, ascorbate (AsA), glutathione (GSH) and carotenoids (Sharma and Dietz 2009; Sharma 2012; Sandalio et al., 2012). Growing evidence indicates that GSH is one of the most studied antioxidant metabolites to cope with oxidative stress caused by arsenic, through its participation in mechanisms related to tolerance, accumulation and distribution of the metalloid (Mrak et al., 2010; Sobrino-Plata et al., 2013; Hernández et al., 2015; Bianucci et al., 2017, 2018).

Given that research about the impact of arsenic on maize are generally focused on non-inoculated plants and that the metalloid concentrations applied are much higher than natural arsenic levels, the extrapolation to field conditions is very difficult (Requejo and Tena 2014; Ghosh et al., 2016; Anjum et al., 2016 a, b; Ghosh et al., 2017; Upadhyay et al., 2018). In a previous research we set out to evaluate the effect of an average natural As^V concentration found in Cordoba´s groundwater on the growth of maize plants and the metalloid translocation to leaves (Peralta et al., 2019). We found that 3 µM As^V had a negative impact on maize growth and the inoculation with *Azospirillum brasilense* strains (Az39 or CD) conditioned the growth variables and restricted the metalloid translocation to edible parts. Specifically, all growth variables as well as nitrogen content were diminished by metalloid addition. Remarkably, *Azospirillum* strains allowed greater biological nitrogen fixation (BNF) in plants compared to non-inoculated plants. On the other hand, in inoculated maize plants, metalloid accumulation was mainly detected in roots compared with leaves, a completely opposite response compared to non-inoculated plants. An interesting fact was that the CD *Azospirillum brasilense* strain increased phytostabilization on maize plants, reducing in a significant way metalloid translocation to shoots when compared to Az39 inoculation (Peralta et al., 2019). Based on these results, our experiments were performed in order to unravel the redox response and the implication of the antioxidant system of maize plants exposed to a natural As^V concentration, when inoculated with *Azospirillum brasilense* Az39 or CD strains. This first report will allow deciphering key aspects of the oxidative and antioxidative mechanisms triggered in the maize- *Azospirillum* interaction exposed to a natural concentration of the metalloid and the implication of *Azospirillum* strains to modulate this response on the plant.

2. Material and methods

2.1 Bacterial strains and cell viability under As exposure

The *Azospirillum brasilense* strains Az39 and CD (ATCC 29710) were obtained from IMIZA-INTA Castelar (Argentina) and EMBRAPA (Brazil), respectively. Bacterial cultures were grown on liquid Nfb medium (Döbereiner 1989) and incubated as described in Peralta et al., (2020).

The growth of *A. brasilense* Az39 or CD strains was evaluated in Nfb liquid culture medium supplemented with increasing $Na_2HAsO_4 \cdot 7H_2O$ (As^V) concentrations (0, 750, 1500, 2250 or 3000 µM). Medium without metalloids was considered as control. The culture flasks were inoculated with an appropriate volume of each strain cultures previously grown in Nfb medium without As^V, to achieve an initial OD_{620nm}value of 0.1. The strains were incubated on a gyratory shaker at 150 rpm and 28 ± 2°C. The OD_{620nm} was monitored at different times until stationary phase. Cell viability was determined by the microdroplet method according to Somasegaran and Hoben (1994).

2.2 Plant management and growth conditions

Hybrid maize seeds DK72-10 VT3pro (DEKALB, Argentina) were superficially disinfected and germinated following the method described by Vincent (1970). Then, seedlings were transferred to a Leonard Jar system as described in Peralta et al., (2020). Hoagland´s nutrient solution (Hoagland and Arnon 1950) devoid of As^V (control) or containing 3 µM As^V (a natural concentration of the metalloid found in groundwater of Córdoba; Cabrera et al., 2005) were the established treatments. The As^V was supplied as $Na_2HAsO_4 \cdot 7H_2O$ and the liquid level in the lower cup remained constant throughout the entire experience. At seven days post-emergence, maize seedlings were inoculated with *A. brasilense* Az39 or *A. brasilense* CD. Plants were grown in a plant chamber as described in Peralta et al., (2020) for 30 days post-inoculation. At harvest, leaves and roots were used for different analysis.

2.3 Inspection of root anatomy

For anatomical analysis, fresh maize adventitious roots were cut into 5 mm long portions, at 1 cm from the root tip according to Travaglia et al., (2012). Samples were processed using microtechnical methods described by Johansen (1940), which included dehydration, infiltration and paraffin embedding, rotary microtome (which model? Company?) cutting and staining. The evaluation of histological preparations was carried out as reported by Peralta et al., (2020).

2.4 Indicators of ROS production

2.4.1 Determination of NADPH oxidase activity and hydrogen peroxide (H_2O_2)

NADPH oxidase activity was determined spectrophotometrically according to Sagi and Fluhr (2001) with some modifications as described in Peralta et al., (2020). Fresh plant material (roots or leaves) was homogenized

following the procedure reported by Sobrino-Plata et al., (2009). One unit of NADPH oxidase was defined as the quantity of enzyme necessary to reduce 1 µmol of NADPH min^{-1}.

Hydrogen peroxide content was spectrophotometrically determined according to the procedure described by Alexieva et al., (2001) with modifications mentioned by Peralta et al., (2020). The amount of H_2O_2 content was calculated from a standard curve of H_2O_2.

2.4.2 Histochemical detection of ROS by fluorescence microscopy in roots

The sample collections for the histological ROS detection in maize roots was carried out according to Travaglia et al., (2012). The fluorescence observation was achieved as in the methods in Sandalio et al., (2008) and Rodríguez-Serrano et al., (2006), using dihydroethidium (DHE; Fluka Biochemika) and 2´-7´-dichlorofluorescein diacetate (DCF-DA; Calbiochem) for $O_2^{\cdot-}$ and H_2O_2 detection, respectively. Although the specific DHE probe was used (Fink et al., 2004), specificity of the reaction was deepened using the tetramethyl piperidinooxy superoxide scavenger (TMP; 1 mM). Likewise, as mentioned in Tarpey et al., (2004), DCF-DA is not a specific probe, therefore ascorbate 1 mM (H_2O_2 scavenger) was used following the Peralta et al., (2020) procedure. The observations were made in a stereomicroscope ($O_2^{\cdot-}$: excitation at 488 nm, emission at 520 nm; H_2O_2: excitation at 485 nm, emission at 530 nm).

2.4.3 Histochemical detection of ROS in leaves

Visual detection of $O_2^{\cdot-}$ was accomplished by incubating fresh plant material in 1 mM NBT, following the procedure described by Frahry and Schopfer (2001). Histological detection of H_2O_2 was performed by incubating fresh plant tissue in 1 mg ml^{-1} 3.3-diaminobenzidine (DAB) according to Orozco-Cárdenas and Ryan (1999). Photographs were then taken using a Dell® scanner.

Oxidative stress markers

The extraction of photosynthetic pigments from fresh leaves was carried out using 80% ethanol, according to Mac Kinney (1938) with modifications described by Peralta et al., (2020). Chlorophyll and carotenoid amounts were calculated using the formula chlorophyll *a* = 11.63 (OD_{665nm}) - 2.39 (OD_{650nm}); chlorophyll *b* = 20.11 (OD_{650nm}) - 5.18 (OD_{665nm}); Total chlorophyll = chlorophyll *a* + chlorophyll *b*; Carotenoids = 0.02 (OD_{450nm}).

Lipid peroxidation and protein carbonylation were evaluated in roots and leaves, as oxidative damage indexes using a UV-visible light spectrophotometer (Spectronic® Genesys 2). Damage to lipid structures was evaluated by quantification of thiobarbiturate-reactive substance (TBARS) concentrations as described by Heath and Packer (1968) with modifications reported by Peralta et al., (2020).

Protein oxidation was analyzed by quantification of the reactive carbonyl groups to 2,4-dinitrophenylhydrazine (DNPH) to form hydrazones (Levine et al., 1990) with modifications reported by Peralta et al., (2020). The number of protein carbonyls was calculated using the extinction coefficient 22 000 mol^{-1} cm^{-1} and the values were expressed in nmol mg^{-1} of protein.

2.6 Antioxidants

2.6.1 Enzymatic activities

The root samples were ground in liquid nitrogen and the powder obtained was homogenized according to the enzyme evaluated. The supernatant from the homogenates for superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), GPX / PRX (EC 1.11.1.9), GR (EC 1.6.4.2), and GST (EC 2.5.1.18) were obtained according to the methodology detailed by Bianucci et al., (2017), and for MDHAR (EC 1.6.5.4) and DHAR (EC 1.8.5.1) as reported by Peco et al., (2020). Protein concentration in plant extracts was determined according to Bradford (1976), using bovine serum albumin as standard. Enzymatic activities were tested spectrophotometrically according to the following methods: SOD activity as reported by Beauchamp and Fridovich (1973), CAT activity was measured using the method described by Aebi (1984), GPX/PRX activity was determined as detailed by Flohé and Gunzler (1984), this enzyme also acts as a TRX-linked thiol peroxidase (Navrot et al., 2006). GR activity was determined as described by Sheadle and Bassham (1977) and GST activity was determined according to Habig et al., (1974). Finally, APX activity was evaluated as reported by Nakano and Asada (1987), the DHAR and MDHAR activities were measured according to Dalton et al., (1993) and Nakano and Asada (1981), respectively.

2.6.2 Gene expression analysis using qRT-PCR

Following the acid guanidine thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (1987), total RNA was determined using a Trizol reagent kit according to the manufacturer's instructions. DNase (Ambion DNA free, Ambion) was used according to the manufacturer's protocol. RNA quality was checked by means on 0.8% agarose gel electrophoresis. An aliquot of RNA of 1 µg was used as a template for the reverse transcriptase (RT) reaction using the iScript ™ cDNA Synthesis Kit (Bio-Rad, Hercules). The program was set at 25°C for 5 min, 42°C for 30 min, 85°C for 5 min and it was kept at 4°C. Primer sequences are described in Supplemental Table S1. Quantitative real-time PCR was programmed on an iCycler iQ5 (Bio-Rad). Each 25 µl reaction contained either 1 µl of the cDNA or a dilution (1: 10 000) for the amplification of the *act1* gene, 200 nM of each primer, and iQ SyBrGreen Supermix (Bio-Rad). Control PCR reactions of the RNA samples not treated with RT were also performed to confirm the absence of contaminating genomic DNA. Initial denaturation of the samples was carried out heating at 95°C for 3 min followed by a 35-cycle amplification and quantification program (95°C for 30 s, 55°C for 45 s and 72°C for 45 s). A melting curve was performed to ensure the amplification of a single product. Amplification efficiency was calculated using the formula ($E = [10 (1 / a) - 1] \times 100$) where a is the slope of the standard curve, the relative expression of each gene was normalized to that of actin (*act1*) and the analysis of the results was performed using the comparative critical threshold method ($\Delta\Delta CT$; Nogales et al.,, 2010).

2.6.3 Quantification of glutathione

Extracts were obtained from maize roots homogenized in 5% (w/v) 5-sulfosalicylic acid and centrifuged at 10 000 *g* for 10 min at 4ºC to remove cell debris. The supernatant was used to measure total GSH content as

determined by Anderson (1985) using baker's yeast GR. GSSG was determined by the same method in the presence of 2-vinylpyridine and GSH content was calculated as the difference between the two forms. The reaction was followed at 412 nm and calibration curves were carried out using GSH (0-30 µM) and GSSG (0–10 µmol) samples. The intracellular GSH content was expressed as nmol GSH g^{-1} fresh weight (FW). The GSH:GSSG ratio was expressed as GSH/(GSH+GSSG).

2.7 Statistical analysis

Experiments were carried out in a completely randomized design with seven replicates and three independent experiments. The data were analyzed using the *InfoStat* software (Di Rienzo et al., 2018). Differences among treatments were analyzed using 2-way ANOVA, taking $P < 0.05$ as significant according to the Duncan test. Before 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 Cell viability of *Azospirillum brasilense* strains exposed to arsenate

In order to evaluate to what extent As^V affects *A. brasilense* strains growth, the number of viable cells of both strains were measured. *A. brasilense* Az39 and *A. brasilense* CD were able to grow with up to 2250 µM As^V, showing a short lag phase and reaching viability higher than 1x 10^9 CFU ml^{-1} at the end of the exponential phase. Survival to the metalloid by both strains studied was markedly decreased at 1500 µM As^V in comparison with the control treatment (Figure 1).

3.2 Effects of arsenate on root anatomy

In all treatments, regardless of the inoculated strain, the three tissue systems dermal, fundamental and vascular were recognized. This allowed us to classify the roots as polyarch types, determined by the projection numbers of the protoxylem inside the vascular cylinder (Figure 2A-D). Although the addition of As^V did not generate changes in the total cross-sectional area of maize roots (Figure 2E, F), it was identified that the parenchymal cells of the exodermis had thicker walls with respect to the control, regardless of the strain tested.

3.3 Influence of arsenate on photosynthetic parameters

The addition of As^V modified the photosynthetic pigment content of the maize - *A. brasilense* CD interaction with respect to control treatment. Specifically, the total chlorophyll content represented mainly by chlorophyll *a* (31.4%) was declined, together with a significant decrease of the carotenoid contents (33.3%). The maize- *A. brasilense* Az39 interaction exposed to As^V did not show significant variations in pigment composition (Table 1).

3.4 ROS production and oxidative damage in arsenate-treated plants

3.4.1 NADPH oxidase activity

To begin dissecting the ROS generation in maize plants, we were interested in evaluating the participation of the NADPH oxidase complex. Addition of As^V increased NADPH oxidase enzyme activity (44%) in leaves of maize plants inoculated with *A. brasilense* Az39, without changes in roots (Figure 3A). Meanwhile, this enzyme activity was significantly decreased (20%) in roots of plants inoculated with *A. brasilense* CD with respect to the control conditions (Figure 3B).

3.4.2 Detection, localization and quantification of H_2O_2 by fluorescence microscopy, histochemistry and spectrophotometry

Figure 4 shows H_2O_2 detection and quantification in roots and leaves of maize plants. Maize roots exposed to As^V showed a higher fluorescence emission compared to the control treatment, irrespective of the inoculated strain (Figure 4A). Similarly, DAB staining of maize leaves revealed that, regardless of the strain used as inoculant, an increase in H_2O_2–dependent dark brown precipitates was observed when arsenate was added compared to the control treatment (Figure 4B). However, the quantitative determination of H_2O_2 revealed that it increased (43.5%) only in leaves from maize plants inoculated with *A. brasilense* CD (Figure 4C). The H_2O_2 histochemical observation in roots was consistent with the quantitative analysis of this species in which significant increases were observed upon As^V exposure, regardless of the inoculated strain. The H_2O_2 increase detected in roots of maize plants inoculated with *A. brasilense* Az39 was significantly higher (44%) than that obtained when *A. brasilense* CD was added as an inoculant (18%; Figure 4D).

3.4.3 In vivo histochemical detection of $O_2^{\cdot -}$ in maize plants

A higher red fluorescence emission was observed in maize roots exposed to As^V, compared to the control treatment, highlighting those roots of maize plants inoculated with the Az39 strain (115,6%; Figures 5A,C). Likewise, irrespective of the inoculated strain, the leaves of maize plants grown under arsenate exposure, ...wed blue precipitate points indicating $O_2^{\cdot -}$ presence (Figure 5B).

3.5 Arsenate effect on lipid and protein oxidation

To determine the extent of the oxidative burst induced by arsenate, we evaluated oxidative damage indexes. As^V increased lipid peroxidation in leaves and roots of maize plants, irrespective of the inoculated strain, compared to the control treatment (Figures 6A,C). Regarding protein oxidation, the results indicated that As^V triggered an increase on the content of carbonylated proteins in all evaluated tissues, with respect to plants devoid of arsenate and irrespective of the inoculated strain (Figures 6B,D).

3.6 Antioxidant response of inoculated maize plants to arsenate

We found that arsenate caused significant increases in superoxide dismutase (SOD) and glutathione S-transferase (GST) activities compared to the control treatment in the roots of maize plants inoculated with *A. brasilense* CD (Figures 7A,C). On the other hand, the specific activities of catalase (CAT) and glutathione peroxidase/peroxirredoxine (GPX/PRX) did not show significant changes with either of the tested strains

(Figures 7B,F). Concerning the enzymatic activities involved in the Foyer-Halliwell–Asada cycle, the metalloid addition caused an increase in GR activity in roots of the maize-*A. brasilense* CD interaction with respect to those plants inoculated with *A. brasilense* Az39 (Figure 7E). Furthermore, while monodehydroascorbate reductase (MDHAR) and ascorbate peroxidase (APX) activities decreased significantly, the dehydroascorbate reductase (DHAR) activity remained without changes, irrespective of the assayed strain (Figures 7G,D,H). Interestingly, the transcript level of *GST23* was upregulated by arsenate stress, whereas *GR* and *APX1* remained unaltered, regardless of the inoculated strain (Figure 7I).

3.7 Glutathione content is altered by arsenate

According to the analysis of glutathione content, As^V diminished the total glutathione (GSH_T) and reduced glutathione (GSH) contents in the roots of maize plants inoculated with *A. brasilense* Az39, while those inoculated with *A. brasilense* CD showed a significant decrease in the oxidized glutathione (GSSG) content. However, the GSH/(GSH+GSSG) ratio remained unalterable in either interaction evaluated (Table 2, Figure S2).

In order to improve the understanding of the results a resume of all the presented result is shown in Table S2.

4. Discussion

The impact of arsenic on plant growth and metabolism has been reported for different plant species (Ghosh et al., 2016, 2017; Souri et al., 2017; Abbas et al., 2018; Bianucci et al., 2017, 2018, 2019). Nevertheless, research about the effect of natural concentrations of the metalloid on maize plants establishing non-symbiotic interactions with members of the edaphic microbiome is scarce, making it difficult to extrapolate the findings to field conditions. CD and Az39 *Azospirillum brasilense* strains are PGPB that have been widely studied in several crops due to their capability to increase crop yield. However, information regarding their behavior when inoculated on maize plants growing under arsenic stress is scarce. Moreover, it is known that the response of plants to metal(loid)s could be differentially modulated by the inoculated strain, even though they belong to the same species. It was extensively observed in plants that establish a symbiotic interaction like soybean and peanut exposed to As or Cd (Bianucci et al., 2012, 2013, 2018, 2019). In this regard, previous work showed that maize inoculation with CD restrained arsenic translocation to aerial parts, limiting grain contamination when compared to Az39 (Peralta et al., 2019). In this work we described the oxidative stress and antioxidant responses of maize plants exposed to a natural arsenate concentration when inoculated with CD or Az39 *Azospirillum brasilense* strains.

Our first approach was to recognize arsenic tolerance through *Azospirillum brasilense* strains thus, in vitro tests were performed. Both bacterial strains showed similar behavior when exposed to increasing As^V concentrations. Although these findings deserve further study in the future, it is known that the reduction of cell viability agrees with previous reports (Mandal et al., 2008; Panigrahi et al., 2013) and could be due to As-induced oxidative

damage. Moreover, defensive strategies to overcome this stress are usually strain-specific, these include formation of As-GSH complexes, efflux systems that eject the metalloid outside the cell, processes of oxidation and/or anaerobic reduction and methylation (Verbruggen et al., 2009; Sharma 2012; Yang and Rosen 2016).

Plants have the ability to acclimate in response to abiotic environmental stresses through physiological and biochemical processes, as well as adjusting morphological and developmental patterns (Schikora and Schmidt 2001; Pasternak et al., 2005). Among these adaptations, roots are the first organs in contact with metals and metalloids in the soil, therefore several anatomical alterations of root tissues can be expected (Deng et al., 2019). However, 3 µM As^V treatment did not modify the cross-section area of adventitious roots of maize plants and did not change the number of cortical layers. Nevertheless, a thickening of plant cell walls when exposed to metal(loid) stress was observed. Similar results were observed in other plant species (Sujkowska-Rybkowska et al., 2015; Lafuente et al., 2015; Bianucci et al., 2017; Sujkowska-Rybkowska and Ważny 2018). Thus, the thickening in root cell walls and exodermis changes could restrict the arsenic permeability avoiding metalloid translocation (Gall et al., 2015).

Less data is available about the mechanisms involved in arsenic toxicity at photosynthesis level. However, it is suggested that it could be related to an alteration of the redox homeostasis (Finnegan and Chen 2012). According to our results, As^V induced a significant decrease in the content of photosynthetic pigments in maize plants inoculated with *A. brasilense* CD, while *A. brasilense* Az39 kept that variable stable. This result was in agreement with our previous findings in which inoculation of maize with the Az39 strain promoted plant growth even when the metalloid translocation to the aerial part was higher, compared to maize plants inoculated with *A. brasilense* CD (Peralta et al., 2019). Thus, under arsenic treatment, we suggest that the maintenance of pigment contents observed in Az39 inoculated plants, could be associated to a priming response induced by the microorganism (probably associated with ROS production). Furthermore, despite the unfavorable growing condition that these plants were exposed to and the a high content of As that was detected in the leaves, maize plants were able to maintain carbon fixation process. Under stress conditions, an overload of the electron transport chain in the photosynthesis process produces a leak of ferredoxin electrons to O_2, giving rise to an oxidative burst which may alter chlorophyll content and integrity (Suneja et al., 2014; Emamverdian et al., 2015; Hasanuzzaman et al., 2017). Thus, some authors proposed the determination of chlorophyll content as a redox status index (Miteva 2002; Shaibur et al., 2009). While arsenic-induced chlorophyll damage is a widely reported response (Stoeva et al., 2003; Abbas et al., 2018), a study carried out by Mascher et al., (2002) showed no modification of the photosynthetic pigments in red clover plants, when exposed to a low arsenic dose. In addition, an increment in chlorophyll content was observed in tomato, onion and maize plants (non-inoculated) exposed to low As^V concentrations (Miteva 2002; Sushant and Ghosh 2010; Mallick et al., 2011). On the other hand, carotenoids protect light-harvesting complexes against light over-excitation and photo-oxidative damage. It is well documented that they dissipate the excess of absorbed energy, thereby preventing or reducing the formation of ROS such as $^1O_2$ (Strzałka et al., 2003) even in plants growing under arsenic exposure (Neill et al., 2002; Sinha et al., 2013).

To investigate the production of ROS that takes place in response to arsenic (Ahsan et al., 2008; Mallick et al., 2011; Sharma 2012; Islam et al., 2015), we evaluated the NAPDH oxidase activity and the detection of $O_2^{\cdot -}$, •OH and H_2O_2 species. Our results showed that the exposition of maize plants to 3 µM As^V, for a period of 30 days in a semi-hydroponic system, induced a significant oxidative burst. The redox imbalance depends on several variables such as, the phenological stage and the plant species, the time of exposure, the type of substrate used and the concentration of the contaminant, among others. For example, Gupta et al., (2013) reported an increase in H_2O_2 content in *Arabidopsis thaliana* plants grown for five days in a hydroponic system, supplemented with 50 µM As^V. In the same way, peanut plants exposed to 20 and 100 µM As^V for fifteen days, showed an increase in $O_2^{\cdot -}$ and H_2O_2 contents compared to the control condition, without significant ROS production at the lower concentration tested (6 µM As^V; Bianucci et al., 2017).

It is known that NADPH oxidase associated with the plasma membrane is one of the most important sources of ROS under arsenic stress (Gupta et al., 2013; Hernández et al., 2015; Bianucci et al., 2017, 2019). Nonetheless, this work revealed that while the activity of NADPH oxidase was enhanced by arsenate in leaves of interaction maize-*A. brasilense* Az39, the enzymatic activity decreased roots of those plants inoculated with the CD strain. This result was particularly intriguing because the radical $O_2^{\cdot -}$ was prominent in leaves and roots of maize and, the H_2O_2 content was significantly increased too. A possible explanation could be directly related to the specific interaction established with maize and the *Azospirillum* strain. In this regard, it was demonstrated that inoculation of maize with the CD strain allowed the plant to phytostabilize the metalloid on roots, while Az39 translocated it to the shoot (Peralta et al., 2019). Thus, the differential arsenic accumulation pattern in maize could modify the oxidative response specially on enzymes due to a direct alteration of the activity induced by ROS or/and post-transductional modifications. In addition, recent research has shown that the inoculated strain could modulate the oxidative response of plants exposed to metal(loid)s in different ways, even when they belong to the same genus as observed in peanut plants exposed to As and Cd (Bianucci et al., 2012a, 2013; Peralta et al., 2020).

Deeper analysis of oxidative metabolism research shows that oxidative stress is a common response to metal(oid)s in plants, which is manifested by the oxidation of essential biomolecules (Scandalios et al., 2002). Among these, lipid peroxidation (Clemens and Ma 2016; Souri et al., 2017a, b; Abbas et al., 2018) mainly represented by the TBARS content is widely known (Gill and Tuteja 2010). We observed an increase in membrane lipid peroxidation of maize exposed to arsenic, independently of the *A. brasilense* strain inoculated. Similarly, Talukdar (2013) and Gupta et al., (2013) reported lipid peroxidation in fenugreek exposed to 90 µM As^V, and in leaves of *Arabidopsis thaliana* after a long-term exposure to the metalloid, respectively. In a study carried out by Singh et al., (2017b) it was revealed that *Vetiveria zizanoides* plants growing with increasing concentrations of arsenic (0-200 µM) showed a correlated increase of lipid oxidative damage. Another effect of oxidative damage is the modification of protein structures demonstrated by the release of carbonyl groups (Parkhey et al., 2014). We also found an increase in carbonyl content of As^V-treated maize plants, independently of the *A. brasilense* strain used. So far, particularly in plants, it has not been totally demonstrated that the induction of oxidation/carbonylation of proteins is promoted by arsenic. Several researchers have focused on

other metals such as Zn (Ramakrishna and Rao 2013), Cd (Romero-Puertas et al., 2002; Rodríguez-Serrano et al., 2006; Bianucci et al., 2012a, b; Pérez-Chaca et al., 2014; Xu et al., 2018), Co (Karuppanapandian and Kim 2013) and Cu (Mustafa and Komatsu 2016; Ju et al., 2019).

In an effort to understand the antioxidant response of inoculated-maize plants to As-induced oxidative stress, first we analyzed the activity of SOD and CAT, which constitute the first line of defense against $O_2^{\cdot -}$ and H_2O_2, respectively (You and Chan 2015; Abbas et al., 2018). According to our results, As^V increased the SOD activity only in roots of maize plants inoculated with *A. brasilense* CD, while CAT activity remained stable in both inoculation conditions. This implies another intriguing finding since the Az39 strain increased NADPH activity while the CD strain reduced it, but both interactions showed an increase in the H_2O_2 content. Alternatively, it is known that GPX/PRX cleaves H_2O_2 using GSH or TRX (Navrot et al., 2006). However, our data did not show changes in GPX/PRX activity. Alterations of these enzymatic activities have been reported for several crops exposed to increasing arsenic doses and under different time exposure (Singh et al., 2017b; Ghosh et al., 2016; Souri et al., 2017; Bianucci et al., 2017, 2019). It was reported that the increased of SOD activity by As can be explained by an enhanced production of $O_2^{\cdot -}$ and/or by a direct action of the metalloid on SOD expression gene (Sharma 2012). On the other hand, an increasing CAT activity was observed when plants were exposed to the metalloid (Pandey et al., 2015; You and Chan 2015; Bianucci et al., 2017). However, there is some data suggesting that a reduction in these enzymatic activities occurs too (Mylona et al., 1998; Singh et al., 2007). Usually, literature shows research involving higher doses of arsenic than we used in our work, e.g. sunflower plants exposed to 10 µM As^V in hydroponic conditions (Saidi et al., 2017), hyper-accumulating plants as *Chrysopogon zizanioides* and *Pteris vittata* treated with increasing As^V concentrations (0-50 mg l^{-1}; Tiwari and Saragi 2017), rice and wheat plants exposed to 0-0.5 mM or 0-50 µM As^V, respectively (Dave et al., 2013; Hasanuzzaman and Fujita 2013).

GST enzymes constitute a large and diverse group with a preponderant role in plant tolerance to abiotic stress (Anjum et al., 2012; Kumar and Trivedi 2018). Interestingly, our results showed an increase in GST activity only in the maize-*A brasilense* CD in response to As^V treatment in roots. Similar results were found in non-inoculated maize seedlings after two days of exposure to the metalloid (50 and 100 µM; Ghosh et al., 2016), while no changes were recorded after four days, due to a possible previous ROS detoxification. Bianucci et al., (2017) unveiled a progressive increase in GST activity, arsenic-dose dependent, in roots of non-inoculated peanut plants. The GST over-expression enhances its binding ability to the free metalloid before ROS overproduction or oxidative damage occurs. Additionally, increases in GST activity in response to As^V treatment have also been observed in leaves of inoculated soybean (Bianucci et al., 2019), rice and carob trees (Mokgalaka-Matlala et al., 2009; Tripathi et al., 2012; Singh et al., 2016).

In plants, the Foyer–Halliwell–Asada Cycle (AsA-GSH) cycle has an active role in the detoxification of H_2O_2, GR and APX being crucial enzymes in this process (Foyer and Noctor 2015). In this regard, there are many studies that highlight the increase in GR and APX activities in the presence of arsenic (Dave et al., 2013; Hasanuzzaman and Fujita 2013; Begum et al., 2016; Saidi et al., 2017). However, in brown mustard and peanut

plants a decrease or no alteration of the enzymatic activities was revealed, respectively (Srivastava and D'souza 2010; Bianucci et al., 2017). In our work, As^V decreased MDHAR and APX activities, regardless of the inoculated strain. In addition, no changes of DHAR activity would indicate that total DHA is metabolized by using GSH. Regarding it, a decrease in the total GSH content, could be explained by its direct action on ROS detoxification as well as in the PCs synthesis (Hasanuzzaman et al., 2017). Although the GSH/(GSH+GSSG) ratio remained unchanged under both inoculation conditions, GSH declined in the maize-*A brasilense* Az39 interaction and remained unchanged in plants inoculated with *A. brasilense* CD. Curiously, in the reverse, a GSSG decrease was detected and as expected, the GR activity in roots of maize plants inoculated with the CD strain was increased. In this interaction, we suggest that endogenous GSH is used to supply GST activity and maintain the DHAR activity. Consequently, AsA could be supplied to the AsA-GSH cycle, even when there is a basal APX activity, thus maintaining the cycle functionality. To gain more insight into the antioxidative response of maize plants to arsenate, we performed transcriptomic analysis of some genes that codify for antioxidant enzymes. Interestingly, the correspondence between the transcript level and the enzymatic activity was only observed in GST for the interaction maize-*A. brasilense* CD and for GR in roots inoculated with the Az39 strain.

Taken together, our results revealed that for both inoculations, the oxidative burst in maize plants exposed to a natural concentration of As^V induced oxidative damage, evidenced by lipid peroxidation of membrane lipids and protein carbonylation. One of the most relevant findings in this work was that the antioxidant response was strain-dependent. It is necessary to emphasize that literature has a wide background about high arsenic concentration effects on non-hyperaccumulating plants. Usually, the changes detected are as expected for such concentration (Abbas et al., 2018). Although originally the inoculation of diazotroph microorganisms had a sole and important function, to serve as a nitrogen source for crops in order to improve growth and increase yield, the utilization of such microorganisms as bioremediation tools constitutes an additional function that has gained special attention in the last ten years (Reichman 2007; Mandal et al., 2011; Gómez-Sagasti and Marino 2015). In addition, physico-chemical transformations of metal(oid)s carried out by microorganisms in the rhizosphere depend on their intrinsic capacities (Finnegan and Chen 2012; Lomax et al., 2012; Yang and Rosen 2016). For this reason, the selection of a suitable PGPB for maize requires deep research, since not all bacteria act in the same manner when inoculated with plants exposed to different arsenic concentrations, as observed in our research. In particular, the associative rhizocenosis could not only modify the growth variables of the plant, but also enhance the phytostabilization process serving as a promising biotechnological strategy to avoid metal(oid)s translocation to edible parts (Bianucci et al., 2018; Peralta et al., 2019; Bianucci et al., 2020). Accordingly, crop inoculation should be carefully controlled, especially in maize whose grains and their post-processing waste are used to produce food for humans or livestock (Gómez-Sagasti and Marino 2015). The key contribution of this work is the elucidation that a low concentration of As^V, naturally present in groundwater of cropping areas from Argentina, negatively impacts the oxidative metabolism of maize plants, which is a novel observation. In addition, each bacterial strain participates in the modulation of the redox response in the non-

symbiotic system in a specific way, conditioning the crop growth and the arsenic translocation to the edible parts of maize, as previously observed in our studies. Particularly, maize plant inoculation with the CD strain further restricted the arsenic translocation towards the leaves, although it accumulated a higher total metalloid concentration with respect to those plants inoculated with *A. brasilense* Az39 (Peralta et al., 2019). Thereby, the translocation restriction of As by the CD strain gives it a remarkable potential in addition to its PGPRs activities in maize.

5. Concluding remarks

In order to decipher the arsenate impact on maize plants inoculated with *Azospirillum brasilense* strains, the present work made several discoveries, among which, a realistic concentration of the metalloid, naturally present in groundwater from agronomic fields, generates an oxidative burst by increased ROS levels and the consequent oxidative stress due to lipid and protein damage. We proposed a model in which it is highlighted that the antioxidant response of the maize-diazotrophs system is modulated depending on the inoculated bacterial strain (Figure 8). Particularly, GSH plays a central role through its participation in ROS scavenging and chelation of arsenate acting mainly as a substrate for the GST, an essential enzyme to maintain cell viability. These findings together with our previous data provide evidence of a sustainable tool to counteract arsenic-effects on crops. Besides the widely known PGPB activities of CD strain on maize, the capability of this microorganism to restrict As translocation in plants remarks it potential use as inoculant on metalloid affected cropping areas. In addition, considering the background of *A. brasilense* Az39 as bioinoculant for maize, research windows are opened for the study of bacterial consortia among the strains studied here. Thus, this research generated knowledge for the selection of the appropriate PGPB, which can be a key component of a potential microbial inoculant for maize crops exposed to naturally adverse growth conditions.

6. Acknowledgments

This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Secretaría de Ciencia y Tecnología-Universidad Nacional de Río Cuarto (SECYT-UNRC), a grant from Ministry of Science, Innovation and Universities together with the European Regional Development Fund (MCIU/AEI/ERDF; PGC2018-098372-B-100). JMP currently holds a doctoral scholarship from CONICET. CT, EB and AF are members of the research career from CONICET. MCRP is research scientist at EEZ-CSIC. SC is a researcher-teacher at UNRC. JMP is grateful to the Programa de Movilidad entre Instituciones Asociadas a la Asociación Universitaria Iberoamericana de Postgrado (AUIP, 2017) for supporting a short-term scholarship in EEZ-CSIC and Universidad de Granada (Spain). The authors are grateful to Dr. Adela Olmedilla and Eliana Molina-Moya and for their technical assistance.

Please add the Author Contributions here:

J.M.P. carried out all the experiments and write the manuscript. E.B., C.T. and S.C. designed the experimental system and supervised assays carried out in Argentina. M.C.R.P. supervised the ROS detection and gene expression assays carried out in Granada, Spain. All authors read and corrected the manuscript.

References

Abbas G, Murtaza B, Bibi I, Shahid M, Niazi N, Khan M, Hussain M (2018) Arsenic uptake, toxicity, detoxification, and speciation in plants: physiological, biochemical, and molecular aspects. Int J Environ Res Public Health 15(1): 59.

Aebi H (1984) Catalase in vitro. In: Methods in enzymology 105: 121-126. Academic Press.

Ahsan N, Lee DG, Lee KW, Alam I, Lee SH, Bahk JD, Lee BH (2008) Glyphosate-induced oxidative stress in rice leaves revealed by proteomic approach. Plant Physiol Biochem 46(12): 1062-1070.

Alexieva V, Sergiev I, Mapelli S, Karanov E (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ 24(12): 1337-1344.

Alka S, Shahir S, Ibrahim N, Chai TT, Bahari ZM, Abd Manan F (2020) The role of plant growth promoting bacteria on arsenic removal: A review of existing perspectives. Environ Technol Innov 17: 100602.

Anderson ME (1985) Determination of glutathione and glutathione disulfide in biological samples. In: Methods in enzymology. Academic Press 113: 548-555.

Anjum NA, Ahmad I, Mohmood I, Pacheco M, Duarte AC, Pereira E, Prasad MNV (2012) Modulation of glutathione and its related enzymes in plants' responses to toxic metals and metalloids—a review. Environ Exp Bot 75: 307-324.

Anjum NA, Sharma P, Gill SS, Hasanuzzaman M, Khan EA, Kachhap K, Sofo A (2016a) Catalase and ascorbate peroxidase—representative H_2O_2-detoxifying heme enzymes in plants. Environ Sci Pollut Res 23(19): 19002-19029.

Anjum SA, Tanveer M, Hussain S, Shahzad B, Ashraf U, Fahad S, Bajwa AA (2016b). Osmoregulation and antioxidant production in maize under combined cadmium and arsenic stress. Environ Sci Pollut Res 23(12): 11864-11875.

Beauchamp CO, Fridovich I (1973) Isozymes of superoxide dismutase from wheat germ. Biochim Biophys Acta Proteins Proteom 317(1): 50-64.

Begum MC, Islam MS, Islam M, Amin R, Parvez MS, Kabir AH (2016) Biochemical and molecular responses underlying differential arsenic tolerance in rice (*Oryza sativa* L.). Plant Physiol Biochem 104: 266-277.

Bianucci E, Fabra A, Castro S (2012a) Involvement of glutathione and enzymatic defense system against cadmium toxicity in *Bradyrhizobium* sp. strains (peanut symbionts). Biometals 25(1): 23-32.

Bianucci E, Furlan A, Hernández LE, Castro S (2019) Insights into the effect of a natural arsenate dose on growth, nodulation and redox metabolism of soybean plants. Pedosphere 29(4): 527-533.

Bianucci E, Furlan A, Tordable MC, Hernández LE, Carpena-Ruiz RO, Castro S (2017) Antioxidant responses of peanut roots exposed to realistic groundwater doses of arsenate: Identification of glutathione S-transferase as a suitable biomarker for metalloid toxicity. Chemosphere 181: 551-561.

Bianucci E, Godoy A, Furlan A, Peralta JM, Hernández LE, Carpena-Ruiz RO, Castro S (2018) Arsenic toxicity in soybean alleviated by a symbiotic species of *Bradyrhizobium*. Symbiosis 74(3): 167-176.

Bianucci E, Peralta JM, Furlan A, Hernández LE, Castro S (2020) Arsenic in Wheat, Maize, and Other Crops, in: Srivastava, S. (Eds.) Arsenic in Drinking Water and Food. Springer, Singapore, 279-306.

Bianucci E, Sobrino-Plata J, Carpena-Ruiz RO, Tordable MC, Fabra A, Hernández LE, Castro S (2012b) Contribution of phytochelatins to cadmium tolerance in peanut plants. Metallomics 4(10): 1119-1124.

Blarasin M, Cabrera A, Matteoda E (2014) Aguas subterráneas de la provincia de Córdoba¸ Universidad Nacional de Río Cuarto, Argentina. (1ra ed.), UniRío, Río Cuarto, Córdoba. [Groundwater of Córdoba province, National University of Río Cuarto]. http://www.unirioeditora.com.ar/wp-content/uploads/2020/02/978-987-688-091-6.pdf

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72(1-2): 248-254.

Bustingorri C, Lavado RS (2014) Soybean as affected by high concentrations of arsenic and fluoride in irrigation water in controlled conditions. Agric Water Manag (144): 134-139.

Cabrera A, Blarasin M, Matteoda E, Villalba G, Gómez GM (2005) Argentine Site of Animal Production. Available at www.produccion-animal.com.ar (Accessed July 2015).

Cassán F, Coniglio A, López G, Molina R, Nievas S, de Carlan CLN, Mora V (2020) Everything you must know about *Azospirillum* and its impact on agriculture and beyond. Biol Fert Soil 56: 461-479.

Catarecha P, Segura MD, Franco-Zorrilla JM, García-Ponce B, Lanza M, Solano R, Leyva A (2007) A mutant of the Arabidopsis phosphate transporter PHT1; 1 displays enhanced arsenic accumulation. Plant Cell 19(3): 1123-1133.

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159.

Clemens S, Ma JF (2016) Toxic heavy metal and metalloid accumulation in crop plants and foods. Annu Rev Plant Biol 67: 489-512.

Coniglio A, Mora V, Puente M, Cassán F (2019) *Azospirillum* as biofertilizer for sustainable agriculture: *Azospirillum brasilense* AZ39 as a model of PGPR and field traceability. In: Microbial Probiotics for Agricultural Systems 45-70. Springer, Cham.

Dalton DA, Langeberg L, Treneman NC (1993) Correlations between the ascorbate-glutathione pathway and effectiveness in legume root nodules. Physiol Plant 87(3): 365-370.

Dave R, Tripathi RD, Dwivedi S, Tripathi P, Dixit G, Sharma YK, Chakrabarty D (2013) Arsenate and arsenite exposure modulate antioxidants and amino acids in contrasting arsenic accumulating rice (*Oryza sativa* L.) genotypes. J Hazard Mater 262: 1123-1131.

Deng F, Yu M, Martinoia E, Song WY (2019) Ideal cereals with lower arsenic and cadmium by accurately enhancing vacuolar sequestration capacity. Front Genet 10.

Di Rienzo J, Casanoves F, Balzarina M, Gonzalez L, Tablada M, Robledo C. Infostat versión 2018. Centro de Transferencia Infostat, FCA. Universidad Nacional de Córdoba. Argentina.

Döbereiner J (1988) Isolation and identification of root associated diazotrophs. In: Nitrogen fixation with non-legumes (pp. 103-108). Springer, Dordrecht.

Fagorzi C, Checcucci A, DiCenzo GC, Debiec-Andrzejewska K, Dziewit L, Pini F, Mengoni A (2018) Harnessing rhizobia to improve heavy-metal phytoremediation by legumes. Genes 9(11): 542.

FAOSTAT 2012. Available at http://faostat3.fao.org/faostat-gateway/go/to/home.E (Accessed on 1 June 2014).

FAOSTAT 2016. Available at http://faostat.fao.org/site/339/default.aspx (Accessed 2 May 2019).

Fink B, Laude K, McCann L, Doughan A, Harrison DG, Dikalov S (2004) Detection of intracellular superoxide formation in endothelial cells and intact tissues using dihydroethidium and an HPLC-based assay. Am J Physiol Cell Physiol 287(4): C895-C902.

Finnegan P, Chen W (2012) Arsenic toxicity: the effects on plant metabolism. Front Physiol 3:182.

Flohé L, Günzler WA (1984) Assays of glutathione peroxidase. In: Methods in enzymology. Academic Press 105: 114-120.

Foyer CH, Noctor G (2015) Defining robust redox signaling within the context of the plant cell. Plant Cell Environ 38(2): 239-239.

Frahry G, Schopfer P (2001) NADH-stimulated, cyanide-resistant superoxide production in maize coleoptiles analyzed with a tetrazolium-based assay. Planta 212(2): 175-183.

Gall H, Philippe F, Domon JM, Gillet F, Pelloux J, Rayon C (2015) Cell wall metabolism in response to abiotic stress. Plants 4(1): 112-166.

Ghosh S, Shaw AK, Azahar I, Adhikari S, Jana S, Roy S, Hossain Z (2016) Arsenate (AsV) stress response in maize (*Zea mays* L.). Environ Exp Bot 130: 53-67.

Ghosh S, Singh K, Shaw AK, Azahar I, Adhikari S, Ghosh U, Hossain Z (2017) Insights into the miRNA-mediated response of maize leaf to arsenate stress. Environ Exp Bot 137: 96-109.

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48(12): 909-930.

Gómez-Sagasti MT, Marino D (2015) PGPRs and nitrogen-fixing legumes: a perfect team for efficient Cd phytoremediation? Front Plant Sci 6: 81.

Gupta DK, Inouhe M, Rodríguez-Serrano M, Romero-Puertas MC, Sandalio LM (2013) Oxidative stress and arsenic toxicity: role of NADPH oxidases. Chemosphere 90(6): 1987-1996.

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249(22): 7130-7139.

Hasanuzzaman M, Fujita M (2013) Exogenous sodium nitroprusside alleviates arsenic-induced oxidative stress in wheat (*Triticum aestivum* L.) seedlings by enhancing antioxidant defense and glyoxalase system. Ecotoxicology 22(3): 584-596.

Hasanuzzaman M, Nahar K, Anee TI, Fujita M (2017) Glutathione in plants: biosynthesis and physiological role in environmental stress tolerance. Physiol Mol Biol Plants 23(2): 249-26.

Hasanuzzaman M, Nahar K, Rahman A, Al Mahmud J, Hossain S, Alam K, Oku H, Fujita M (2017) Actions of biological trace elements in plant abiotic stress tolerance. In: Naeem, M., Ansari, A., Gill, S. (Eds.), Essential Plant Nutrients. Springer, Berlin, Germany, 213–274.

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 25(1): 189-198.

Hernández LE, Sobrino-Plata J, Montero-Palmero MB, Carrasco-Gil S, Flores-Cáceres ML, Ortega-Villasante C, Escobar C (2015) Contribution of glutathione to the control of cellular redox homeostasis under toxic metal and metalloid stress. J Exp Bot 66(10): 2901-2911.

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Circular. California agricultural experiment station 347 (2nd edit).

Iqbal S, Bhanger MI (2006) Effect of season and production location on antioxidant activity of *Moringa oleifera* leaves grown in Pakistan. J Food Compost Anal 19(6-7): 544-551.

Islam MR, Chowdhury K, Rahman MM, Rohman MM (2015) Comparative investigation of glutathione S-transferase (GST) in different crops and purification of high active GSTs from onion (*Allium cepa* L.). J Plant Sci 3(3): 162-170.

Johansen DA (1940) Plant microtechique, McGraw-Hill Book Company, Inc., London.

Jozefczak M, Remans T, Vangronsveld J, Cuypers A (2012) Glutathione is a key player in metal-induced oxidative stress defenses. Int J Mol Sci 13(3): 3145-3175.

Ju W, Liu L, Fang L, Cui Y, Duan C, Wu H (2019) Impact of co-inoculation with plant-growth-promoting rhizobacteria and rhizobium on the biochemical responses of alfalfa-soil system in copper contaminated soil. Ecotoxicol Environ Saf 167: 218-226.

Karuppanapandian T, Kim W (2013) Cobalt-induced oxidative stress causes growth inhibition associated with enhanced lipid peroxidation and activates antioxidant responses in Indian mustard (*Brassica juncea* L.) leaves. Acta Physiol Plant 35(8): 2429-2443.

Kumar S, Trivedi PK (2018) Glutathione S-transferases: role in combating abiotic stresses including arsenic detoxification in plants. Front Plant Sci 9.

Lafuente A, Pérez-Palacios P, Doukkali B, Molina- Sánchez MD, Jménez- Zurdo J, Caviedes MA, Pajuelo E (2015) Unraveling the effect of arsenic on the model Medicago–Ensifer interaction: a transcriptomic meta-analysis. New Phytol 205(1): 255 -272.

Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Stadtman ER (1990) Determination of carbonyl content in oxidatively modified proteins, In: Methods in enzymology. 186, 464-478.

Lomax C, Liu WJ, Wu L, Xue K, Xiong J, Zhou J, Zhao FJ (2012) Methylated arsenic species in plants originate from soil microorganisms. New Phytol 193(3): 665-672.

Mac Kinney G (1938) Some absorption spectra of leaf extracts. Plant Physiol 13(1): 123.

Mallick S, Sinam G, Sinha S (2011) Study on arsenate tolerant and sensitive cultivars of *Zea mays* L.: differential detoxification mechanism and effect on nutrients status. Ecotoxicol Environ Saf 74(5): 1316-1324.

Mandal SM, Gouri SS, De D, Das BK, Mondal KC, Pati BR (2011) Effect of arsenic on nodulation and nitrogen fixation of blackgram (*Vigna mungo*). Indian J Microbiol 51(1): 44-47.

Mandal SM, Pati BR, Das AK, Ghosh AK (2008) Characterization of a symbiotically effective Rhizobium resistant to arsenic: Isolated from the root nodules of *Vigna mungo* (L.) Hepper grown in an arsenic-contaminated field. J Gen Appl Microbiol 54(2): 93-99.

Marwa N, Mishra N, Singh N, Mishra A, Saxena G, Pandey V, Singh N (2020) Effect of rhizospheric inoculation of isolated arsenic (As) tolerant strains on growth, As-uptake and bacterial communities in association with *Adiantum capillus-veneris*. Ecotoxicol Environ Saf 196: 110498.

Mascher R, Lippmann B, Holzinger S, Bergmann H (2002) Arsenate toxicity: effects on oxidative stress response molecules and enzymes in red clover plants. Plant Sci 163(5): 961-969.

Mishra S, Dubey RS (2006) Inhibition of ribonuclease and protease activities in arsenic exposed rice seedlings: role of proline as enzyme protectant. J Plant Physiol 163(9): 927-936.

Miteva E (2002) Accumulation and effect of arsenic on tomatoes. Commun Soil Sci Plant Anal 33(11-12): 1917-1926.

Mittler R (2017) ROS are good. Trends Plant Sci 22(1): 11-19.

Mittler R, Vanderauwera S, Suzuki N, Miller GAD, Tognetti VB, Vandepoele K, Van Breusegem F (2011) ROS signaling: the new wave? Trends Plant Sci 16(6): 300-309.

Mokgalaka-Matlala NS, Flores-Tavizon E, Castillo-Michel H, Peralta-Vide JR, Gardea-Torresdey JL (2009) Arsenic tolerance in mesquite (*Prosopis* sp.): Low molecular weight thiols synthesis and glutathione activity in response to arsenic. Plant Physiol Biochem 47(9): 822-826.

Molina R, Rivera D, Mora V, López G, Rosas S, Spaepen S, Cassán F (2018) Regulation of IAA biosynthesis in *Azospirillum brasilense* under environmental stress conditions. Curr microbiol 75(10): 1408-1418.

Mrak T, Jeran Z, Batič F, Di Toppi LS (2010) Arsenic accumulation and thiol status in lichens exposed to As(V) in controlled conditions. Biometals 23(2): 207-219.

Mustafa G, Komatsu S (2016) Toxicity of heavy metals and metal-containing nanoparticles on plants. Biochim Biophys Acta Proteins Proteom 1864(8): 932-944.

Mylona PV, Polidoros AN, Scandalios JG (1998) Modulation of antioxidant responses by arsenic in maize. Free Radic Biol Med 25: 576–585.

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22(5): 867-880.

Nakano Y, Asada K (1987) Purification of ascorbate peroxidase in spinach chloroplasts; its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. Plant Cell Physiol 28(1): 131-140.

Navrot N, Collin V, Gualberto J, Gelhaye E, Hirasawa M, Rey P, Knaff DB, Issakidis E, Jacquot JP, Rouhier N (2006) Plant glutathione peroxidases are functional peroxiredoxins distributed in several subcellular compartments and regulated during biotic and abiotic stresses. Plant Physiol 142: 1364e1379.

Neill SJ, Desikan R, Clarke A, Hurst RD, Hancock JT (2002) Hydrogen peroxide and nitric oxide as signalling molecules in plants. J Exp Bot 53(372): 1237-1247.

Noctor G, Mhamdi A, Chaouch S, Han YI, Neukermans J, Marquez-Garcia B, Foyer CH (2012) Glutathione in plants: an integrated overview. Plant Cell Environ 35(2): 454-484.

Nogales J, Dominguez Ferreras A, Amaya Gomez CV, Van Dillewijn P, Cuellar V, San Juan J, Soto MJ (2010) Transcriptone prolifing of a *Sinorhizobium melilotis* fadD mutant reveals the role of rhizobactin 1021 biosynthesis and regulation genes in the control of swarming. BMC Genomics 8: 157.

Nuss ET, Tanumihardjo SA (2010) Maize: a paramount staple crop in the context of global nutrition. Compr Rev Food Sci Food Saf 9(4): 417-436.

Orozco-Cárdenas M, Ryan CA (1999) Hydrogen peroxide is generated systemically in plant leaves by wounding and system in via the octadecanoid pathway. Proc Natl Acad Sci USA 96(11): 6553-6557.

Pandey C, Raghuram B, Sinha AK, Gupta M (2015) miRNA plays a role in the antagonistic effect of selenium on arsenic stress in rice seedlings. Metallomics 7(5): 857-866.

Panigrahi DP, Sagar A, Dalal S, Randhawa GS (2013) Arsenic resistance and symbiotic efficiencies of alfalfa and cowpea rhizobial strain isolated from arsenic agricultural fields. J Exp Biol 3: 322-333.

Parkhey S, Naithani SC, Keshavkant S (2014) Protein metabolism during natural ageing in desiccating recalcitrant seeds of *Shorea robusta*. Acta Physiol Plant 36(7): 1649-1659.

Peco JD, Campos JA, Romero-Puertas MC, Olmedilla A, Higueras P, Sandalio LM (2020) Characterization of mechanisms involved in tolerance and accumulation of Cd in *Biscutella auriculata* L. Ecotoxicol Environ Saf 201: 110784.

Pedraza RO, Motok J, Salazar SM, Ragout AL, Mentel MI, Tortora ML, Díaz-Ricci JC (2010) Growth-promotion of strawberry plants inoculated with *Azospirillum brasilense*. World J Microbiol Biotechnol 26(2): 265-272.

Peralta JM, Travaglia CN, Gil RA, Furlan A, Castro S, Bianucci EC (2019) An effective rhizoinoculation restraints arsenic translocation in peanut and maize plants exposed to a realistic groundwater metalloid dose. In: Zhu Y, Guo H, Bhattacharya P, Ahmad A, Bundschuh J, Naidu R (Eds.), Environmental Arsenic in a Changing World: Proceedings of the 7th International Congress and Exhibition on Arsenic in the Environment (AS 2018). CRC Press, Beijing, PR China, p. 283.

Peralta JM, Travaglia CN, Romero-Puertas MC, Furlan A, Castro S, Bianucci E (2020) Unraveling the impact of arsenic on the redox response of peanut plants inoculated with two different *Bradyrhizobium* sp. strains. Chemosphere 127410.

Pérez-Chaca MV, Vigliocco A, Reinoso H, Molina A, Abdala G, Zirulnik F, Pedranzani H (2014) Effects of cadmium stress on growth, anatomy and hormone contents in *Glycine max* (L.) *Merr.* Acta Physiol Plant 36(10): 2815-2826.

Perrig D, Boiero ML, Masciarelli OA, Penna C, Ruiz OA, Cassán FD, Luna MV (2007) Plant-growth-promoting compounds produced by two agronomically important strains of *Azospirillum brasilense*, and implications for inoculant formulation. Appl Microbiol Biotech 75(5): 1143-1150.

Ramakrishna B, Rao SSR (2013) Preliminary studies on the involvement of glutathione metabolism and redox status against zinc toxicity in radish seedlings by 28-Homobrassinolide. Environ Exp Bot 96: 52-58.

Reichman SM (2007) The potential use of the legume–rhizobium symbiosis for the remediation of arsenic contaminated sites. Soil Biol Biochem 39(10): 2587-2593.

Requejo R, Tena M (2014) Intra-specific variability in the response of maize to arsenic exposure. Environ Sci Pollut Res 21(18): 10574-10582.

Rodríguez-Serrano M, Romero- Puertas MC, Zabalza A, Corpas FJ, Gomez M, Del Rio LA, Sandalio LM (2006) Cadmium effect on oxidative metabolism of pea (*Pisum sativum* L.) roots. Imaging of reactive oxygen species and nitric oxide accumulation in vivo. Plant Cell Environ 29(8): 1532-1544.

Romero-Puertas MC, Palma JM, Gómez M, Del Rio LA, Sandalio LM (2002) Cadmium causes the oxidative modification of proteins in pea plants. Plant Cell Environ 25(5): 677-686.

Sagi M, Fluhr R (2001) Superoxide production by plant homologues of the gp91phox NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection. Plant Physiol 126(3): 1281-1290.

Saidi I, Yousfi N, Borgi MA (2017) Salicylic acid improves the antioxidant ability against arsenic-induced oxidative stress in sunflower (*Helianthus annuus*) seedling. J Plant Nutr 40(16): 2326-2335.

Sandalio LM, Rodríguez-Serrano M, Gupta DK, Archilla A, Romero-Puertas MC, Luis A (2012) Reactive oxygen species and nitric oxide in plants under cadmium stress: from toxicity to signaling. In: Environmental adaptations and stress tolerance of plants in the era of climate change. Springer, New York, NY, 199-215.

Sandalio LM, Rodríguez-Serrano M, Romero- Puertas MC, Luis A (2008) Imaging of reactive oxygen species and nitric oxide in vivo in plant tissues. Methods in Enzymology 440: 397-409.

Scandalios JG (2002) The rise of ROS. Trends Biochem Sci 27(9): 483-486.

Schikora A, Schmidt W (2001) Acclimative changes in root epidermal cell fate in response to Fe and P deficiency: a specific role for auxin? Protoplasma. 218(1-2): 67-75.

Shahid M, Dumat C, Khalid S, Schreck E, Xiong T, Niazi NK (2017) Foliar heavy metal uptake, toxicity and detoxification in plants: A comparison of foliar and root metal uptake. J Hazard Mater 325: 36-58.

Shaibur MR, Kitajima N, Huq SI, Kawai S (2009) Arsenic–iron interaction: Effect of additional iron on arsenic-induced chlorosis in barley grown in water culture. Soil Sci Plant Nutr 55(6): 739-746.

Sharma I (2012) Arsenic induced oxidative stress in plants. Biologia 67(3): 447-453.

Sharma SS, Dietz KJ (2009) The relationship between metal toxicity and cellular redox imbalance. Trends Plant Sci 14(1): 43-50.

Sheadle M, Bassham J (1977) Chloroplast glutathione reductase. Plant Physiol 59:1011–1012.

Sies H, Berndt C, Jones DP (2017) Oxidative stress. Annu Rev Biochem 86: 715-748.

Singh AP, Dixit G, Kumar A, Mishra S, Kumar N, Dixit S, Singh PK, Dwivedi S, Trivedi PK, Pandey V (2017a) A protective role for nitric oxide and salicylic acid for arsenite phytotoxicity in rice (*Oryza sativ*a L.). Plant Physiol Biochem 115: 163–173.

Singh HP, Batish DR, Kohli RK, Arora K (2007) Arsenic-induced root growth inhibition in mung bean (*Phaseolus aureus* Roxb.) is due to oxidative stress resulting from enhanced lipid peroxidation. Plant Growth Regul 53(1): 65-73.

Singh R, Singh S, Parihar P, Mishra RK, Tripathi DK, Singh VP, Prasad SM (2016) Reactive oxygen species (ROS): beneficial companions of plants' developmental processes. Front Plant Sci 7: 1299.

Singh S, Sounderajan S, Kumar K, Fulzele DP (2017b) Investigation of arsenic accumulation and biochemical response of in vitro developed *Vetiveria zizanoides* plants. Ecotoxicol Environ Saf 145: 50-56.

Sinha K, Das J, Pal PB, Sil PC (2013) Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. Arch Toxicol 87(7): 1157-1180.

Sistema Nacional Argentino de Vigilancia y Monitoreo de Plagas 2019. Available at https://www.sinavimo.gov.ar/cultivo/zea-mays (Accessed October 2020).

Smedley PL, Kinniburgh DG (2002) A review of the source, behavior and distribution of arsenic in natural waters. Appl Geochem 17: 517-568.

Sobrino-Plata J, Herrero J, Carrasco-Gil S, Pérez-Sanz A, Lobo C, Escobar C, Hernández LE (2013) Specific stress responses to cadmium, arsenic and mercury appear in the metallophyte *Silene vulgaris* when grown hydroponically. RSC advances 3(14): 4736-4744.

Sobrino-Plata J, Ortega-Villasante C, Flores-Cáceres ML, Escobar C, Del Campo FF, Hernández LE (2009) Differential alterations of antioxidant defenses as bioindicators of mercury and cadmium toxicity in alfalfa. Chemosphere 77(7): 946-954.

Somasegaran P, Hoben HJ (1994) Quantifying the growth of rhizobia, in: Handbook for Rhizobia. Springer, New York 47-57.

Souri Z, Karimi N, Sandalio LM (2017a) Arsenic hyperaccumulation strategies: an overview. Front Cell Dev Biol 5: 67.

Souri Z, Karimi N, Sarmadi M, Rostami E (2017b) Salicylic acid nanoparticles (SANPs) improve growth and phytoremediation efficiency of *Isatis cappadocica* Desv., under As stress. IET Nanobiotechnology 11(6): 650-655.

Srivastava S, D'souza SF (2010) Effect of variable sulfur supply on arsenic tolerance and antioxidant responses in *Hydrilla verticillata* (Lf) Royle. Ecotoxicol Environ Saf 73(6): 1314-1322.

Stoeva N, Berova M, Zlatev Z (2003) Physiological response of maize to arsenic contamination. Biol Plantarum 47(3): 449-452.

Strzałka K, Kostecka-Gugała A, Latowski D (2003) Carotenoids and environmental stress in plants: significance of carotenoid-mediated modulation of membrane physical properties. Russ J Plant Physiol 50(2): 168-173.

Sujkowska-Rybkowska M, Borucki W (2015) Pectins esterification in the apoplast of aluminum-treated pea root nodules. J Plant Physiol 184: 1-7.

Sujkowska-Rybkowska M, Ważny R (2018) Metal resistant rhizobia and ultrastructure of *Anthyllis vulneraria* nodules from zinc and lead contaminated tailing in Poland. Int J Phytoremediation 20(7): 709-720.

Suneja Y (2014) Physio-biochemical responses and allelic diversity for water deficit tolerance related traits in *Aegilops tauschii* and *Triticum dicoccoides*. Ph.D. Thesis, Punjab Agricultural University, Ludhiana, India.

Sushant KS, Ghosh AK (2010) Effect of Arsenic on Photosynthesis, Growth and its Accumulation in the Tissues of *Allium cepa* (Onion). Environ Eng Manag J 1(1): 39-50.

Talukdar D (2013) Arsenic-induced changes in growth and antioxidant metabolism of fenugreek. Russ J Plant Physiol 60(5): 652-660.

Tarpey MM, Wink DA, Grisham MB (2004) Methods for detection of reactive metabolites of oxygen and nitrogen: *in vitro* and *in vivo* considerations. Am J Physiol Regul Integr Comp Physiol 286(3): R431-R444.

Tiwari S, Sarangi BK (2017) Comparative analysis of antioxidant response by *Pteris vittata* and *Vetiveria zizanioides* towards arsenic stress. Ecol Eng 100: 211-218.

Travaglia C, Balboa G, Espósito G, Reinoso H (2012) ABA action on the production and redistribution of field-grown maize carbohydrates in semiarid regions. Plant Growth Regul 67(1): 27-34.

Tripathi RD, Tripathi P, Dwivedi S, Dubey S, Chakrabarty D (2012) Arsenomics: omics of arsenic metabolism in plants. Front Physiol 3: 275.

Upadhyay MK, Yadav P, Shukla A, Srivastava S (2018) Utilizing the potential of microorganisms for managing arsenic contamination: A feasible and sustainable approach. Front Environ Sci 6: 24.

Verbruggen N, Hermans C, Schat H (2009) Mechanisms to cope with arsenic or cadmium excess in plants. Curr Opin Plant Biol 12(3): 364-372.

Vincent JM (1970) A manual for the practical study of the root-nodule bacteria. A manual for the practical study of the root-nodule bacteria.

Xu Q, Pan W, Zhang R, Lu Q, Xue W, Wu C, Du S (2018) Inoculation with *Bacillus subtilis* and *Azospirillum brasilense* produces abscisic acid that reduces Irt1-mediated cadmium uptake of roots. J Agr Food Chem 66(20): 5229-5236.

Yadav V, Arif N, Kováč J, Sing VP, Tripathi DK, Chauhan DK, Vaculík M (2020) Structural modifications of plant organs and tissues by metals and metalloids in the environment: a review. Plant Phys Biochem.

Yang HC, Rosen BP (2016) New mechanisms of bacterial arsenic resistance. Biomed J 39(1): 5-13.

You J, Chan Z (2015) ROS regulation during abiotic stress responses in crop plants. Front Plant Sci 6: 1092.

Zhao FJ, Ma JF, Meharg AA, McGrath SP (2009) Arsenic uptake and metabolism in plants. New Phytol 181(4): 777-794.

Figure legends

Figure 1. Bacterial viability at different arsenate concentrations. (A) *Azospirillum brasilense* Az39, (B) *Azospirillum brasilense* CD. Data represent the means ± SE of three independent experiments.

Figure 2. Effect of arsenate on the morphology of inoculated maize roots. Cross sections of adventitious maize root in control treatment (A and B) and exposed to 3 µM As^V (C and D), inoculated with *Azospirillum brasilense* Az39 (A, C) or *Azospirillum brasilense* CD (B, D). Abbreviations: c, cortex; cc, central cylinder; en, endodermis; ep, epidermis; ex, exodermis. Magnification: 10X. Effect of arsenate on total cross section area (E) and central cylinder area (F). Data represent the mean ± SE (n = 10). Different letters indicate significant differences between *A. brasilense* strains for the same As^V dose. Different numbers indicate significant differences between As^V doses for each inoculated strain ($P < 0.05$) according to Duncan's test.

Figure 3. Effect of arsenate on NADPH oxidase activity in leaves (A) and roots (B) of maize plants. Data represent the mean ± SE (n = 7). Different letters indicate significant differences between *A. brasilense* strains for the same As^V dose. Different numbers indicate significant differences between As^V doses for each inoculated strain ($P < 0.05$) according to Duncan´s test.

Figure 4. Detection of hydrogen peroxide (H_2O_2) in maize plants. (A) Representative images of H_2O_2- DCF-DA dependent fluorescence in maize roots devoid of As^V or exposed to 3 µM As^V. As negative control the roots were incubated in 1 mM ascorbate, a H_2O_2 scavenger. Magnification: 5X. (B) Distribution of H_2O_2 in maize leaf blade from plants devoid of As^V and treated with 3 µM As^V. The arrows indicate the dark deposits resulting from the reaction of H_2O_2 with DAB. H_2O_2 content in maize leaves (C) and roots (D). Data represent the mean ± SE (n = 10). Different letters indicate significant differences between *A. brasilense* strains for the same As^V dose. Different numbers indicate significant differences between As^V doses for each inoculated strain ($P < 0.05$) according to Duncan´s test.

Figure 5. Superoxide radical ($O_2^{\bullet -}$) production in maize plants. (A) Histochemical detection of $O_2^{\bullet -}$ by fluorescence in an adventitious root of maize plants. Representative fluorescence images of $O_2^{\bullet -}$ DHE dependent staining are shown in plants devoid of As^V or in the presence of 3 µM As^V. As a negative control, the roots were incubated with TMP 1 mM ($O_2^{\bullet -}$ scavenger). (B) Distribution of $O_2^{\bullet -}$in maize leaf blade from plants devoid of As^V and treated with 3 µM As^V. The arrows indicate reduced formazan blue deposits caused by the reduction of NBT with $O_2^{\bullet -}$. (C) Quantification of $O_2^{\bullet -}$ fluorescence intensity (F. Int.) from images in arbitrary units (a.u). Magnification: 5X. Data represent the mean ± SE (n = 10). Different letters indicate significant differences between *A. brasilense* strains for the same As^V dose. Different numbers indicate significant differences between As^V doses for each inoculated strain ($P < 0.05$) according to Duncan´s test.

Figure 6. Oxidative stress induced due to arsenic in inoculated maize plants. Lipid peroxidation and carbonyl groups content in leaves (A, B) and roots (C, D) of maize plants. Data represent the mean ± SE (n = 10). Different letters indicate significant differences between *A. brasilense* strains for the same As^V dose. Different numbers indicate significant differences between As^V doses for each inoculated strain ($P < 0.05$) according to Duncan´s test.

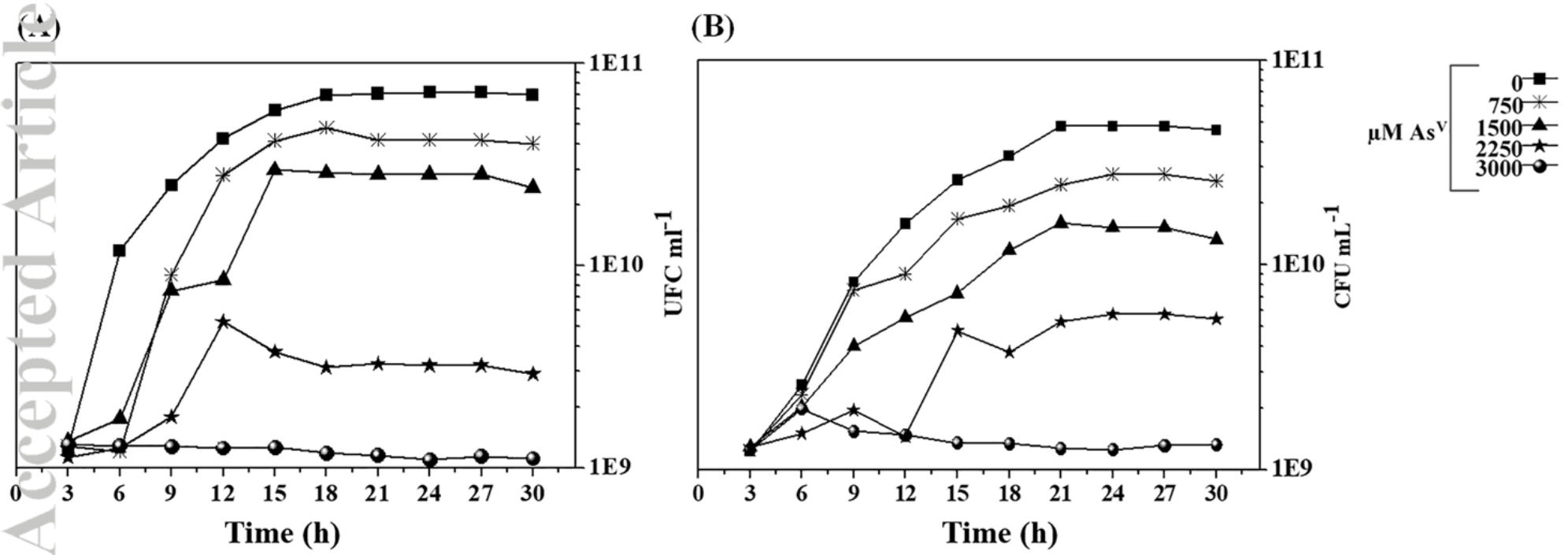
Figure 7. Arsenate effect on antioxidative enzymes in roots of maize plants inoculated. Activities of (A) SOD, (B) CAT, (C) GST, (D) APX, (E) GR, (F) GPX/PRX, (G) MDHAR and (H) DHAR. Analysis of mRNA relative expression of *GR* (I), *APX* (J) and *GST* (K) by qRT-PCR. Each bar represents the mean ± SE (n = 7). Different letters indicate significant differences between *A. brasilense* strains for the same As^V dose. Different numbers indicate significant differences between As^V doses for each inoculated strain ($P < 0.05$) according to Duncan's test.

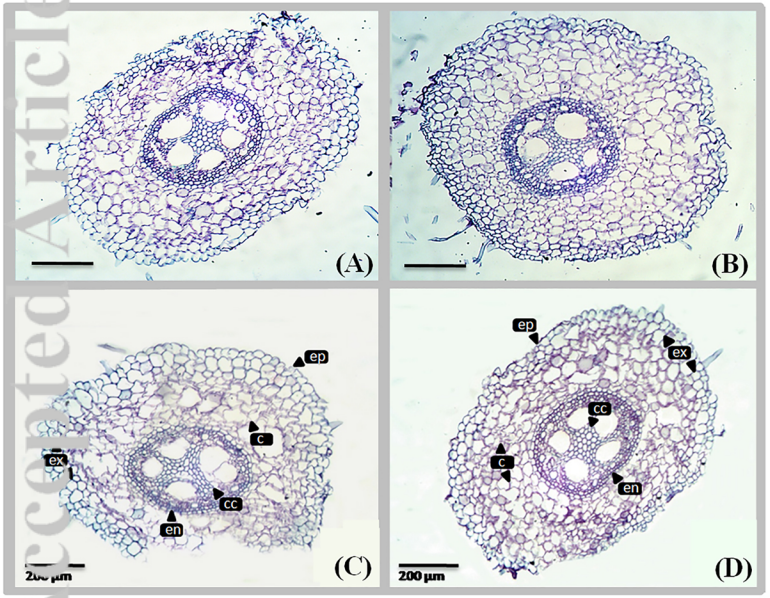
Figure 8. Proposed response model for maize-*A. brasilense* interaction exposed to a realistic As^V dose. Abbreviations: Ct, Total chlorophyll; Ca, Chlorophyll *a*; Cb, Chlorophyll *b*; NADPHox, NADPH oxidase; SOD, Superoxide dismutase; CAT, Catalase; GST, Glutathione S-transferase; APX, Ascorbate peroxidase; GR, Glutathione reductase; GPX / PRX, Glutathione peroxidase/peroxiredoxin; MDHAR, Monodehydroascorbate reductase; DHAR, Dehydroascorbate reductase; GSH_T, Total Glutathione; GSH_R, Reduced Glutathione; GSSG, Oxidized Glutathione; TBARS, Thiobarbituric acid reactive substances; C = O, free carbonyl groups.

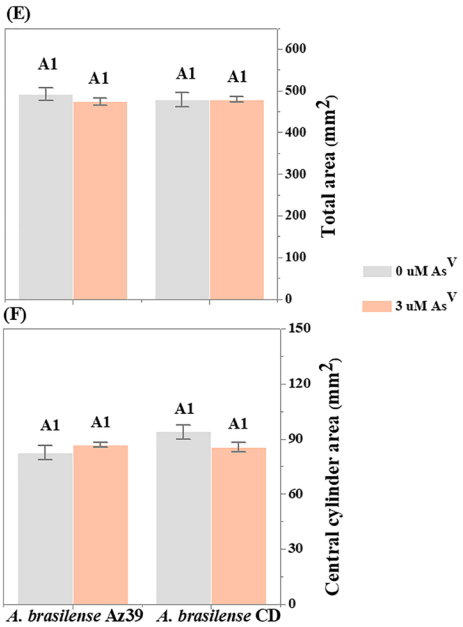
Supplementary material

Figure S1. Quantification of H_2O_2 fluorescence intensity (F. Int.) from images in arbitrary units (a.u.).

Figure S2. Relative concentration of GSH and GSSG species in inoculated maize plant roots exposed to As^V.

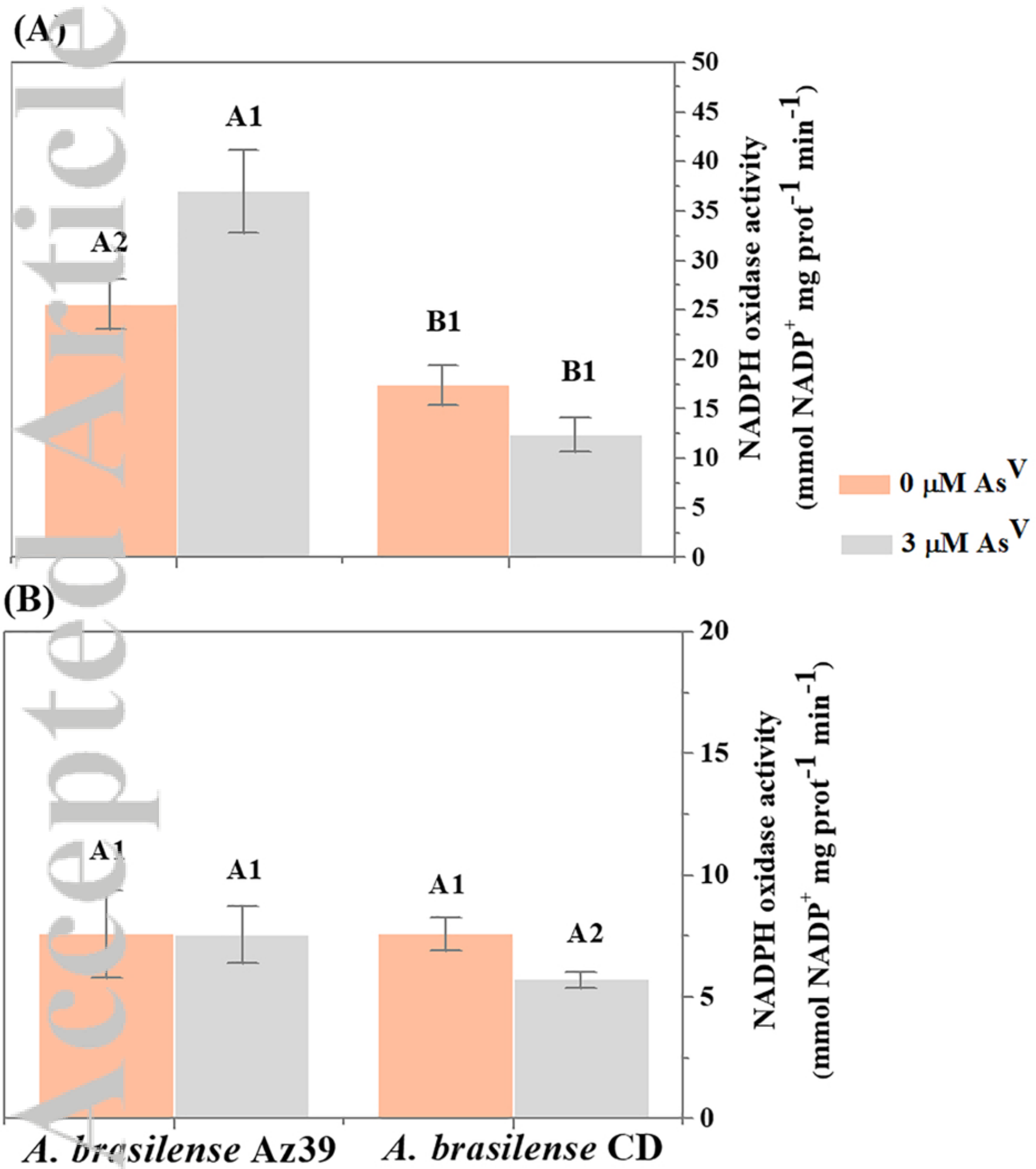




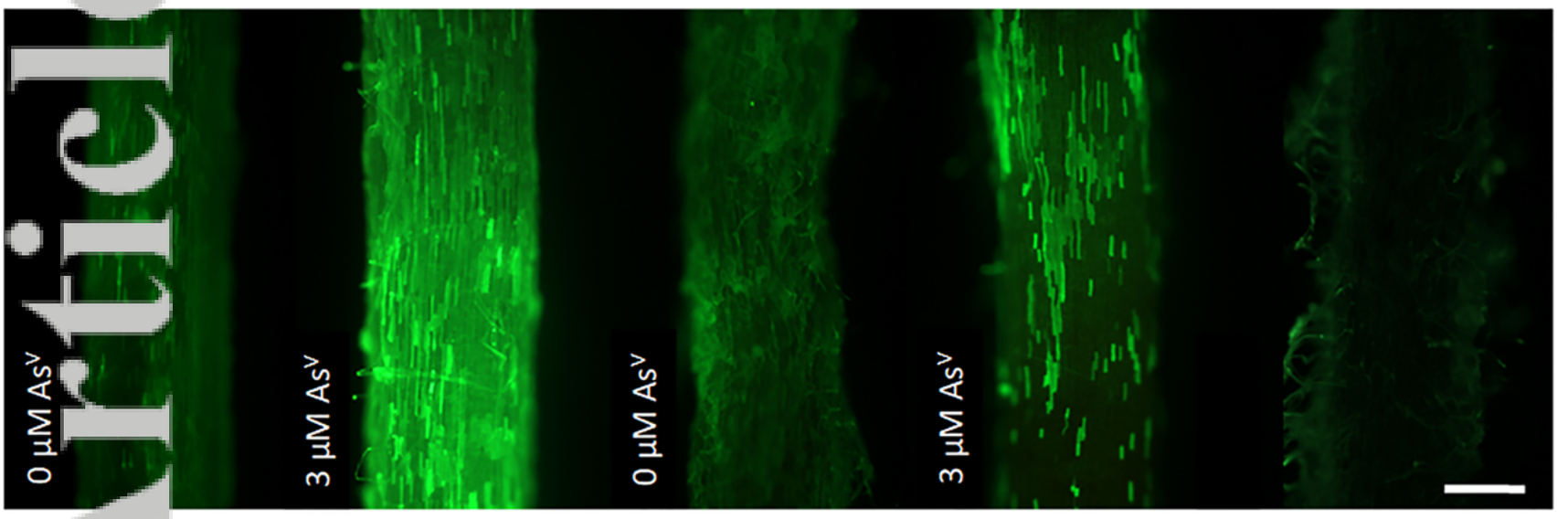


A. brasilense Az39

A. brasilense CD



(A)

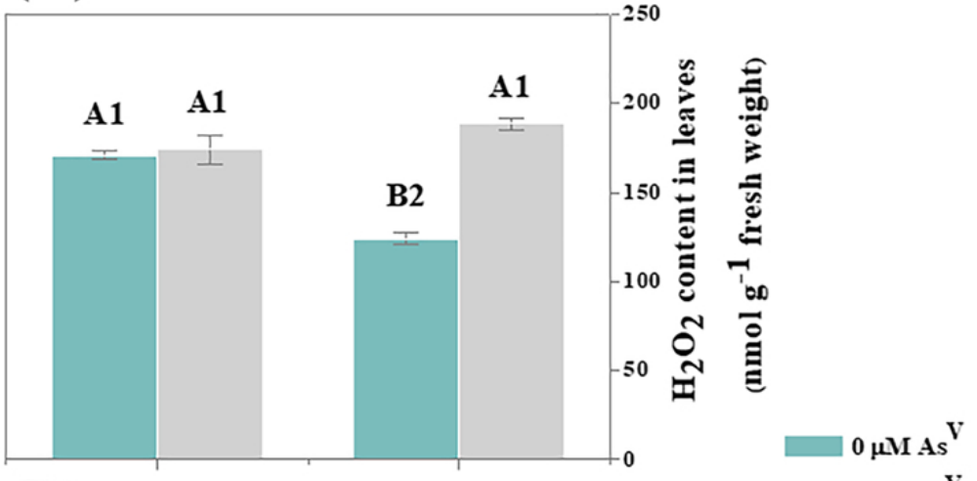


A. brasilense Az39 *A. brasilense* CD Control

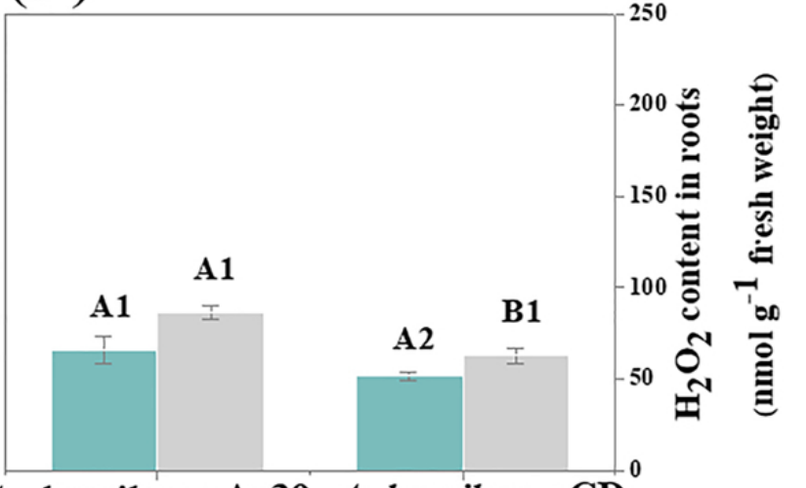
(B)

A. brasilense Az39 *A. brasilense* CD

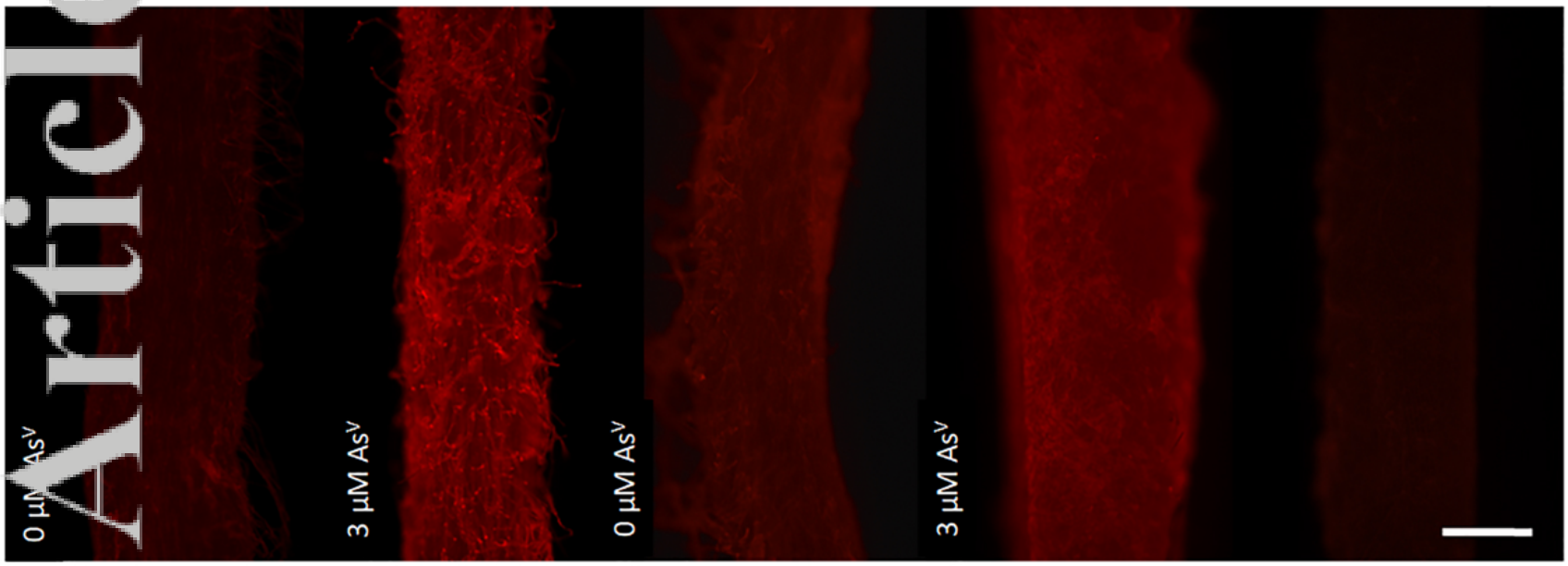
(C)



(D)



(A)

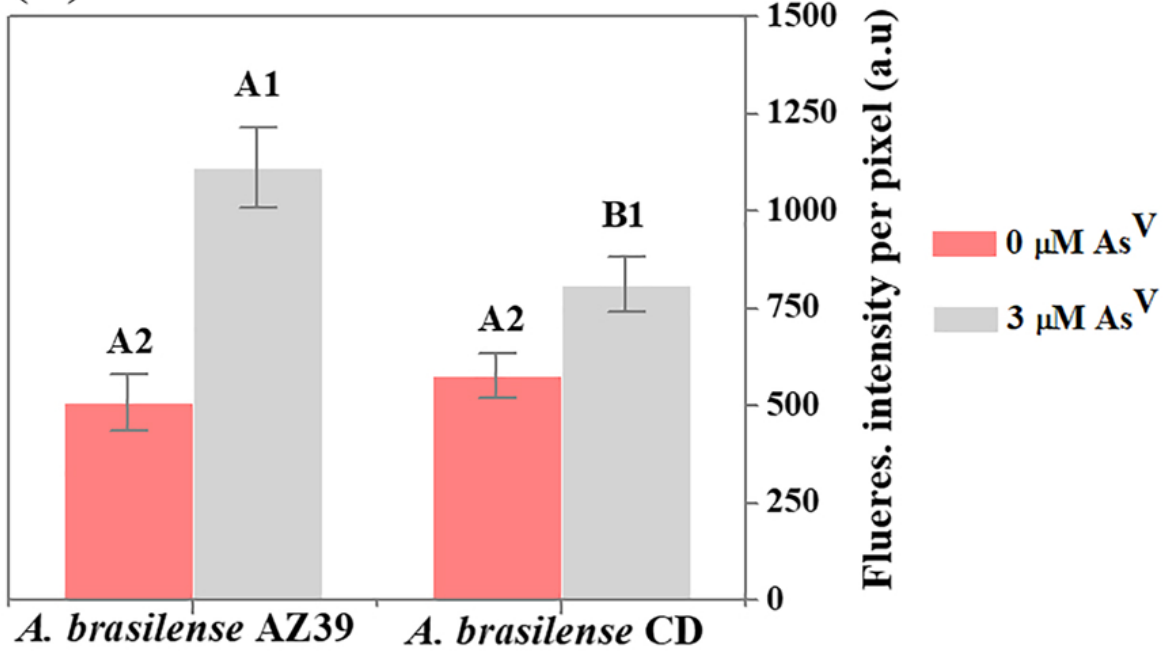


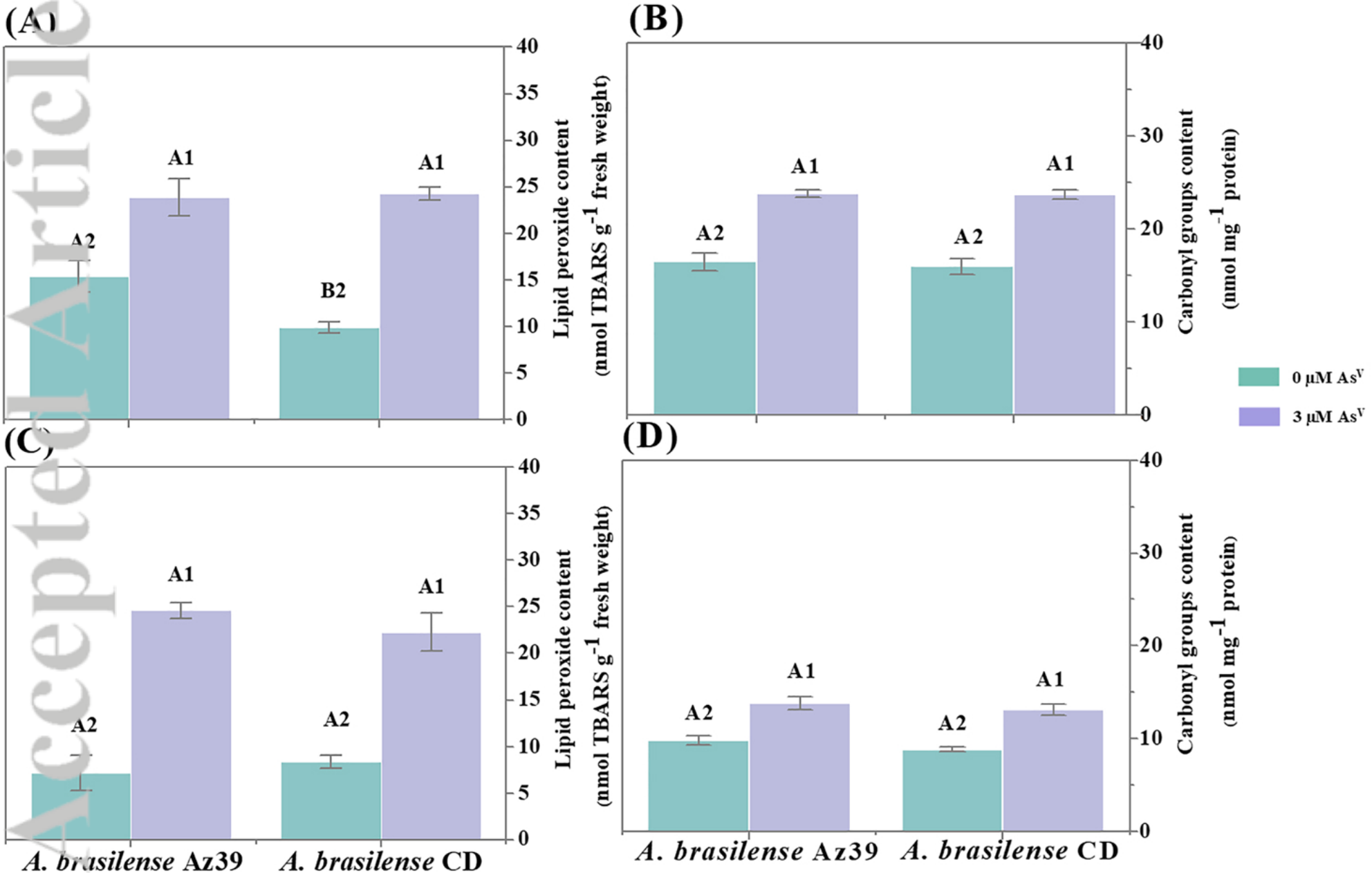
A. brasilense Az39 | *A. brasilense* CD | Control

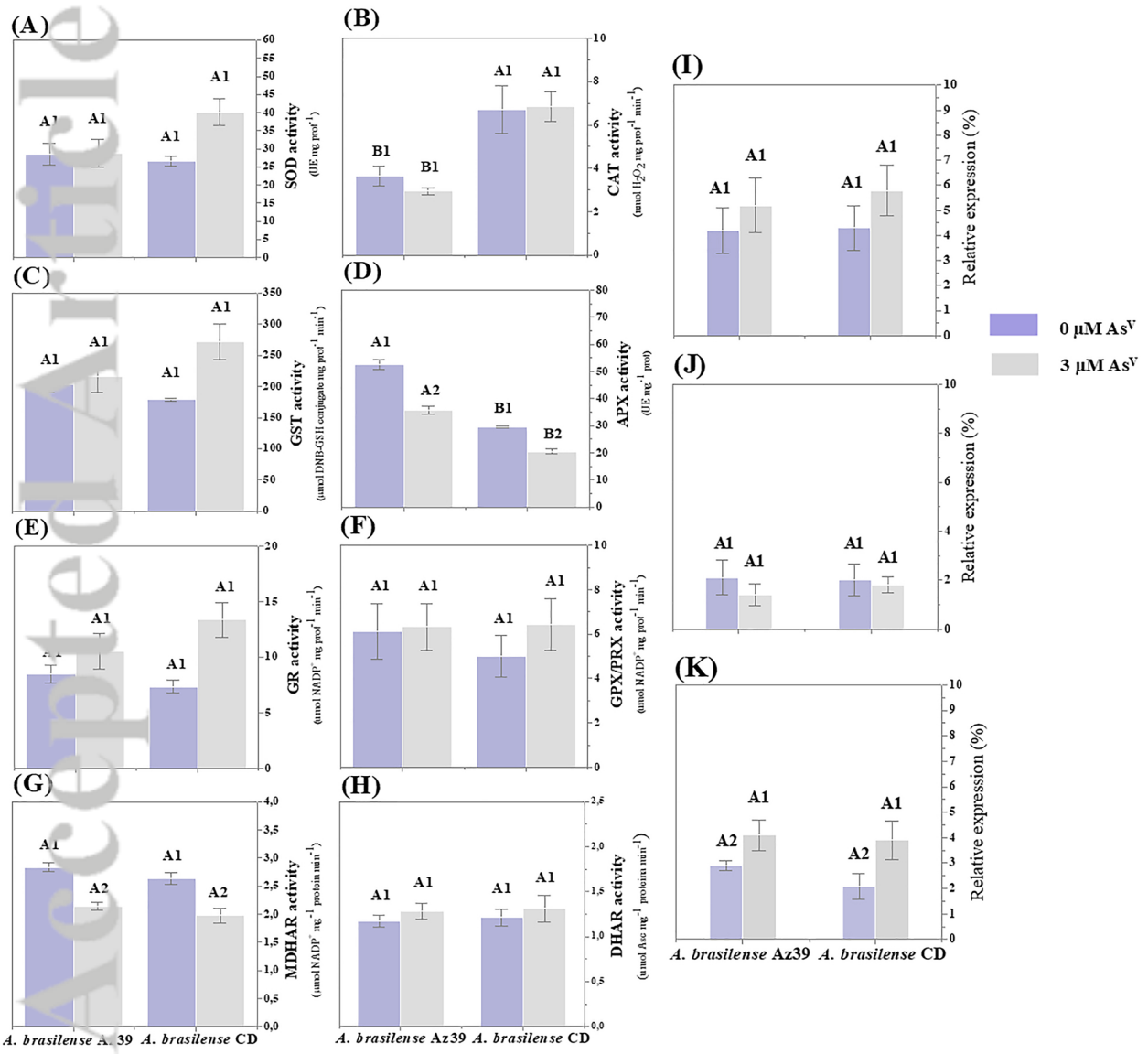
(B)

A. brasilense Az39 | *A. brasilense* CD

(C)






(A)
SOD activity
(UE mg prot-1)
(B)
CAT activity
(umol H2O2 mg prot-1 min-1)
(C)
GST activity
(umol DNB-GSH conjugate mg prot-1 min-1)
(D)
APX activity
(UE mg-1 prot)
(E)
GR activity
(umol NADP+ mg prot-1 min-1)
(F)
GPX/PRX activity
(umol NADP+ mg prot-1 min-1)
(G)
MDHAR activity
(umol NADP+ mg-1 protein min-1)
(H)
DHAR activity
(umol Asc mg-1 protein min-1)
(I)
(J)
(K)
Relative expression (%)
0 µM AsV
3 µM AsV
A. brasilense Az39
A. brasilense CD

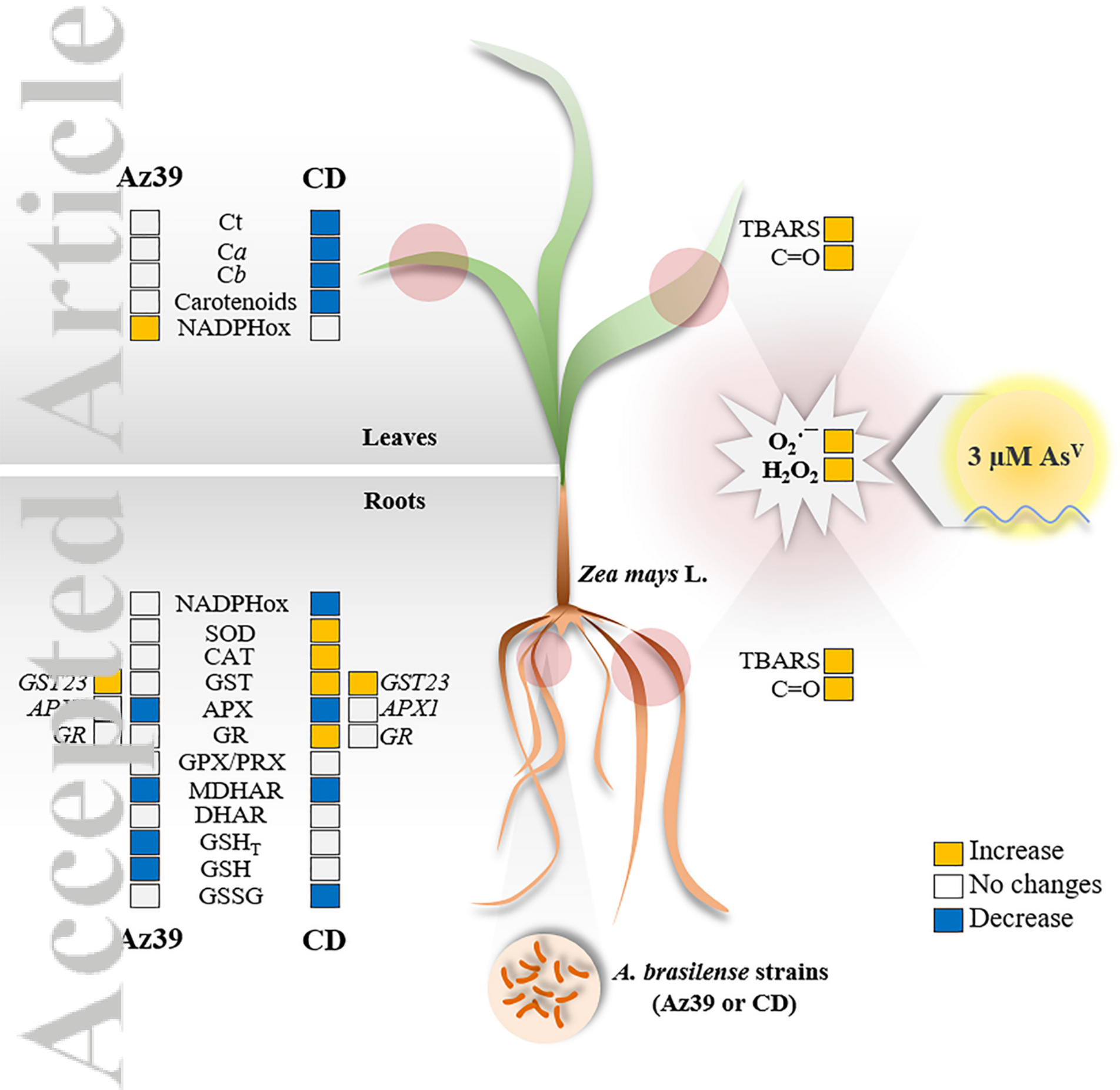


Table 1. Photosynthetic pigment contents in maize plants exposed to As^V.

Azospirillum brasilense strains	Photosynthetic pigments content (mg g^{-1} dry weight)							
	Total Chlorophyll		Chlorophyll *a*		Chlorophyll *b*		Carotenoids	
	0 µM As^V	3 µM As^V	0 µM As^V	3 µM As^V	0 µM As^V	3 µM As^V	0 µM As^V	3 µM As^V
Az39	1.30 ± 0.17^{A1}	1.10 ± 0.19^{B1}	0.92 ± 0.11^{A1}	0.78 ± 0.13^{B1}	0.38 ± 0.05^{A1}	0.33 ± 0.06^{B1}	$2.1\ E^{-3} \pm 2.6\ E^{-4}$ A1	$1.8\ E^{-3} \pm 2.7\ E^{-4}$ A1
CD	1.23 ± 0.09 A1	1.06 ± 0.05 B2	0.88 ± 0.06 A1	0.74 ± 0.02 B2	0.33 ± 0.03 A1	0.33 ± 0.03 B1	$1.9\ E^{-3} \pm 1.3\ E^{-4}$ A1	$1.3\ E^{-3} \pm 2.3\ E^{-4}$ B2

Data represent the mean ± SE (n = 7). Different letters on each column indicate significant differences between strains for each treatment and different numbers on each row indicate significant differences between treatment for each strain according to the Duncan`s test ($P < 0.05$)

Table 2. Effect of As^V on glutathione content in maize roots.

	GSH_T (nmol g^{-1} FW)		GSH (nmol g^{-1} FW)		GSSG (nmol g^{-1} FW)		GSH/(GSH+GSSG)	
Azospirillum brasilense strains	0 µM As^V	3 µM As^V	0 µM As^V	3 µM As^V	0 µM As^V	3 µM As^V	0 µM As^V	3 µM As^V
Az39	149.88 ± 16.23^{A1}	89.55 ± 6.5^{B2}	144.90 ± 16.23^{A1}	83.96 ± 6.21^{B2}	4.98 ± 0.01^{B1}	5.47 ± 0.50^{A1}	0.96 ± 0.01^{A1}	0.94 ± 0.01^{A1}
CD	151.24 ± 8.08^{A1}	133.71 ± 4.23^{A1}	$144,28 \pm 7.46^{A1}$	133.83 ± 6.83^{A1}	8.08 ± 0.62^{A1}	5.46 ± 0.48^{A2}	0.95 ± 0.01^{A1}	0.96 ± 0.01^{A1}

Data represent the mean ± SE (n = 7). Different letters on each column indicate significant differences between strains for each treatment and different numbers on each row indicate significant differences between treatment for each strain according to the Duncan`s test ($P < 0.05$). GSH_T: glutathione total; GSH: glutathione reduced; GSSG: glutathione disulfide; FW: fresh weight.