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Arsenite and arsenate stress differently affect auxin distribution in rice roots and brassinosteroids restore it sustaining root system plasticity

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ABSTRACT

Rice is a worldwide cultivated crop that serves as an important source of food for the human population, but it is also the simplest route for arsenic (As) contamination of the food chain. The As inorganic forms, arsenate [$As^{(V)}$] and arsenite [$As^{(III)}$], are the highly toxic As species found in the soil and the most easily absorbed by the roots. The absorption of $As^{(V)}$ prevails in aerobic soils while that of $As^{(III)}$ is favored in anaerobic soils. $As^{(V)}$ is converted to $As^{(III)}$ in the roots, although small amounts of $As^{(V)}$ also remain in the plant organs. The root system is the first target of the action of both As forms. The mechanisms of action of $As^{(V)}$ and $As^{(III)}$ are still widely unknown. Understanding them is essential for selecting rice genotypes with a lower capacity of As uptake and transport to the caryopses, thus improving food safety. Auxin is the phytohormone necessary for the development and plasticity of the root system, and its action is modulated by endogenous/exogenous brassinosteroids (BRs), mainly under stress conditions. The research aim was to deepen the knowledge of the mechanisms triggered by $As^{(III)}$ or $As^{(V)}$ in rice roots with particular attention to the role played by the interaction between auxin transport and BRs. We show that $As^{(III)}$ is the main As species present in rice roots regardless of the $As^{(III)}$ or $As^{(V)}$ forms supplied to the growing medium. Arsenic alters auxin distribution in both adventitious and lateral roots, but strongly in the latter ones. The application of an exogenous BR, the 24-epibrassinolide (eBL), combined with $As^{(III)}$ or $As^{(V)}$ strongly increases the expression of the *OsPIN2* and *OsAUX1* genes involved in auxin transport, thus contributing to restore the correct auxin distribution altered by As, and mainly by $As^{(III)}$, with higher effects on the LRs. Moreover, eBL increases the antioxidant activity in the roots in the presence of As, but only when combined with $As^{(V)}$.

1. Introduction

Rice production in the world is threatened by the presence, in rice paddies, of high concentrations, often far above the permitted levels by law, of the carcinogenic metalloid arsenic (As). Rice is a staple food of a large part of humanity and the presence of As is endangering the health of the world population, especially of people with the lowest economic income. Many studies have been performed to understand the mechanisms of action of As toxicity in various crops, particularly in rice. Progress has been made to shed light on the uptake and transport mechanisms of As, on the damage that the metalloid causes to the cells and the whole plant, and on how the plant counteracts the metalloid toxicity (Geng et al 2023 and references therein; Piacentini et al. 2020a, 2020b, 2020c; Ronzan et al. 2018). However, it is important to further our understanding of the regulatory mechanisms underlying As detoxification in rice. This knowledge is crucial for the selection of rice genotypes that exhibit greater tolerance to metalloid toxicity, lower As accumulation in the aerial organs, and improvement of both quality and food safety of the caryopses. The As absorbed by the roots causes serious damages to the growth and productivity of the plant with most of the damage resulting from oxidative stress associated with overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Piacentini et al., 2020c).

Arsenic is present in soil and water in inorganic and organic forms. The inorganic forms arsenate [$As^{(V)}$] and arsenite [$As^{(III)}$] are highly toxic and the most likely to be absorbed by the roots (Finnegan and

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Chen, 2012). In environments with a reduced presence of oxygen (anoxic environments), such as stagnant aquatic environments or soils where water persists for an extended period (paddy fields) the redox condition (reduced environment) favors the presence of $As^{(III)}$, whereas the $As^{(V)}$ form predominates in aerobic conditions (Wu et al., 2017). However, the As speciation in paddy fields, and mainly in the rice rhizosphere, is complex and is subjected to strong spatio-temporal variations (Stroud et al., 2011). Higher oxygen levels around the roots induce the oxidation of $As^{(III)}$ to $As^{(V)}$ and the iron plaque, that characterizes rice roots, provides a strong store for $As^{(V)}$ (Liu et al., 2006). There is evidence that $As^{(III)}$ is more toxic than $As^{(V)}$ (Ashraf et al., 2019; Coelho et al., 2020; Piacentini et al., 2020c) and the latter is easily converted to $As^{(III)}$ when absorbed by the root cells (Abbas et al., 2018). However, an amount of $As^{(V)}$ may remain in the root cells and be transported to the aerial organs (Geng et al., 2023). The greater toxicity of $As^{(III)}$ in comparison with $As^{(V)}$ is due to its ability to bind to the sulfhydryl groups of proteins, resulting in the disruption of redox processes and cell metabolism (Shen et al., 2013). Furthermore, $As^{(III)}$ uses proteins of the aquaglyceroporin family to enter cells (Abbas et al., 2018). These proteins are highly abundant in both the plasma membrane and the tonoplast, facilitating the extensive presence of this As form in different cell compartments. In fact, five aquaporin subfamilies have been identified in plant cells and three of them, namely nodulin 26-like intrinsic proteins (NIPs), plasma membrane intrinsic proteins (PIPs), and tonoplast intrinsic proteins (TIPs) have been shown to be involved in $As^{(III)}$ translocation into plant cells and vacuoles (Maciaszczyk-Dziubinska et al., 2012). Many aquaporins have bidirectional cellular transport properties for $As^{(III)}$, thus their action may facilitate and speed up the cellular influx and efflux of this As form (Xu et al., 2015).

The toxicity of $As^{(V)}$ is due to its similarity with the inorganic phosphate, and consequently to the competition for phosphate anion transporters, and to the ability to replace phosphate in some biochemical reactions such as in the ATP synthesis, forming unstable ADP-As molecules that interrupt energy flows (Huang and Mitchell, 1972). In Arabidopsis, the phosphate transporters AtPHT1;1 and AtPHT1;4 are involved in taking up $As^{(V)}$ from the environment (Shin et al., 2004). Similarly, in rice, OsPT1, OsPT4, and OsPT8 are involved in $As^{(V)}$ uptake and transport (Ye et al., 2017, and references therein).

However, both the As inorganic forms coexist in the soils and in the plants and are harmful to plant metabolism and development probably through different mechanisms (Sinha et al., 2023). Therefore, it is important to deepen the studies on the mechanisms of action of two highly toxic species of As to shed full light on the damage caused during the growth and development phases of the plants and their defense strategies.

In particular, the root is the organ primarily affected by soil toxicity and the one in which the first damage and the first defense responses occur. While it is well established that As is responsible for the alteration of the root architecture in many plants, including Arabidopsis and rice (Fattorini et al., 2017; Kumar et al., 2020; Piacentini et al., 2020a; Ronzan et al., 2018), it remains unclear how the two different forms of inorganic As affect the root system development. Understanding this is crucial because the root system modulates its growth in the presence of $As^{(III)}$ and $As^{(V)}$ with implications for variety/hybrid selection in agronomically important plants such as rice.

We previously demonstrated that As negatively impacts the synthesis and transport of the phytohormone auxin with different effects on different typologies of roots, e.g. primary root, lateral roots (LRs), adventitious roots (ARs), in Arabidopsis and in rice, despite their differences in root system architecture (Fattorini et al., 2017; Piacentini et al., 2020b; Ronzan et al., 2018). Moreover, in Arabidopsis, it was also demonstrated that $As^{(III)}$ uses the PIN FORMED 2 (PIN2) auxin efflux transporter to move from cell to cell (Ashraf et al., 2020). Auxin, the main plant phytohormone, regulates the morphological, biochemical, and physiological response of the plant. In particular, root formation and development are strictly controlled by auxin (Della Rovere et al., 2013), and auxin counteracts As toxicity in Arabidopsis (Krishnamurthy and Rathinasabapathi, 2013). However, in the case of rice, the latter aspect needs to be better studied as it could represent one of the crucial strategies to reduce the stress induced by As, especially by its two highly toxic inorganic species, and the yield loss of this agronomically important plant.

As previously mentioned, As causes cellular nitro-oxidative stress resulting in the overproduction of ROS and RNS. It has been demonstrated that treatments with exogenous phytohormones (i.e., jasmonates, auxin, brassinosteroids, abscisic acid) reduce the metalloid-induced oxidative burst in numerous plants, including *Oryza sativa, Arabidopsis thaliana*, and *Solanum lycopersicum* (Nahar et al., 2022 and references therein; Singh et al., 2021). Auxin modulates the metalloid-induced stress by controlling RNS/ROS production in various intracellular organelles of the root cells, including mitochondria, plastids, peroxisomes, and in the cytoplasm (Kolbert et al., 2018; Krishnamurthy and Rathinasabapathi, 2013; Parveen et al., 2022; Piacentini et al., 2020a). Thus, auxin, a key hormone for plant growth and development and modulator of plant defense responses is, at the same time, a target of As toxicity.

Proper plant growth and development are the result of complex, and finely regulated, interactions of multiple hormones as well as their involvement in plant stress responses. Besides auxin, an increasing interest has been focusing on brassinosteroids (BRs), steroidal phytohormones, due to their roles in both plant growth/development and stress responses (Hafeez et al., 2021 and references therein; Nazir et al., 2019; Wei and Li, 2016). Several studies have highlighted that BRs not only are essential for development but also improve the plant defense system to counteract the stress. In fact, exogenous treatments with 24-epibrassinolide (eBL), a biologically active BR, increase the antioxidant activities in *Carthamus tinctorius* L. exposed to water stress, and treatments with exogenous BRs induce higher levels of antioxidants in *Linum usitatissimum* L. subjected to drought stress (Aghaee and Rahmani, 2020; Zafari et al., 2020). However, to date, the role of BRs in plant responses to As toxicity needs further investigation.

An interaction between auxin and BRs has been reported to regulate plant growth and development (Tian et al., 2018), with the root apical meristem (RAM) as a specific target of this interaction (Durbak et al., 2012). Furthermore, BRs and auxin play synergistic roles also in root system architecture affecting the formation of LRs (Tian et al., 2018) and enhancing plant defense against toxic metal stress (Betti et al., 2021; Kour et al., 2021). However, a recent study highlights that the interaction between auxin and BRs is complex and still largely unknown, with the same target genes possibly involved (Betti et al., 2021). Above all, the auxin-BR interaction in the responses of rice roots exposed to the toxicity of the most widespread and highly toxic As species, $As^{(III)}$ and $As^{(V)}$, needs to be elucidated.

In the present paper, we show that $As^{(III)}$ is the main As species present in rice roots regardless of the $As^{(III)}$ or $As^{(V)}$ salt supplied to the growing medium. Both As forms alter auxin distribution in ARs and LRs, with deleterious effects on the latter ones, in particular. However, the exogenous BR (eBL) treatment repairs the stress. In fact, eBL restores the regular auxin distribution in $As^{(III)}$ and $As^{(V)}$ ARs and LRs, with greater effects in the latter ones. The eBL treatment strongly increases the expression of both auxin influx and auxin efflux carrier genes when combined with $As^{(V)}$, but mainly with $As^{(III)}$. Moreover, eBL increases the antioxidant activity in the roots, but only when combined with $As^{(V)}$.

Finally, the role of BRs and their interaction with auxin in the responses of the rice root system to either $As^{(III)}$ or $As^{(V)}$ toxicity is discussed.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *Oryza sativa* L. ssp. Japonica cv. Nihonmasari (wild-type, wt) and of *OsDR5::GUS* (Wang et al., 2014) transgenic line were surface sterilized with ethanol 70 % (v/v) for 1.30 min, rinsed three times with ultrapure water (Milli-Q water), soaked in a solution of 40 % (v/v) NaClO for 25 min, and again rinsed three times in sterile Milli-Q water. Then, the seeds were sown in sterile Phytatray-type vessels (Phytatray™ II, Sigma-Aldrich, Saint Louis, USA) containing a half-strength MS (Murashige and Skoog, 1962), 1 % sucrose, and 0.8 % agar, at pH 5.6–5.8 (Control), and were kept for 10 days in long-day conditions (16/8 h light/dark, 210 mmol photons $m^{-2}s^{-1}$ and at 27 °C). To the Control medium composition, either 100 μM $Na_2HAsO_4{\cdot}7H_2O$ [i.e., $As^{(V)}$] or 25 μM $NaAsO_2$ [i.e., $As^{(III)}$] was alternatively added in combination or not with 0.1 μM of 24-epibrassinolide (i.e., eBL) (Sigma-Aldrich, Saint Louis, USA). The eBL concentration was chosen because the most effective concentration in mitigating the toxic effects induced by As on the rice root system, based on our previous results (Piacentini et al., 2023). The $As^{(V)}$ and $As^{(III)}$ concentrations were chosen according to Piacentini et al. (2020c).

2.2. Arsenic extraction, determination, and speciation

2.2.1. Root system digestion for total As determination

The roots from 30 seedlings cultured on Control medium or in the presence of $As^{(V)}$ or $As^{(III)}$ alone or combined with eBL were dried at 35 °C for 4 days and then homogenized with a mortar and pestle. An aliquot of the homogenized powder was weighed (≅ 20 mg) and digested with 5 mL HNO_3 (65 % Suprapur, Merck) and 1 mL H_2O_2 (30 % Suprapur, Merck) with microwave-assisted Digestor (Ethos, Milestone Advanced Microwave Labstation). The digestion program was: 10 min 650 W 80 °C; 10 min 550 W 80 °C; 15 min 950 W 80 °C; 15 min 950 W 160 °C. After being digested, the samples were appropriately diluted to 50 mL with MilliQ and then analyzed by ICP-OES (5800, Agilent).

2.2.2. Extraction for As speciation

For As speciation, a second aliquot of the previously homogenized samples (≅ 20 mg) was ultrasonically treated with a 10 mL methanol: Milli-Q solution (1:1 v/v) at 60 °C for 3 h, and then centrifuged, and the supernatants recovered. The procedure was repeated with the residual pellet and the two extracts were combined to get 20 mL of extraction solution (Tu et al., 2004). Five mL of the extraction solution were evaporated at 60 °C on a heating plate to dryness to remove the presence of methanol that interferes with ICP-OES analysis. Then, 10 mL of 2 % HNO_3 in Milli-Q were added to recover As and analyzed to determine the total extracted As. Further 5 mL of extraction solution (MeOH 50 %) were diluted 1:5 with Milli-Q to 25 mL final volume (10 % MeOH) (pH ≅ 7). From the latter solution, 12 mL were passed through As-speciation cartridges (Metal Soft Center, Highland Park, NJ), which retain $As^{(V)}$ (Tu et al., 2004). The first 2 mL were discarded, while the following 10 mL were collected to determine the $As^{(III)}$ fraction. To avoid MeOH interference, also the $As^{(III)}$ fraction was evaporated and recovered with 2 % HNO_3 in Milli-Q, then analyzed by ICP-OES. The percentage of $As^{(III)}$ was calculated with respect to the total extracted As. Standard solutions of 100 and 500 μg/L of both $As^{(V)}$ and $As^{(III)}$ were treated with the same extraction and separation procedures and recovery obtained were in the range of 90–95 %. The limit of detection (LOD) for As determination was 1 μg/L under experimental conditions.

2.3. GUS detection analysis

The root systems of 30 seedlings of the *DR5::GUS* transgenic line, grown in the presence of $As^{(V)}$ or $As^{(III)}$ combined or not with eBL, were processed for β-glucuronidase (GUS) staining according to Ronzan et al. (2018). The roots were cleared with a solution of chloral hydrate/glycerol/water (8:1:2, w/v/v) (Weigel and Glazebrook, 2002) and observed with a Nomarski optical microscope (DMRB, Leica, Wetzlar, Germany) equipped with a digital camera (C-P20CC, Optika, Italy). Three adventitious roots (ARs) per root system, along with their lateral root primordia (LRPs) and elongated lateral roots (LRs), from ten randomly chosen seedlings were examined per treatment. Images at different magnifications of both ARs, LRPs, and LRs were acquired and analyzed through a professional image analysis software (OPTIKA PROView version May 2021, Optika, Italy). Among the three biological replicates, the GUS expression pattern was highly homogeneous. Roots with evident artefacts in GUS staining were less than 5 % per root category and treatment and for this reason were excluded from the analyzes.

2.4. Quantitative RT-PCR analysis of OsPIN2 and OsAUX1 genes

The root system of 10 wt seedlings grown in the presence/absence of $As^{(V)}$/$As^{(III)}$, combined or not with 0.1 μM eBL was harvested, frozen in liquid nitrogen, and stored at -80 °C before RNA extraction. Total RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, Saint Louis, USA) according to the manufacturer's instructions. After checking RNA integrity on 1.2% (w/v) agarose gel, we proceeded with DNA removal through a TURBO DNA—free kit (Invitrogen, Waltham, MA, USA). For cDNA synthesis, a leveling of RNA quantity was necessary (5 μg in 10 μL) optimized also for a first normalization of the following expressions analysis. The retro transcription was made with SuperScript IV (Invitrogen, Waltham, MA, USA) and with random hexamers according to the manufacturer's instructions (Chomczynski and Sacchi, 2006). SYBR-Green-based quantitative assays on CFX Opus 96 RT-PCR (BioRad, Hercules, CA, USA) were performed in triplicate using 1 μL from the cDNA prepared as described above. The analysis was repeated twice for each biological replicate. Rice *actin-1* was selected as the housekeeping gene (Ronzan et al., 2018). Gene-specific primers are reported in Supplementary Table 1 and were designed with Primer3web (https://primer3.ut.ee, accessed on September 2023), with a melting temperature of 59 °C. The two-step PCR conditions were the following: one cycle at 98 °C for 3 min, then 40 cycles at 98 °C for 15 min, and at 59 °C for 3 min. Melting curve analysis was performed at the end to check the specificity of the amplification reactions. After validation tests, normalization to *actin-1* was performed using the ΔΔCT method by the "PCR" library performed on RStudio (1.3.1093).

2.5. Antioxidant activity detection by the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) analysis

The antioxidant activity of the root extracts against 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined according to the procedure described by Sanna et al. (2012) with slight modifications. Briefly, about 0.5 g of roots of the wt seedlings grown in the presence of the different treatments were ground with liquid nitrogen and mixed with 5 mL of 95 % ethanol. The mixtures were stored at 4 °C for 3 h and shaken to ensure complete extraction. Then, the supernatants were collected after a 10-minute centrifugation at 6500 rpm (CL10, Thermo Fisher Scientific, Sunnyvale, CA, USA). To perform the DPPH assay, 1.5 mL of the supernatant collected was added to 2 mL of ethanolic DPPH (0.1 mM; 96 % EtOH). The resulting mixture was shaken for 30 min by rotating agitation (60 rpm; Glas-Col, Terre Haute, IN, USA) at room temperature in the dark. The absorbance of the solution was measured at the wavelength of 517 nm using a UV–vis spectrophotometer (Varian Cary 50 Bio UV–Vis; Varian Inc., Palo Alto, CA, USA). By measuring the sample absorbance decrease with respect to that of a blank solution, the DPPH radical scavenging activity was determined. The antioxidant activity of each sample was calculated in terms of the percentage consumption of DPPH according to the following equation:

$$DPPH\ [\%] = \frac{(A_0 - A_s)}{A_0} \times 100$$

where A_0 represents the absorbance of the blank solution and A_s is the absorbance of the sample (Miliauskas et al., 2004).

2.6. Statistical analysis

Statistical analysis was performed using either a one-way ANOVA test followed by Tukey's post-test (at least at $P < 0.05$) or a two-tailed Student's *t*-test through GraphPad Prism 9.3.0 or RStudio software. Percentage data from the DPPH analysis were transformed using the arsine[sqrt(x)] method to achieve normality before conducting ANOVA (Clewer and Scarisbrick, 2001). All experiments were conducted in triplicate with similar results and the data from the first or the third experiment are presented.

3. Results

3.1. Arsenite is the main As species present in rice roots regardless of the As salt supplied to the growing medium

Total As was quantified in the root system of ten-days-old rice seedlings grown with each of the two As forms either alone or in combination with 0.1 μM eBL. Despite the seedlings were grown in the presence of different salts of supplied As at different concentrations, their root systems showed similar total As accumulation (Fig. 1). This was not due to the saturation of the extraction solution of MeOH, as the As levels within the roots were similar in the two treatments even following the acid digestion process (Supplementary Fig. S1). No endogenous As was detected neither in the Control nor in the root system of the eBL alone treated seedlings.

When eBL was combined with As, there was a general reduction in the total root As content compared to treatments with As alone, though non-statistically significant (Fig. 1). In addition, the reduction in the root As content was more pronounced when eBL was combined with $As^{(III)}$ than with $As^{(V)}$. The As speciation analysis revealed that regardless of the oxidation state of the As in the salts added to the growing medium, $As^{(III)}$ was the prevailing form accumulated in the rice roots. Indeed, approximately 94 % and 92 % of the total As accumulated in the rice roots, grown in the presence of $As^{(III)}$ or $As^{(V)}$ salt, respectively, was in the form of $As^{(III)}$, which maintained its prevalence even when eBL was added (Fig. 1).

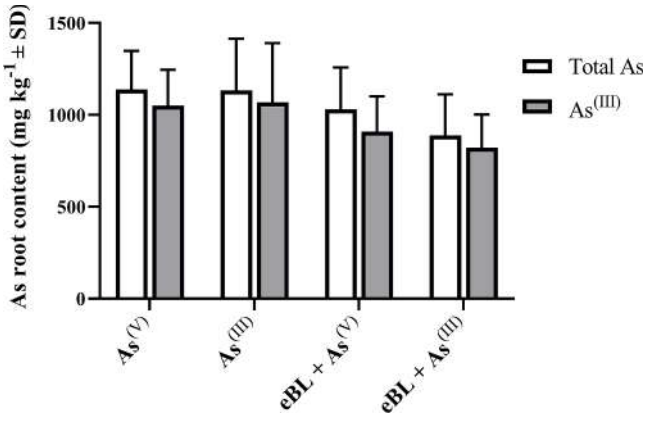

Fig. 1. Total arsenic and $As^{(III)}$ content (mg kg^{-1} ± SD) in the root system of 10 d old wt rice seedlings. The seedlings were treated with 100 μM $Na_2HAsO_4{\cdot}7H_2O$ [$As^{(V)}$], or with 25 μM $NaAsO_2$ [$As^{(III)}$], or with 0.1 μM of 24-epibrassinolide plus 100 μM $Na_2HAsO_4{\cdot}7H_2O$ [eBL + $As^{(V)}$], or with 0.1 μM of 24-epibrassinolide plus 25 μM $NaAsO_2$ [eBL + $As^{(III)}$].

3.2. Arsenic delocalizes the auxin maximum in the apex of the adventitious roots, but eBL restores its positioning and the correct auxin distribution in $As^{(V)}$ and $As^{(III)}$-stressed adventitious roots

In ten-days-old rice seedlings, the rice root system consists of ARs of embryonic origin and their LRPs and developed LRs.

We analyzed the auxin distribution in the root system of *OsDR5::GUS* seedlings exposed to $As^{(V)}$ or $As^{(III)}$, alone or combined with eBL (Fig. 2). The macroscopic images provide an overall view of the rice root system and GUS signal in all the treatments (Fig. 2). The GUS signal was evident in both ARs and LRs (Fig. 2A–F). However, compared to the Control, a reduction in the growth of the root system due to $As^{(V)}$ or $As^{(III)}$ treatments was observed (Fig. 2C and E) . The exogenous eBL associated with the metalloid (both forms) only slightly increased the root system growth compared to the treatments with the metalloid alone (Fig. 2D and F).

In the ARs, the GUS signal was localized in the root apical meristem (RAM) and the proximal elongating/differentiating zones, gradually decreasing towards the mature (differentiated) zones, regardless of the treatment (Fig. 3). The Control ARs apices showed a high GUS signal in all cap cells, quiescent center (QC) and surrounding initials, collectively forming the stem cell niche (Fig. 3B, circle), and in the procambium (Fig. 3A).

24-epibrassinolide alone had no impact on auxin signal intensity or localization in the AR apex compared to the Control (Fig. 3C and D). However, there was a lack of signal in the proximal part of the niche corresponding to the immediate derivatives of the procambial initial cells (Fig. 3D). Under $As^{(V)}$-alone treatment, the ARs showed a RAM with a signal intensity similar to the Control, but more diffuse (Fig. 3E and F). However, the signal localization differed, because the highest GUS signal, i.e. the auxin maximum, was delocalized to the basal columella (Fig. 3F, oval). In this treatment, as in the Control, the GUS signal rapidly and uniformly decreased towards the AR differentiated regions remaining quite evident in the procambium of the elongating/differentiating zones (Fig. 3E). When $As^{(V)}$ was combined with eBL, the signal remained in the procambium with intensity comparable to the $As^{(V)}$ alone treatment (Fig. 3G, and 3E in comparison). However, in the apex, it was strongly reduced compared to the $As^{(V)}$-alone, Control, and eBL treatments, with the lateral root cap showing no signal (Fig. 3H). The auxin signal marked the stem cell niche (Fig. 3H, oval), differently from the $As^{(V)}$ alone, but, interestingly, there was again a lack of GUS expression in the proximal niche corresponding to the immediate derivatives of the procambial initials (Fig. 3H) as in the eBL alone treatment (Fig. 3D).

The apex of the ARs exposed to $As^{(III)}$ alone showed a higher in intensity GUS signal than $As^{(V)}$ (Fig. 3I and E in comparison). It was shown by the root cap, RAM, and procambium (Fig. 3I and J). The most intense auxin signal was in the root cap also in this case, and more evidently than in the $As^{(V)}$ treatment (Fig. 3J). However, the stem cell niche showed a strong reduction in signal and a delocalization of the auxin maximum to the basal columella (Fig. 3J, oval). No difference occurred in the procambial expression between the two As treatments (Fig. 3I and E in comparison). Similarly to the $As^{(V)}$ plus eBL treatment, in the $As^{(III)}$ plus eBL the GUS signal was present in the procambium of the elongating/differentiating zones, with no difference from $As^{(III)}$ alone treatment (Fig. 3K). However, in the RAM the signal was lower than in $As^{(III)}$ alone, but correctly localized in the apical stem cell niche, as in the $As^{(V)}$ plus eBL treatment, and with the same lack of expression in correspondence with the procambial derivative cells (Fig. 3L).

3.3. Arsenic causes a delay and an alteration in auxin distribution in the lateral roots, but eBL restores the correct auxin localization independently on the $As^{(V)}$ or $As^{(III)}$-induced stress

The distribution of the GUS signal in the LRPs and the LRs was investigated in *OsDR5::GUS* rice seedlings. In the Control, the signal was

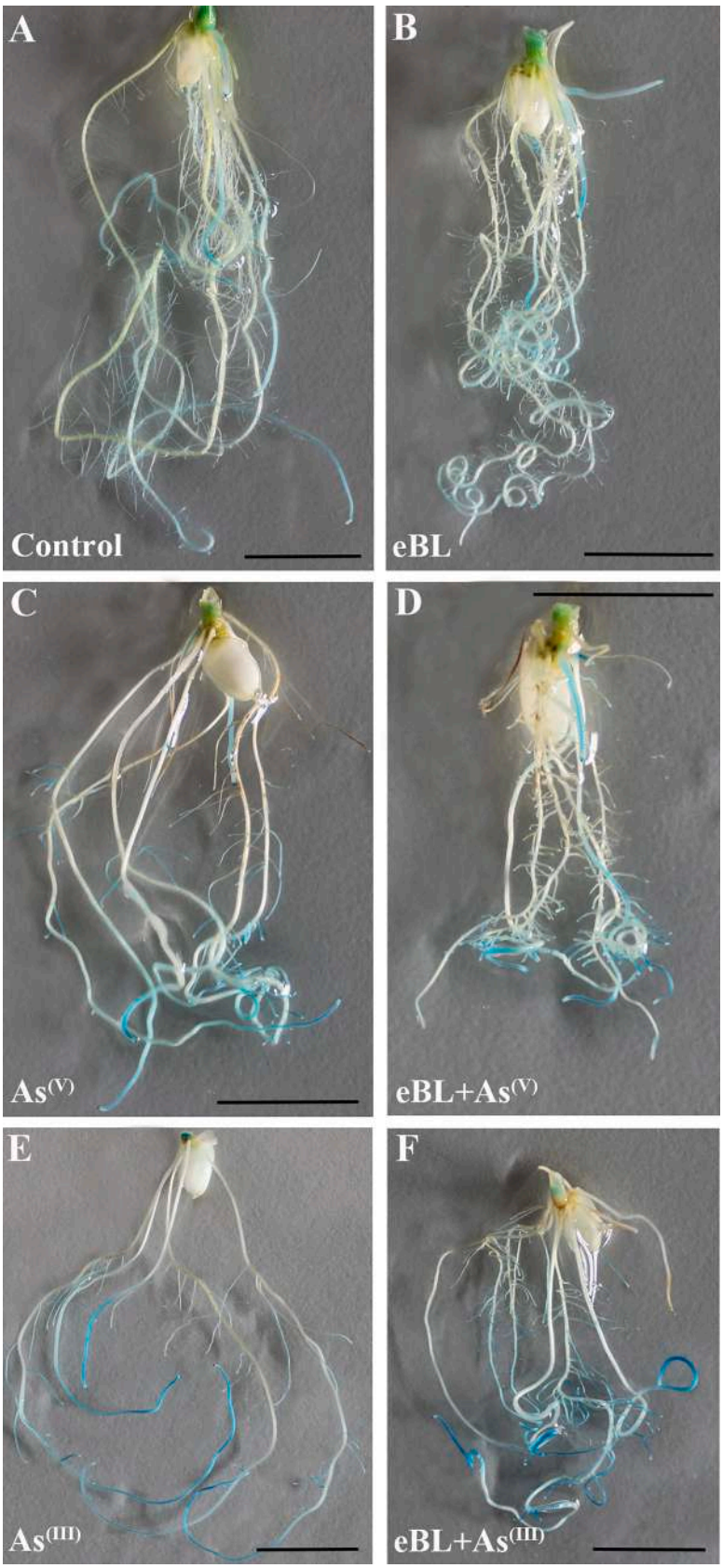

Fig. 2. Macroscopic images showing auxin distribution in the root system of 10 d old *OsDR5::GUS* rice seedlings. (A) Untreated (Control). (B) Treated with 0.1 μM of 24-epibrassinolide (eBL). (C) Treated with 100 μM $Na_2HAsO_4{\cdot}7H_2O$ [$As^{(V)}$]. (D) Treated with 0.1 μM of 24-epibrassinolide plus 100 μM $Na_2HAsO_4{\cdot}7H_2O$ [eBL+$As^{(V)}$]. (E) Treated with 25 μM $NaAsO_2$ [$As^{(III)}$]. (F) Treated with 0.1 μM eBL plus 25 μM $NaAsO_2$ [eBL+$As^{(III)}$]. Representative images from 30 root systems per treatment. Bars = 1 cm.

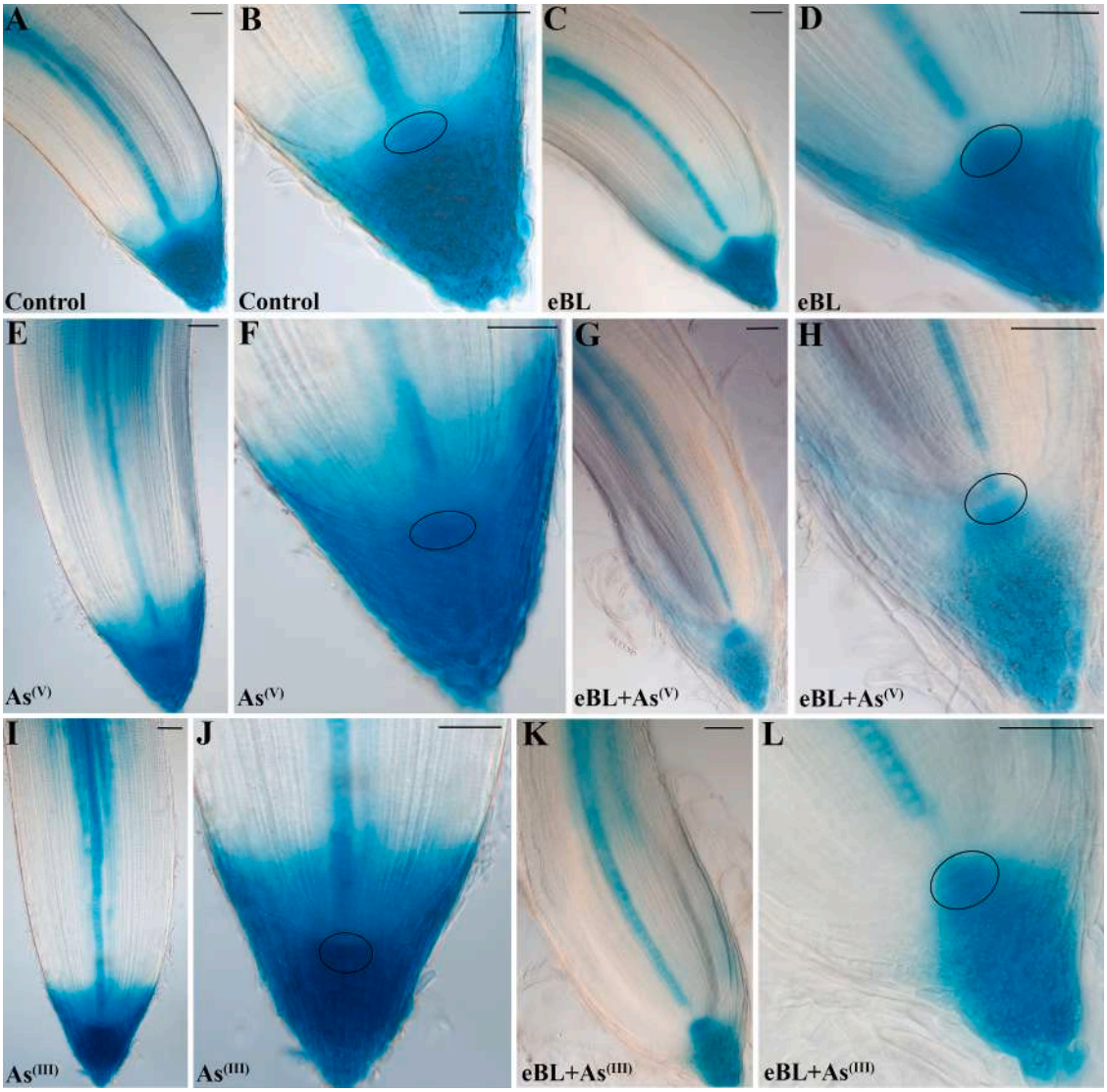

Fig. 3. Bright-field microscopy images of adventitious roots of 10 d old *OsDR5::GUS* seedlings showing auxin distribution in the apical regions. The seedlings were untreated (Control) or treated with 100 μM $Na_2HAsO_4{\cdot}7H_2O$ [$As^{(V)}$] or 25 μM $NaAsO_2$ [$As^{(III)}$] with/without 0.1 μM of 24-epibrassinolide (eBL). B,D,F,H,J,L are magnifications of the apex. The auxin maximum is marked by an oval. Representative images of 30 ARs from ten randomly chosen root systems per treatment. Bars = 50 μm (A-K), 25 μm (L).

observed in both the basal and apical parts of the forming LRP and in the QC niche starting from its formation (Fig. 4A, arrowhead). During the primordium growth, the auxin signal remained confined at the same positions (Fig. 4B, oval). In the elongated LRs, the signal was still present in the cap and RAM, QC stem cell niche enclosed (Fig. 4C, oval) and more intense in all the differentiating tissues except for the developing rhizodermis and exodermis (Fig. 4C). Differently, in the $As^{(V)}$-alone treatment there was no GUS signal in the apical part of young LRPs, suggesting no QC niche formation and related auxin maximum definition (Fig. 4D). The signal remained strongly reduced at further developmental stages of the LRP growth, appearing, in the basal procambium and in the cap, but not in the apical niche (Fig. 4E). The same signal localization occurred in the LRs (Fig. 4F). Even if more intense at any stage in comparison with the $As^{(V)}$ alone treatment, the $As^{(III)}$ alone did not change the GUS signal localization in either young LRPs (Fig. 4G) or developing LRPs (Fig. 4H), with a similar delay in auxin signal appearance and a delocalization of the auxin maximum to the cap, respectively. The GUS signal remained weak in the LRs (Fig. 4I).

Under the eBL alone treatment, the forming LRPs showed a Control-like signal, particularly marking their QC niche (Fig. 5A, arrowhead). A strongly increased GUS signal, in comparison with the Control, characterized both the developing LRPs and the mature LRs. In the former, the signal occurred throughout the entire LRP, and particularly in its QC niche (Fig. 5B, oval); in the latter, it was observed in the whole apical meristem and root cap, including the QC niche (Fig. 5C, oval). When eBL was combined with $As^{(V)}$, the GUS signal in the young LRPs became higher than with $As^{(V)}$ alone, in particular in the apical niche and the base of the organ, like in the Control (Fig. 5D, arrowhead). Also, the tissues of the elongating LRPs and the LRs showed a slight recovery of the signal, in comparison with the $As^{(V)}$ alone (Figs. 5E, F and 4E, F, in comparison), with a slight signal also in the QC niche (Fig. 5E and F, ovals). When eBL was combined with $As^{(III)}$, the signal strongly increased in the forming and elongating LRPs becoming higher than in the Control and the $As^{(V)}$ plus eBL treatment. However, it similarly marked the cap cells, the correctly positioned QC niche (Fig. 5G and H, arrowhead and oval), and the forming procambium (Fig. 5H). The signal became even higher in the LRs, where the elongating/differentiating tissues except for the rhizodermis and exodermis, showed a very high expression, the same as the apical stem cell QC niche (Fig. 5I, oval).

Collectively, results show that both the As forms stressed the LRs

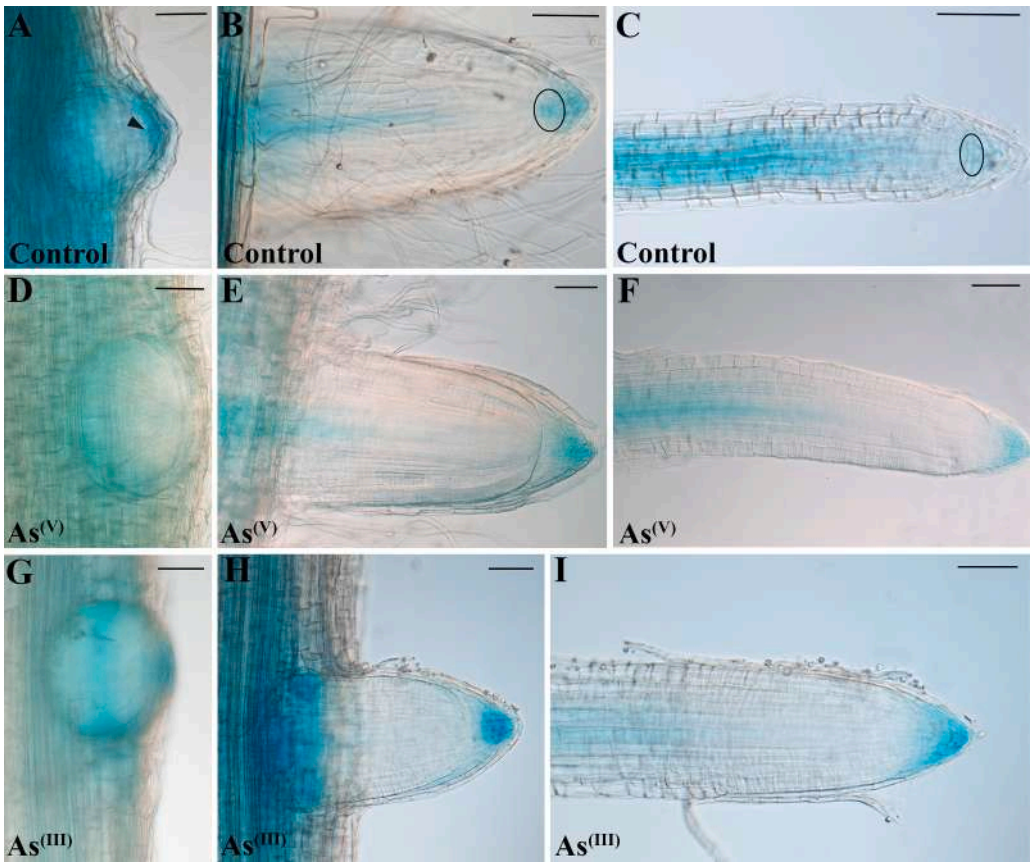

Fig. 4. Bright-field microscopy images showing auxin distribution in young lateral root primordia (A,D,G), elongating primordia (B,E,H) and in the apical region of developed lateral roots (C,F,I) of 10 d old *OsDR5::GUS* rice seedlings. The seedlings were untreated (Control) or treated with 100 μM $Na_2HAsO_4{\cdot}7H_2O$ [$As^{(V)}$] or 25 μM $NaAsO_2$ [$As^{(III)}$]. The auxin maximum corresponding to the quiescent center niche is marked by an arrowhead in A, and by an oval in B and C. Representative images of LRPs or LRs from 30 ARs per treatment. Bars = 50 μm.

from their early stages, causing delays and alterations in their QC niche and related auxin maximum. These effects persisted with the further LR development, and with an increase of the auxin signal intensity caused by the reduced As form. However, eBL bypassed these alterations restoring the normal development of the LRs through a correct auxin distribution, albeit with variations in the GUS signal intensity, which was higher when the hormone was combined with $As^{(III)}$ than with $As^{(V)}$.

3.4. eBL strongly increases the expression of both auxin influx and auxin efflux carrier genes when combined with $As^{(V)}$ or $As^{(III)}$

The auxin influx carrier gene *OsAUX1* and the auxin efflux carrier gene *OsPIN2* expression in seedling roots were analyzed after 10 days under the different treatments (Fig. 6). The treatment with eBL alone significantly ($P < 0.001$) reduced *OsAUX1* expression but not *OsPIN2* expression compared to the Control (Fig. 6). Arsenate alone reduced ($P < 0.01$) *OsAUX1* expression but not that of *OsPIN2*. Arsenite alone significantly ($P < 0.05$) reduced *OsAUX1* expression but increased ($P < 0.05$) *OsPIN2,* in comparison with the Control. Collectively, none of the treatments significantly enhanced auxin transport through the tested carriers, except for $As^{(III)}$ to some extent. By contrast, important changes occurred when eBL was combined with each As form. Increases in expression occurred when $As^{(V)}$ was combined with eBL even if they were less significant ($P < 0.01$ for *AUX1* only) in comparison with the Control and with the $As^{(V)}$ or eBL alone treatments (Fig. 6). The combined treatments of $As^{(III)}$ and eBL also induced significant, and similar, increases in the expression of the two genes ($P < 0.01$ for *OsAUX1* and $P < 0.05$ for *OsPIN2*) in comparison with the Control (Fig. 6), but also in comparison with $As^{(III)}$ or eBL alone treatments (Fig. 6).

Collectively, data show that the exogenous BR enhanced the auxin transport in the presence of As stress in the roots, and mainly in the presence of the reduced As form.

3.5. Exogenous eBL increases the antioxidant activity in rice roots exposed to arsenate but not to arsenite

The root aqueous extracts from the different treatments were evaluated for antioxidant activity using the DPPH assay. The Control showed the highest level of antioxidant activity, with a consumption of about 50 % of DPPH per gram of root (Fig. 7). The eBL treatment resulted in an approx. 30 % reduction in antioxidant activity compared to the Control (Fig. 7), suggesting a slight oxidative stress of eBL *per se*. Both $As^{(V)}$ and $As^{(III)}$ treatments strongly reduced ($P < 0.001$) the antioxidant activity by approximately 90 % and 73 %, respectively, compared to the Control (Fig. 7), showing that their deleterious effects on AR and LR morphology and auxin distribution (Figs. 2–4) also involved oxidative stress. The combination of eBL with $As^{(V)}$, led to a significant ($P < 0.001$) increase in the antioxidant activity, reaching approx. 30 % of DPPH consumption per gram of root compared to $As^{(V)}$ treatment alone (Fig. 7). However, supplementing eBL with $As^{(III)}$ resulted in an antioxidant activity similar to that of roots grown in the presence of the pollutant alone (Fig. 7).

Altogether, results show that the eBL role in counteracting As-induced root stress involves an increase in antioxidant activity, which is more pronounced in the presence of $As^{(V)}$, suggesting its lower toxicity or greater susceptibility to counteraction compared to the other form of As applied.

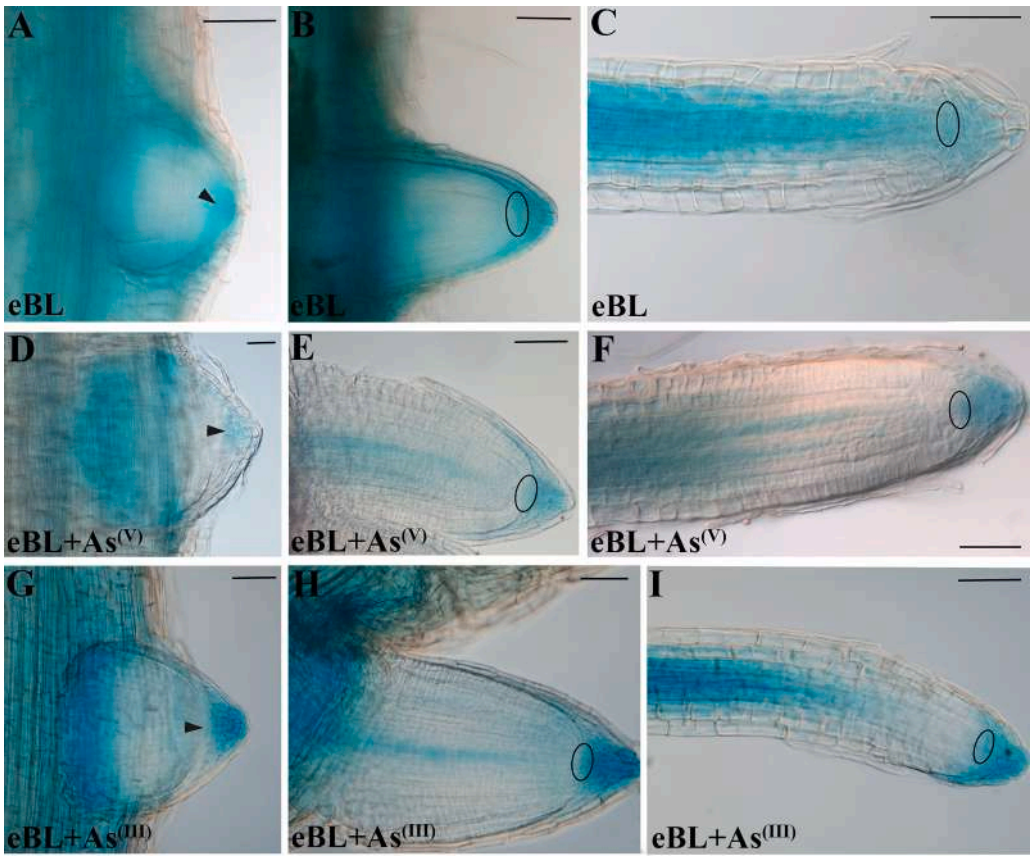

Fig. 5. Bright-field microscopy images showing auxin distribution in young lateral root primordia (A,D,G), elongating primordia (B,E,H) and in the apical region of developed lateral roots (C,F,I) of 10 d old *OsDR5::GUS* rice seedlings. The seedlings were treated with 0.1 μM of 24-epibrassinolide (eBL) (A,B,C), 100 μM $Na_2HAsO_4{\cdot}7H_2O$ with eBL [eBL + $As^{(V)}$] (D,E,F) or 25 μM $NaAsO_2$ with eBL [eBL + $As^{(III)}$] (G,H,I). The auxin maximum corresponding to the quiescent center niche is marked by an arrowhead in A,D,G and by an oval in B,C,E,F,H,I. Representative images of LRPs or LRs from 30 ARs per treatment. Bars = 50 μm.

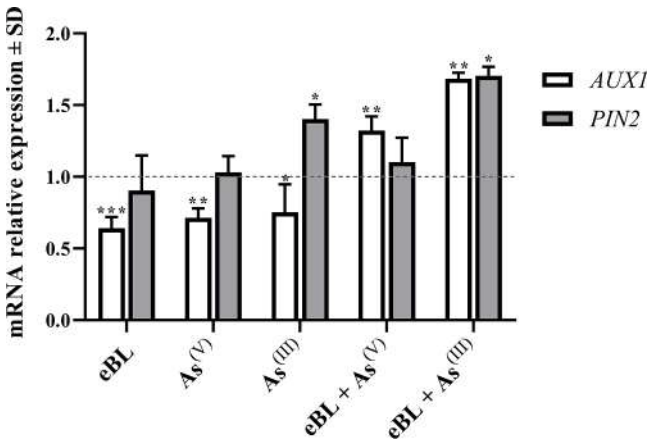

Fig. 6. Relative expression of *OsAUX1* and *OsPIN2* genes (RT-qPCR analysis) (± SD) in the root system of 10 d old wt rice seedlings. The seedlings were untreated (Control) or treated with 0.1 μM of 24-epibrassinolide (eBL), 100 μM $Na_2HAsO_4{\cdot}7H_2O$ [$As^{(V)}$], 25 μM $NaAsO_2$ [$As^{(III)}$], 0.1 μM of 24-epibrassinolide plus 100 μM $Na_2HAsO_4{\cdot}7H_2O$ [eBL+$As^{(V)}$], 0.1 μM of 24-epibrassinolide plus 25 μM $NaAsO_2$ [eBL+$As^{(III)}$]. The expression levels of the two genes in the Control were set to 1 (dashed line). Symbols show significant differences, for the same gene in comparison with the Control, for at least $P < 0.05$.

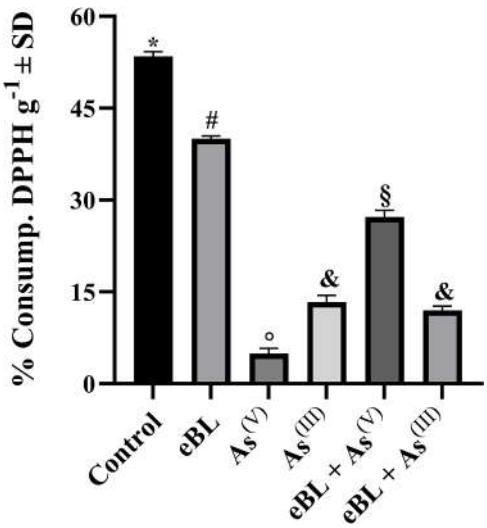

Fig. 7. Antioxidant activity in the root system of 10 d old wild rice seedlings. The antioxidant activity was detected by the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) analysis and expressed as percentage consumption (± SD) of DPPH. The seedlings were untreated (Control) or treated with 0.1 μM of 24-epibrassinolide (eBL), 100 μM $Na_2HAsO_4{\cdot}7H_2O$ [$As^{(V)}$], 25 μM $NaAsO_2$ [$As^{(III)}$], 0.1 μM of 24-epibrassinolide plus 100 μM $Na_2HAsO_4{\cdot}7H_2O$ [eBL + $As^{(V)}$], 0.1 μM of 24-epibrassinolide plus 25 μM $NaAsO_2$ [eBL + $As^{(III)}$]. Different symbols show statistical differences for at least $P < 0.01$ among the treatments. The same symbol shows no significant difference.

4. Discussion

This study explored the effects of toxicity of the inorganic As species, $As^{(III)}$ and $As^{(V)}$, on the rice root system and the potential mitigation role

of exogenous brassinosteroids through an interaction with auxin. We show that roots exposed for 10 days to $As^{(III)}$ or $As^{(V)}$ salts accumulated mainly $As^{(III)}$, in accordance with previous works (Chen et al., 2022; Navarro et al., 2021). Despite the accumulation of various As forms in rice organs strongly depends on the variety tested (Zheng et al., 2023), it is noteworthy that the roots accumulate an As content higher than the shoot and for an extended period (Pan et al., 2020). No major effect on As accumulation was observed in rice roots when As salts were combined with 24-epibrassinolide (eBL), except for a weak reduction in total As when eBL was combined with $As^{(III)}$. Similarly, a weak reduction in As accumulation has been previously observed in rice roots exposed to the same As form combined with eBL, although under different conditions of cultivation (Xu et al., 2018), suggesting that BR might be involved in reducing the $As^{(III)}$ uptake in the roots. Arsenite uses aquaporins to enter plant cells, and the involvement of BRs in regulating specific aquaporins at the transcriptional and translational levels has been demonstrated in barley under stress-inducing temperatures (Sadura et al., 2020). Our results show that both As species, without any difference between the two, influence the root auxin distribution. It is known that the auxin levels are affected, in rice, by abiotic stresses, as cold, heat, drought and toxic metals (Du et al., 2013; Ronzan et al., 2018). As for other hormones, the correct cellular levels of auxin and its distribution and activity are due to its correct synthesis, transport, and signaling, and inorganic As, particularly its oxidized form, is known to negatively affect the hormone biosynthesis, transport, and signaling (Ronzan et al., 2018; Singh et al., 2021). In accordance, we previously demonstrated that the expression of the auxin influx gene *OsAUX1* is reduced by $As^{(V)}$ in the rice roots (Ronzan et al., 2018), and current data demonstrate a similar reduction caused by $As^{(III)}$. Altogether, both current and previous data support the idea that this carrier is a target of As toxicity, regardless of its form, and that it affects overall auxin levels and distribution in the root system. Differently from *OsAUX1*, the present data show that *OsPIN2* is significantly over-expressed by $As^{(III)}$ treatment and non-affected by $As^{(V)}$. The *PIN* genes are numerous in plants, i.e., at least 12 in the rice genome and 8 in Arabidopsis, and their roles in plant development, responsiveness to abiotic stress, and formation of auxin-dependent root architecture have been reported in rice and in other plants (Fattorini et al., 2017; Manna et al., 2022; Ronzan et al., 2018). In Arabidopsis, it was demonstrated that AtPIN2 is responsible for the auxin transport from the root apex to the elongation zone, for regulating the maximum auxin gradient at the root tip (Ashraf et al., 2020) and for correct gravitropic responses (Rahman et al., 2010). It is possible that *OsPIN2,* which is the unique homolog of *AtPIN2* in rice, has a similar role as also highlighted by Wang et al. (2018). In addition, OsPIN2 seems to play a key role in the regulation of auxin distribution in rice roots and in the control of AR elongation and LR formation (Inahashi et al., 2018). Recent studies have demonstrated *OsPIN2* upregulation during drought stress, suggesting its potential role in modulating auxin transport and in contributing to rice tolerance to this stress (Manna et al., 2022). Moreover, it has been also demonstrated that PIN2 is involved in the stress response to $As^{(III)}$, particularly in regulating arsenite transport in Arabidopsis (Ashraf et al., 2020). Altogether, these findings support our results, which demonstrate a positive correlation between *OsPIN2* over-expression in response to As stress and the resulting alteration in auxin distribution, ultimately affecting AR apical structure and LR formation and development. Furthermore in the conditions of our experiments, $As^{(III)}$ was the predominant As form in the roots, regardless of the exogenously applied As salt, and with a small difference in comparison with the total As detected. The result suggests that in the $As^{(V)}$-treated seedlings the small fraction of the endogenous As that does not convert into $As^{(III)}$ might be the one responsible for the absence of the *OsPIN2* over-expression occurring in this treatment. Alternatively, differences in *OsPIN2* transcription in the roots between the two As treatments may have occurred before the overall reduction of $As^{(V)}$ into $As^{(III)}$, persisting up to the detection time (day 10 of seedling growth). We show that the exogenous eBL alone reduced the expression of *OsAUX1*, in comparison with the Control, but did not affect *OsPIN2* expression. Similarly, in Arabidopsis, it was demonstrated that BR activity does not affect the expression of *PIN* genes (Hacham et al., 2012), even if more recent data show that eBL signaling is implicated in sorting and steady-state level control of PIN2 in Arabidopsis, but under specific events (Retzer et al., 2019). It is possible that when applied alone, the exogenous BR causes a saturation of the endogenous auxin levels (i.e. those present in the control) rendering necessary a downregulation of the expression of the auxin influx carrier AUX1 as a compensative mechanism to maintain regular root growth.

When eBL was combined with As, especially with $As^{(III)}$, a strong overexpression of *OsAUX1* and *OsPIN2* was observed, sustaining that eBL plays a positive role in inducing the correct distribution of auxin in the ARs and LRs to mitigate As-stress, as a prerequisite for root growth. This last result is in agreement with our previous data obtained by the same eBL and $As^{(III)}$ or $As^{(V)}$ concentrations, which demonstrate that eBL enhances the LR formation in rice possibly counteracting As-stress in this way (Piacentini et al., 2023). It is indeed possible that when eBL is combined with As, particularly with the more toxic and abundant form, i.e., $As^{(III)}$, it can stimulate auxin transport through influx and efflux carriers, including PINs, to restore auxin distribution in the roots to counteract As toxicity. In accordance, BRs are known to interact with auxin for inducing LR formation and for maintaining the correct organization and functionality of the roots (Ackerman-Lavert et al., 2021), and this mutual relationship also exists in plant response to the stress. This occurs through a modulation of the auxin transport (Bhandari and Nailwal, 2020) and is involved in stabilizing the root systems altered by stressful environmental conditions, e.g. As toxicity (Devi et al., 2022; Piacentini et al., 2023). Our results show that in rice roots $As^{(III)}$ and $As^{(V)}$ treatments strongly reduced antioxidant activity. This result is in accordance with the literature because it is known that the toxic elements, including the metalloid As, induce a strong nitro-oxidative stress in plant cells due to an increase of ROS and RNS molecules (Corpas and Barroso, 2013). It is known that high ROS and RNS cellular levels cause the oxidation of proteins, lipids, and nucleic acids, resulting in cytological alterations, mutagenesis, cytotoxicity, and inhibition of the development of the root system (Piacentini et al., 2020c; Prakash et al., 2020; Ronzan et al., 2018). Also, present results show a reduction in the development of the root system which may be related to the oxidative stress induced by the pollutant. Interestingly, exogenous eBL was able to increase the antioxidant activity, when combined with $As^{(V)}$, whereas no increase occurred in the presence of $As^{(III)}$. The higher toxicity of this As form might have caused the exogenous BR inability. The BRs are known to enhance both the enzymatic and the non-enzymatic plant antioxidant systems, as in tomato plants exposed to chromium and in *Brassica juncea* plants exposed to lead (Kohli et al., 2018). The same mechanisms may be here activated in the rice roots, but only when exposed to the less toxic As form, i.e., $As^{(V)}$. Interactions between BRs and nitrogen (RNS) species have also been demonstrated (Hu et al., 2021; Piacentini et al., 2023). Nitric oxide (NO) is a cell signaling RNS able to interact with ROS (Mansoor et al., 2022) in the responses to environmental stresses (Corpas et al., 2009), including those caused by toxic metals (Piacentini et al., 2023 and other references therein). It has been demonstrated that NO can reduce the cellular oxidative stress caused by heavy metals (Emamverdian et al., 2021), and increase As tolerance, as in maize (Kaya et al., 2020). However, its action in rice depends on the As form interacting with exogenous BRs (Piacentini et al., 2023). In fact, at the same concentration here used, eBL restores the $As^{(V)}$-altered NO levels and its cellular distribution alleviating the damages caused by As alone, but this does not occur in the presence of $As^{(III)}$ (Piacentini et al., 2023). Therefore, both current and previous findings support the hypothesis that the severe toxicity of $As^{(III)}$, which also operates through ROS/RNS oxidative stress, hinders the ability of BRs to restore normal cellular functions responsible for root system growth.

5. Conclusion

Present data show that $As^{(III)}$ is the main As species present in rice roots regardless of the $As^{(III)}$ or $As^{(V)}$ salt exogenously supplied. Both As forms alter auxin distribution in the rice root system differently interfering with auxin transporters AUX1 and PIN2. The exogenously applied BR repairs the stress restoring the regular auxin distribution in $As^{(III)}$ and $As^{(V)}$-stressed roots and increasing the antioxidant activity. These reparative effects are similar in the presence of both As salts, even if the two salts exhibit strong differences in their toxic action. These differences, and the repair by BRs, must be taken into consideration in crop improvement programs aimed to select agronomically important varieties/hybrids of rice, whose cultivation areas are at risk due to As pollution worldwide.

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CRediT authorship contribution statement

D. Piacentini: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. **C. Bellini:** Methodology. **A. Peduzzi:** Methodology. **B. Casentini:** Methodology. **C. Tiraboschi:** Methodology. **A. Cacciotti:** Methodology. **M.M. Altamura:** Data curation, Writing – original draft, Writing – review & editing. **G. Falasca:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **F. Della Rovere:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors state that they do not have any identifiable conflicting financial interests or personal relationships that could have been perceived as influencing the work presented in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100418.

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