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Auxin-jasmonate crosstalk in *Oryza sativa* L. root system formation after cadmium and/or arsenic exposure

Marilena Ronzan[a], Diego Piacentini[a], Laura Fattorini[a], Della Rovere Federica[a], Emilia Caboni[b], Elisabeth Eiche[c], Jörg Ziegler[d], Bettina Hause[e], Michael Riemann[f], Camilla Betti[g], Maria Maddalena Altamura[a], Giuseppina Falasca[a,*]

[a] Department of Environmental Biology, "Sapienza" University of Rome, Italy
[b] CREA- Centro di ricerca Olivicoltura, Frutticoltura e Agrumicoltura, Rome, Italy
[c] Institute of Applied Geosciences (AGW), Geochemistry and Economic Geology Group, Karlsruhe Institute of Technology (KIT), Germany
[d] Department of Molecular Signal Processing, Leibniz Institute of Plant Biochemistry, Weinberg 3, Halle, Germany
[e] Department of Cell and Metabolic Biology, Leibniz Institute of Plant Biochemistry, Weinberg, Germany
[f] Botanical Institute, Molecular Cell Biology, Karlsruhe Institute of Technology, Germany
[g] Department of Medicine, University of Perugia, Perugia, Italy

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ABSTRACT

Soil pollutants may affect root growth through interactions among phytohormones like auxin and jasmonates. Rice is frequently grown in paddy fields contaminated by cadmium and arsenic, but the effects of these pollutants on jasmonates/auxin crosstalk during adventitious and lateral roots formation are widely unknown. Therefore, seedlings of *Oryza sativa* cv. Nihonmasari and of the jasmonate-biosynthetic mutant *coleoptile photomorphogenesis2* were exposed to cadmium and/or arsenic, and/or jasmonic acid methyl ester, and then analysed through morphological, histochemical, biochemical and molecular approaches.

In both genotypes, arsenic and cadmium accumulated in roots more than shoots. In the roots, arsenic levels were more than twice higher than cadmium levels, either when arsenic was applied alone, or combined with cadmium. Pollutants reduced lateral root density in the wild -type in every treatment condition, but jasmonic acid methyl ester increased it when combined with each pollutant. Interestingly, exposure to cadmium and/or arsenic did not change lateral root density in the mutant. The transcript levels of *OsASA2* and *OsYUCCA2*, auxin biosynthetic genes, increased in the wild-type and mutant roots when pollutants and jasmonic acid methyl ester were applied alone. Auxin (indole-3-acetic acid) levels transiently increased in the roots with cadmium and/or arsenic in the wild-type more than in the mutant. Arsenic and cadmium, when applied alone, induced fluctuations in bioactive jasmonate contents in wild-type roots, but not in the mutant. Auxin distribution was evaluated in roots of *OsDR5::GUS* seedlings exposed or not to jasmonic acid methyl ester added or not with cadmium and/or arsenic. The *DR5::GUS* signal in lateral roots was reduced by arsenic, cadmium, and jasmonic acid methyl ester. Lipid peroxidation, evaluated as malondialdehyde levels, was higher in the mutant than in the wild-type, and increased particularly in As presence, in both genotypes.

Altogether, the results show that an auxin/jasmonate interaction affects rice root system development in the presence of cadmium and/or arsenic, even if exogenous jasmonic acid methyl ester only slightly mitigates pollutants toxicity.

1. Introduction

In plants, arsenic (As) and cadmium (Cd) induce detrimental effects impairing development, metabolism and productivity. The earliest and greatest damages occur in the roots altering growth and architecture (Zanella et al., 2016; Fattorini et al., 2017a; Bruno et al., 2017; Ronzan

Abbreviations: AR, adventitious root; *cpm2*, *coleoptile photomorphogenesis2* mutant; IAA, indole-3-acetic acid; JA, jasmonic acid; JA-Ile, jasmonoyl-Isoleucine; JAMe, jasmonic acid methyl ester; JAs, jasmonates; LR, ateral root; LRP, lateral root primordium; MDA, malondialdehyde; Nihon, *Oryza sativa* L. ssp. japonica cv. Nihonmasari; OPDA, 12-oxophytodienoic acid; PR, primary root; ROS, reactive oxygen specpeciecies; TF, translocation factor; wt, wild-type

* Corresponding author at: Department of Environmental Biology, Sapienza University of Rome, Italy.
E-mail address: giuseppina.falasca@uniroma1.it (G. Falasca).

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et al., 2018). Changes ranging from complex interactions among phytohormones, e.g., auxin and jasmonates (JAs), to synthesis of various types of stressors, e.g., reactive oxygen species (ROS), affect the expression of numerous genes related to the plant ability to respond and defend to metal/metalloid toxicity.

Oryza sativa L. is a staple food that supports almost half of world's population. Unfortunately, rice is also the most relevant source of Cd and As contamination for humans (Bhattacharya et al., 2010; Uraguchi and Fujiwara, 2013) due to an increase of these pollutants in paddy fields.

Nevertheless, detailed studies on the effect of As and Cd on rice growth and changes in hormonal crosstalk are still poor. It is known that phytohormones do not act independently but are tightly interconnected in regulating plant development and environmental responses. Auxin (indole-3-acetic acid, IAA) is a key phytohormone for root formation and development (Benková et al., 2003; Della Rovere et al., 2013). Its biosynthesis and distribution within the plant, and mainly in the root system, is altered by heavy metals/metalloids (Sofo et al., 2013; Bahmani et al., 2016; Ronzan et al., 2018). Using a *DR5::GUS* auxin-reporter line in Arabidopsis, it has been demonstrated that Cd increases *DR5* signal in both the primary root (PR), lateral (LR) and adventitious (AR) roots (Wang et al., 2015; Fattorini et al., 2017a), and down-regulates the expression of the IAA efflux-carrier *PIN-FORMED1* (*PIN1)* gene (Wang et al., 2015). However, in the same plant species, other authors reported a reduction of auxin level in the root tip after Cd exposure (Hu et al., 2013; Yuan and Huang, 2016; Bruno et al., 2017). By contrast, As, alone or combined with Cd, reduces or totally inhibits *DR5::GUS* expression in Arabidopsis ARs and LRs (Fattorini et al., 2017a), even if an increase in *DR5* signal in the PR was detected after short arsenite exposure (Bahmani et al., 2016).

We have recently demonstrated in rice that As reduced/inhibited the expression of *AUXIN RESISTANT1* (*AUX1)* and *PIN5* genes, coding for IAA influx and efflux carriers respectively, whereas Cd increased *AUX1* expression and strongly inhibited *PIN5*, thus affecting auxin distribution (Ronzan et al., 2018).

Polyunsaturated fatty acids, components of cell membrane lipids, are the main target of the oxidative impact of toxic elements (Demiral and Türkan, 2005). An increase in the malondialdehyde (MDA) level is known to mark membrane oxidative damage (Verma and Dubey, 2003). Lipid peroxidation follows a Cd-mediated increase of lipoxygenase (LOX) activity (Liptáková et al., 2013), thus enhancing the production of oxylipins, including JAs.

Jasmonic acid methyl ester (JAMe), jasmonic acid (JA), and its metabolically active derivatives JA-Isoleucine (JA-Ile) and JA precursor 12-oxophytodienoic acid (OPDA), are signalling molecules involved in plant growth regulation and stress response (Dhakarey et al., 2017, and references therein). In fact, the endogenous levels of JAs increase after wounding, pathogen infection and metal stress (Rao and Davis, 2001; Kanna et al., 2003; Piotrowska et al., 2009). Evidences have shown that the role of JA is crucial in alleviating heavy metal effects in different plant species (Keramat et al., 2010; Yan and Tam, 2013). Furthermore, JAMe, combined with Cd, reduces the oxidative stress in rice seedlings, improving the antioxidant response and lowering Cd accumulation (Singh and Shah, 2014).

The rice mutant coleoptile photomorphogenesis2 (cpm2) is blocked in the conversion of allene oxide to OPDA by Allene Oxide Cyclase (AOC) due to a deletion of 11 bp in the first exon of the *AOC* gene. The mutation results into low endogenous levels of JAs compared to the wild-type (wt) (Riemann et al., 2013). Surprisingly, recent studies on *cpm2* exposed to drought stress conditions have shown that the mutant copes better with stress than the wt, suggesting a negative role for JAs in rice drought tolerance (Dhakarey et al., 2017).

An auxin/JA crosstalk has been identified in the control of root formation and development in both Arabidopsis and rice (Cai et al., 2014; Liu et al., 2015; Fattorini et al., 2018). One level of hormonal interaction is the modulation of each other's homeostasis and transport. JA mediates the expression of IAA biosynthetic genes, as *ANTHRANILATE SYNTHASE -alpha1* (*ASA1*) and some members of the *YUCCA* family (*YUCCA6*, *YUCCA8* and *YUCCA9*), all involved in root growth (Sun et al., 2009; Hentrich et al., 2013; Veloccia et al., 2016; Fattorini et al., 2017b).

So far, the crosstalk between auxin and JAs in the presence of As and Cd has been poorly investigated in rice root development. However, *OsASA2* and *OsYUCCA2* genes have been found to be active in IAA biosynthesis and affected by Cd and/or As (Ronzan et al., 2018), providing a solid basis for further investigating the interactions between auxin and these pollutants in this crop.

The rice root system is fibrous and comprises ARs of embryonic origin and their post-embryonic LRs (Rebouillat et al., 2009). The root is the preferential target of the soil pollutants. Hence, the JAs/auxin relationship after Cd and/or As exposure on the rice root system development was here investigated in ARs and LRs of wt, transgenic line, and *cpm2* mutant seedlings through an integrated morphological, histochemical, biochemical and molecular approach.

The results indicate that As and Cd affect root system morphology in both genotypes, and 1μM JAMe does not alleviate the damages caused by the toxic compounds. In the roots, *OsASA2* and *OsYUCCA2* expression are affected by the pollutants and by JAMe. However, As and Cd affect IAA and JAs levels in different ways, and lipid peroxidation is enhanced by As, independently from the genotype. An auxin/JAs interaction as a fine-tuning mechanism governing the developmental response/adaptation of the rice root system to Cd and/or As is discussed.

2. Material and methods

2.1. Plant material and growth conditions

Oryza sativa L., ssp. japonica cv. Nihonmasari (Nihon) wild type (wt), the mutant *coleoptile photomorphogenesis2* (*cpm2*) and the rice auxin-reporter line (*OsDR5::GUS*), containing the highly active synthetic auxin response element (DR5) fused to β-glucuronidase (GUS) (Ulmasov et al., 1997), were used for the experiments. Heterozygous *cpm2* seeds were used, because homozygous mutant seeds were not obtained due to the male sterility of the mutant. The screening of mutant plants was carried out according to Riemann et al. (2013). The seeds of *OsDR5::GUS* transgenic line were kindly provided by Prof. J. Xu.

The seeds of all genotypes were sterilized according to Ronzan and co-workers (2018) and sown on a medium containing half-strength Murashige and Skoog salts (MS, 1962), 0.1% sucrose and 0.8% agar, at pH 5.6–5.8 (Control). To this medium were added: 100 μM Na_2HAsO_4. $7H_2O$ (100As); 100 μM $CdSO_4$ (100Cd); 100 μM Na_2HAsO_4. $7H_2O$ plus 50 μM $CdSO_4$ (100 As +50 Cd) and 100 μM $CdSO_4$ plus 50 μM Na_2HAsO_4. $7H_2O$ (100 Cd +50 As) according to Ronzan and co-workers (2018).

The high concentrations of Cd and As used were chosen for two reasons: on one side, rice plants grown *in vitro* for 10 days under these conditions show toxicity symptoms but are not completely inhibited in growth (Ronzan et al., 2018); on the other side, in some regions of Earth, very high Cd and As levels are reached in paddy soil (Kosolsaksakul et al., 2014; Patel et al., 2016).

Moreover, wt and *OsDR5::GUS* seeds were also sown on the Control medium with the addition of 1 μM JAMe combined or not with 100 μM Cd, 100 μM As, 100 μM As + 50 μM Cd and 50 μM As + 100 μM Cd. Jasmonic acid methyl ester was used at 1μM concentration based on our preliminary experiments.

cpm2 seeds were exposed to Cd and/or As only because both the long coleoptile in light and the reduced fertility mutant phenotypes can be restored by exogenous application of JAMe; moreover, JA responsive genes can be triggered by exogenous JAs application (Riemann personal communication).

At least 800 wt seeds and 150 transgenic seeds were sown (5 seeds

per Phytatray™ vessel). The wt and *OsDR5::GUS* seeds were kept in dark conditions, at 28 °C, for 2 days until germination. After germination, the vessels containing the seeds were exposed to a photoperiod of 14 h light (210 μmol/m^2s^{-1}) and 10 h dark and relative humidity at 70%, for other 8 days. Twenty seedlings of 10 days from sowing were used for the experiments.

One thousand six hundred heterozygous *cpm2* seeds were sown on medium containing or not 100As, 100Cd, 100 As +50 Cd and 100 Cd +50 As and exposed directly to the long day photoperiod (as above). Two days after germination their phenotype was screened under the stereomicroscope, and seedlings (1:4) showing the mutant phenotype (Riemann et al., 2013) were selected. Twenty mutant seedlings were used per each treatment. Ultra-pure water (Milli-Q) was used for all culture media.

2.2. Morphological analysis and GUS detection

The morphological analysis was carried out on the root system of wt and *cpm2* seedlings. For each root system, the length of the embryonic adventitious roots (ARs) and the number of lateral roots (LRs) and lateral root primordia (LRPs) formed on the ARs were evaluated under a LEICA MZ8 stereomicroscope. Digital images were captured using Zeiss AxioCam camera associated with the AxioVision Release 4.7.2 software. Moreover, roots and shoots of wt and *cpm2* seedlings were separated and weighted.

In each treatment, the root system of *OsDR5::GUS* seedlings was processed for β-glucuronidase (GUS) staining according to Wang et al. (2015). The roots were then cleared with chloral hydrate solution (Weigel and Glazebrook, 2002), mounted on microscope slides, and observed with Nomarski optics applied to a Leica DMRB optical microscope equipped with a Leica DC 500 camera. The image analysis was performed using LEICA IM1000 Image Manager Software. The GUS signal in LRPs, LRs, and ARs was classified as “Regular”, “Reduced”, “Diffused” or “Absent” and the number expressed as mean percentage (± SE) according to Ronzan and co-workers (2018).

2.3. Cadmium and arsenic detection

In each treatment, wild-type and *cpm2* seedlings were harvested, rinsed with ultra-pure water, divided into roots and shoots, and freeze-dried at 0.05 mbar and −20 °C for 24 h to determine plant dry weight. Each sample, consisting of 0.01–0.1 g dried material, was digested according to Ronzan and co-workers (2018).

The As and Cd concentrations in both roots or shoots were measured with ICP-MS (X-Series II, Thermo Fisher) after microwave acid digestion (MLS Start 1500) using HNO_3 (65%, sub-boiled) and H_2O_2 (30%, p.a.). One blank and one certified plant standard material (NIES CRM No. 10-c, unpolished rice) per every eight samples was included in the process to verify digestion quality. The recovery for As and Cd was around 80%. Additionally, the certified reference material CRM-TMDW-A (High-Purity standards, Inc.) was included into the protocol (accuracy: ± 5% for both elements) to check the quality of the ICP-MS measurements.

The translocation factor (TF) for As and Cd was evaluated as the ratio of As and Cd levels in shoots vs roots.

2.4. Hormone analyses

Auxin (IAA), jasmonic acid (JA), jasmonoyl-isoleucine (JA-Ile) and 12-oxophytodienoic acid (OPDA) were quantified in the wt and *cpm2* roots and shoots. The seedlings were grown for 10 days on agarized medium containing the same MS salts and sucrose concentrations as reported above (Control medium), then transferred to a hydroponic medium containing the MS salts and sucrose, and with/without Cd and/or As, for 8 h, 48 h, and 10 days.

IAA was extracted from 20 to 50 mg of frozen samples and quantified using [$^2H_5$]-IAA (0.025 ng/μl extraction solvent) as described by Ziegler et al. (2014). The [$^2H_5$]-IAA specific multiple reaction monitoring parameters are reported in Table Supplementary Information 1.

For simultaneous quantification of OPDA, JA and JA-Ile, 20–50 mg of frozen samples were homogenized in a mortar under liquid nitrogen and extracted with 500 μL methanol containing 0.1 ng/μL of each stable isotope-labeled internal standard ([$^2H_5$]OPDA, [$^2H_6$]JA, [$^2H_2$]JA-Ile). Extracts were purified by solid phase extractions using a strong cation exchange HR-XC material (Macherey & Nagel, Düren,Germany). Hormone contents were determined using a standardized ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)-based method according to Balcke et al. (2012).

2.5. Quantitative RT-PCR analysis of ASA2 and YUCCA2 genes

The root system of 10 seedlings grown in the presence/absence of Cd and/or As and/or JAMe was harvested, frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction. Total RNA was isolated using the Spectrum Plant Total RNA Kits (Sigma-Aldrich) according to the manufacturer’s instructions. RNA concentration and purity were evaluated spectrophotometrically using SmartSpec™ Plus Spectrophotometer (BIORAD).

For cDNA synthesis, 1 μg of total RNA was reversely transcribed with Bioline SensiFAST™ Reverse Transcriptase cDNA synthesis kit according to the manufacturer’s instructions. Relative levels of *OsASA2* and *OsYUCCA2* mRNAs were examined by real-time PCR, using Bioer LineGene 9620 qPCR detection system. Specific primers were designed (Table Supplementary Information 2) using NCBI Primer- BLAST for both the genes of interest; the reference genes used were *OsGAPDH* and *OsUBQ10* according to Ronzan and co-workers (2018).

The RT-qPCR experiments were carried out in 10 μL reactions containing the final concentration of 250 nM for each primer, 1x SYBR green (SensiMIX™ SYBR® No-ROX Kit) and 1 μL of a 1:10 cDNA dilution according to Ronzan et al. (2018). Amplification parameters were: 95 °C for 3 min; 40 amplification cycles (95 °C for 15 s, 60 °C for 30 s).

2.6. Peroxidation analysis

Roots (400 mg of fresh weight per treatment and genotype) of wt and *cpm2* seedlings exposed or not to Cd and/or As for 5 and 10 days were used for peroxidation analysis. Peroxidation of membrane lipids was detected through the quantification of the Thio-Barbituric Acid Reactive Species (TBARS) such as malondialdehyde (MDA). MDA assay was performed according to Taulavuori et al. (2001) with slight modifications. The roots were homogenized in liquid nitrogen and extracted with 6 ml of 0.1% Trichloroacetic acid (TCA). The extracted solution was centrifuged for 5 min at 2268 *g* at room temperature. Per each treatment, 4 ml of the supernatant were added into 4 Falcons (1 ml in each tube), of which only one contained 1 ml of TCA at 20% (blank) and the other 3 contained 1 ml of Thiobarbituric acid (TBA) at 0.5% in TCA at 20%. The solutions were heated at 95 °C for 30 min and cooled down in ice for 10 min to stop the reaction. The solutions were then centrifuged at 1000 *g* for 5 min at room temperature. The absorbance of the supernatants was measured, within the following 30 min, at 440, 532 and 600 nm. The MDA values were calculated according to Hodges et al. (1999) and expressed as nmoles/g of MDA on fresh weight.

2.7. Statistical analysis

Statistical analysis was performed using one or two way ANOVA test followed by Tukey’s post-test ($P < 0.05$). All the experiments were performed in three biological replicates with similar results.

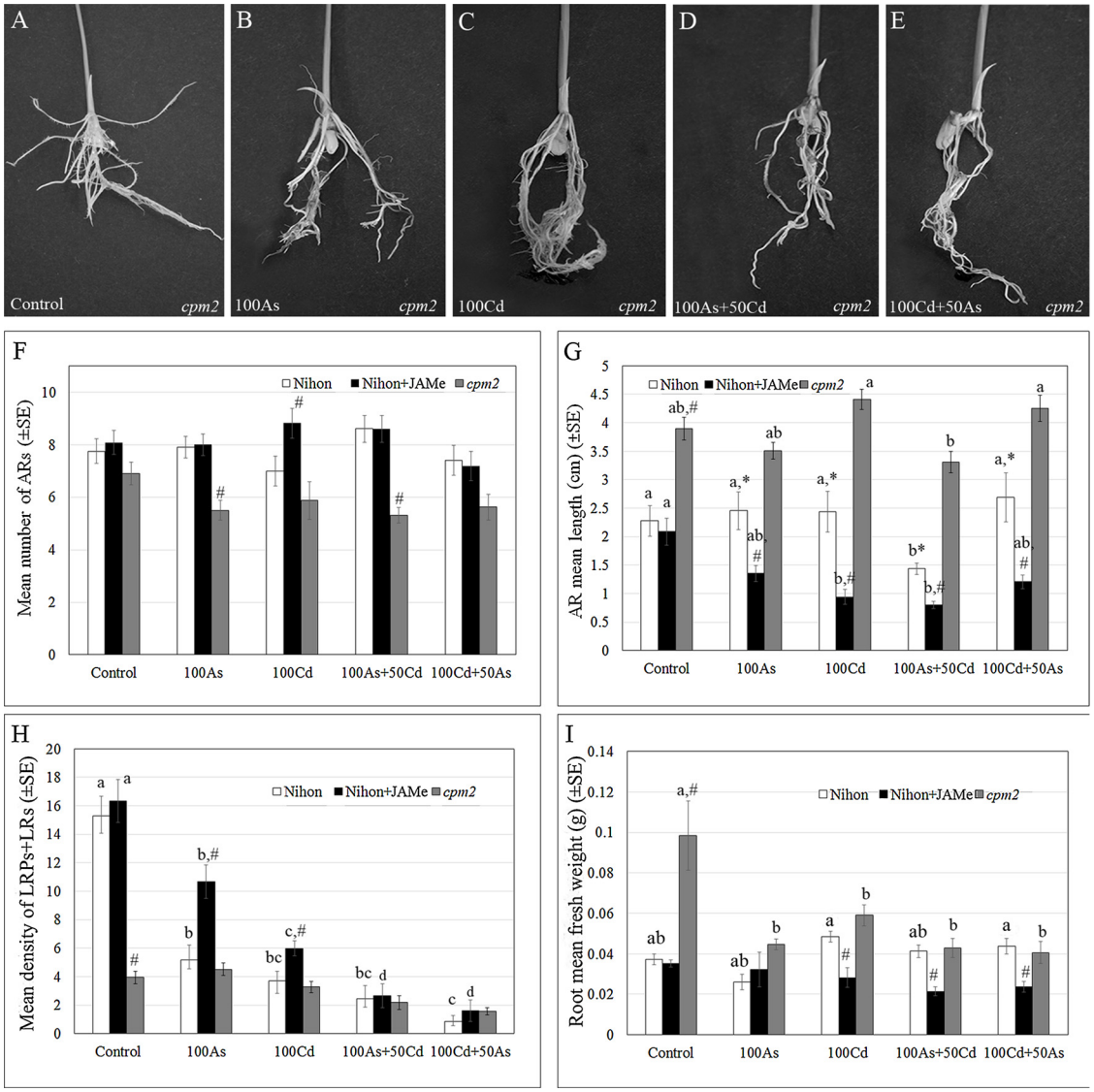

Fig. 1. *cpm2* root system morphology. A-E, Roots of seedlings non exposed to Cd and/or As (Control, A) or treated with 100 μM $Na_2HAsO_4{\cdot}7H_2O$ (100As, B), 100 μM $CdSO_4$ (100Cd, C), 100 μM $Na_2HAsO_4{\cdot}7H_2O$ plus 50 μM $CdSO_4$ (100As + 50Cd, D) and 100 μM $CdSO_4$ plus 50 μM $Na_2HAsO_4{\cdot}7H_2O$ (100Cd + 50As, E) for 10 days. Mean number of adventitious roots (ARs) (F), AR mean length (G), mean density of lateral root primordia (LRPs) and lateral roots (LRs) (H), root mean fresh weight (I) (± SE) in *wt* (Nihon) seedlings not treated (Control) or treated with 100As, 100Cd, 100As + 50Cd and 100Cd + 50As and/or 1 μM jasmonic acid methyl ester (JAMe) for 10 days. Mean number of ARs (F), AR mean length (G), mean density of LRPs and LRs (H), root mean fresh weight (I) (± SE) in *cpm2* seedlings not treated (Control) or treated with 100As, 100Cd, 100As + 50Cd and 100Cd + 50As for 10 days.
Different letters show statistic differences among treatments within Nihon, Nihon + JAMe and *cpm2*, respectively. Different symbols show statistic differences among Nihon, Nihon + JAMe and *cpm2*, within the same treatment. Significant differences at least at $P < 0.05$ level are shown. N = 20.

3. Results

3.1. Arsenic and cadmium affect root system morphology in both wt and cpm2

A morphological analysis was carried out on the root system of wt seedlings, treated or not with Cd and/or As and with or without 1 μM JAMe (JAMe), and of *cpm2* exposed to the toxic compounds (Fig. Supplementary Information 1 and Fig. 1A-E).

Cadmium and As, alone or combined, did not affect the mean number of ARs in the wt (Fig. 1F). Also, the addition of JAMe did not change significantly the AR mean number in wt seedlings exposed to the toxic compounds, except for the Cd treatment in which JAMe significantly ($P < 0.05$) increased ARs (Fig. 1F). On the contrary, 100As, alone or combined with 50Cd, significantly ($P < 0.001$) reduced the mean number of ARs in the *cpm2* mutant in comparison with the wt, treated or not with JAMe (Fig. 1F).

Arsenic or Cd did not affect AR length in the wt (Nihon) (Fig. 1G); however, 100As plus 50Cd significantly ($P < 0.05$) reduced it in comparison with the Control and the other treatments (Fig. 1G). The addition of JAMe did not result into a change in AR length in the wt seedlings not exposed to the toxic compounds, but significantly ($P < 0.001$) decreased it when combined with Cd and/or As (Fig. 1G). In *cpm2* seedlings the AR mean length was significantly ($P < 0.0001$) higher than in the wt, exposed or not to the toxic compounds and JAMe (Fig. 1G).

In addition, Arsenic and Cd, alone or combined, strongly ($P < 0.001$) reduced the density of LRPs/LRs in wt seedlings (Fig. 1H). JAMe significantly ($P < 0.001$) increased LRPs/LRs density when combined with As or Cd alone in comparison to the respective pollutant absence, whereas did not change it in the combined treatments (Fig. 1H). In the Control, LRPs/LRs density of *cpm2* seedlings was

significantly ($P < 0.0001$) reduced in comparison with the wt treated or not with JAMe (Fig. 1H). The exposure to Cd and/or As did not change LRP/LR density in the mutant seedlings (Fig. 1H). The root fresh weight was similar in the wt exposed or not to Cd and/or As (Fig. 1J) and also JAMe did not significantly affect it (Fig. 1I). In the Control *cpm2* seedlings the root fresh weight was higher ($P < 0.001$) than in the wt, independently from JAMe treatment (Fig. 1I). Moreover, As and/or Cd significantly ($P < 0.001$) reduced root fresh weight in *cpm2* in comparison to the Control (Fig. 1I).

We also evaluated the shoot fresh weight in the wt exposed to Cd and/or As and to JAMe, and in the mutant exposed to toxic compounds only (Fig. Supplementary Information 2). The exposure to Cd and As, alone or combined, significantly ($P < 0.01$) reduced mean shoot fresh weight in wt seedlings, and JAMe showed no effect on it (Fig. Supplementary Information 2). The shoot fresh weight of *cpm2* seedlings was significantly ($P < 0.01$) higher than the wt, treated or not with JAMe, in every treatment condition (Fig. Supplementary Information 2).

3.2. Jasmonates slightly influence arsenic and cadmium accumulation in rice roots and shoots

Arsenic and Cd accumulated in the roots more than in the shoots of *cpm2* and wt seedlings (Fig. 2A-B and C–D). Moreover, in the roots of both genotypes, As levels were more than twice higher than Cd levels either when the metalloid was present alone in the culture, or when it was combined with the heavy metal (Fig. 2A-B). Cadmium was transferred to the aerial organs more than As in both genotypes (Fig. 2C–D). In fact, the TF in the wt was 0.20 and 0.06 for Cd and As respectively, and 0.33 and 0.014 for Cd and As in *cpm2*. In wt roots, the As levels reflected its concentrations in the media, whereas, the presence of Cd significantly ($P < 0.001$) reduced As accumulation (Fig. 2A). The presence of JAMe did not change the root As levels, both in the As alone and in 100As plus 50Cd. On the contrary, JAMe significantly ($P < 0.01$) reduced root As levels in 100Cd plus 50As treatment (Fig. 2A).

In the mutant, As levels in the roots were similar for each treatment condition but were significantly lower ($P < 0.001$) than the wt in the As alone treatment (Fig. 2A). However, in the mutant exposed to the combined treatments, the As levels were similar to those in wt roots (Fig. 2A).

Cadmium levels were higher in the single treatment respect to the combined one in wt roots, (Fig. 2B) indicating that when the two elements are simultaneously present in the medium a competition occurs to enter the root cells. The presence of JAMe significantly decreased Cd level in the Cd alone treatment (Fig. 2B). In *cpm2* roots, Cd level reached the highest level ($P < 0.001$) when it was applied alone (Fig. 2B).

The As levels in wt shoots were low for every treatment, except for 100As plus 50Cd, where a highly significant ($P < 0.001$) increase was observed and was generally unaffected by JAMe (Fig. 2C). Arsenic was very low in *cpm2* shoots under all treatments (Fig. 2C). In wt shoots, Cd levels were higher in the seedlings exposed to Cd alone in comparison to the combined treatments (Fig. 2D), as it was in the roots (Fig. 2B). Moreover, JAMe did not change significantly Cd levels in comparison to its absence (Fig. 2D). No differences with the wt were observed for Cd accumulation in *cpm2* shoots (Fig. 2D).

Finally, JAMe did not affect the wt ability to translocate As and Cd from roots to shoots because the TF of As was 0.06 either in the presence or absence of JAMe, and that of Cd was 0.20 and 0.24 in absence and presence of JAMe, respectively.

3.3. Cd and/or As affect auxin levels in the wt more than in the mutant

In the absence of Cd and/or As, IAA levels in wt roots were stable during the whole culture period (Fig. 3A). On the contrary, in *cpm2* roots an increase of IAA was observed at 48 h (Fig. 3C). In wt roots, after 48 h of As and/or Cd treatment, a significant ($P < 0.001$) increase in IAA content was observed. The hormone levels remained high until 10d in roots exposed to As alone and 100Cd plus 50As (Fig. 3A). In *cpm2* roots, exposed to As alone, the IAA level remained low and quite stable during the whole culture period (Fig. 3C). In the same mutant, Cd caused a weak but significant ($P < 0.05$) increase of IAA in roots after 48h of exposure (Fig. 3C); the two combined treatments, instead, induced a significant decrease of hormone levels after 8 h of culture (Fig. 3C).

The IAA levels were overall higher in the shoots compared to the roots of both genotypes (Fig. 3B,D and A,C). In wt shoots, not exposed

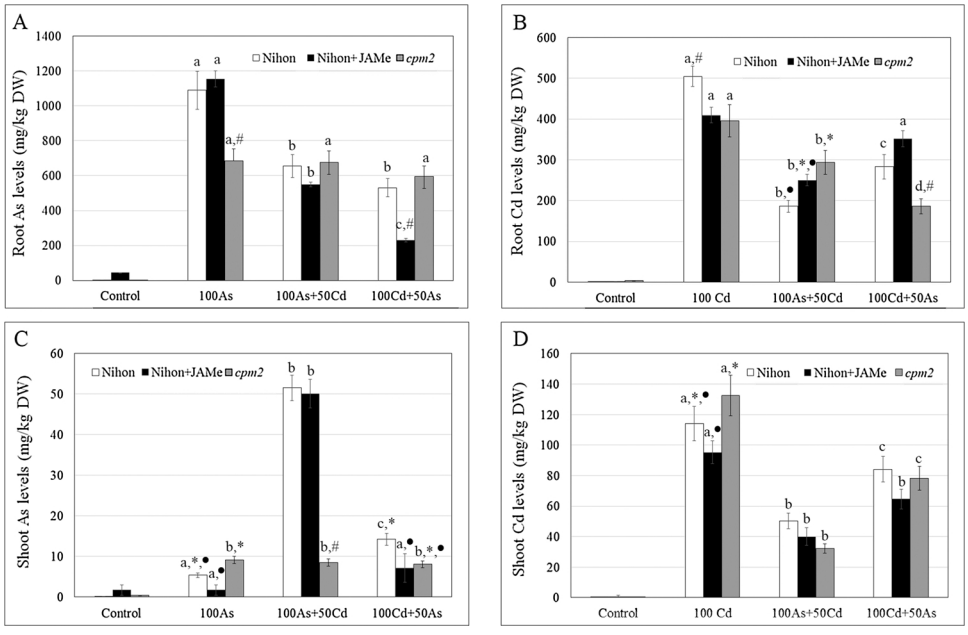

Fig. 2. Arsenic and Cd accumulation in wt (Nihon) and *cpm2* roots and shoots. A- B, As (A) and Cd (B) mean levels (± SE) in Nihon roots non exposed (Control) or exposed to 100 µM $Na_2HAsO_4{\cdot}7H_2O$ (100As), 100 µM $CdSO_4$ (100Cd), 100 µM $Na_2HAsO_4{\cdot}7H_2O$ plus 50 µM $CdSO_4$ (100As + 50Cd) and 100 µM $CdSO_4$ plus 50 µM $Na_2HAsO_4{\cdot}7H_2O$ (100Cd + 50As) and/or to 1 µM jasmonic acid methyl ester (JAMe), and As (A) and Cd (B) mean levels (± SE) in *cpm2* roots non exposed (Control) or exposed to 100As, 100Cd, 100As + 50Cd and 100Cd + 50As for 10 days. C-D, As (C) and Cd (D) mean levels (± SE) in Nihon shoots non exposed (Control) or exposed to 100As, 100Cd, 100As + 50Cd and 100Cd + 50As) and/or to 1 µM JAMe, and As (C) and Cd (D) mean levels (± SE) in *cpm2* shoots non exposed (Control) or exposed to 100As, 100Cd, 100As + 50Cd and 100Cd + 50As for 10 days. Different letters show statistic differences among treatments within Nihon, Nihon + JAMe and *cpm2*, respectively. Different symbols show statistic differences among Nihon, Nihon + JAMe and *cpm2*, within the same treatment. Significant differences at least at $P < 0.05$ level are shown. N = 20.

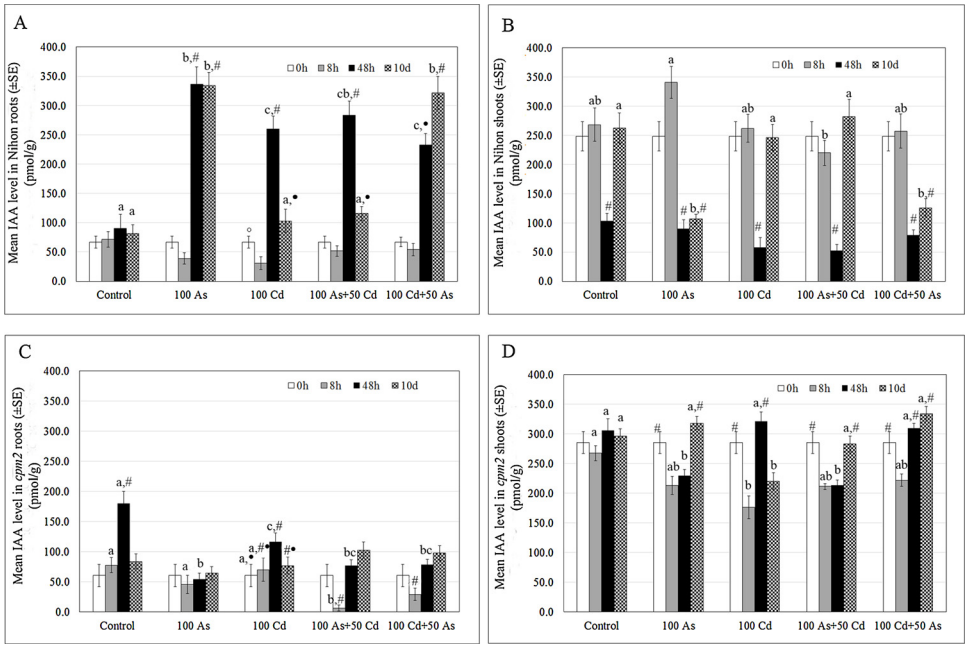

Fig. 3. Endogenous IAA contents in wt (Nihon) and *cpm2* roots and shoots. A-D, IAA level in Nihon roots (A) and shoots (B) and in *cpm2* roots (C) and shoots (D) non-exposed (Control) or exposed to 100 μM $Na_2HAsO_4{\cdot}7H_2O$ (100As), 100 μM $CdSO_4$ (100Cd), 100 μM $Na_2HAsO_4{\cdot}7H_2O$ + 50 μM $CdSO_4$ (100As + 50Cd) and 100 μM $CdSO_4$ + 50 μM $Na_2HAsO_4{\cdot}7H_2O$ (100Cd + 50As) for 10 days. Different letters show statistic differences among treatments at the same time point. Different symbols show statistic differences within the same treatment at different times. Significant differences at least at $P < 0.05$ level are shown. Mean of 3 biological replicates.

or exposed to the toxic compounds, a drastic reduction of IAA was measured at 48 h of culture and was followed by a significant ($P < 0.001$) increase at 10d, except for the As alone or combined with 100Cd treatments in which the hormone levels remained low (Fig. 3B). In *cpm2* shoots, not exposed to Cd or As, IAA levels were more stable during the culture period. However, in the presence of the toxic compounds, alone or combined, a significant ($P < 0.05$) decrease was observed at 8h, followed by an increase up to 10d (Fig. 3D).

3.4. Cadmium and arsenic affect jasmonates levels in the wt more than in cpm2

In general, in wt roots, the OPDA levels were higher than those of JA and JA-Ile during the entire culture period (Fig. 4A–C). In the Control, OPDA and JA-Ile levels significantly ($P < 0.05$) increased after 8h of culture, while JA significantly ($P < 0.05$) increased after 48h (Fig. 4A–C). After 10d of culture, the levels of OPDA, JA and JA-Ile were similar to those at the beginning (Fig. 4A–C). During the culture period, As and Cd, alone or combined, induced weak and non-significant fluctuations in OPDA levels (Fig. 4A). Cadmium induced precocious (8 h–48 h) and significant ($P < 0.001$) increases of JA and JA-Ile (Fig. 4B-C). Arsenic alone, instead, increased JA-Ile levels after 8 h (Fig. 4C), whereas the combined treatments did not change significantly JA and JA-Ile levels (Fig. 4B-C).

In wt shoots not exposed to the toxic compounds, OPDA levels remained similar until 48 h and then significantly decreased (Fig. 4D). On the contrary, JA and JA-Ile significantly ($P < 0.001$) increased during the first 48h and then strongly decreased (Fig. 4E–F). Arsenic alone weakly influenced JA and JA-Ile levels, but reduced significantly ($P < 0.001$) OPDA levels at 8h (Fig. 4D-E). Cadmium alone induced significant increases of JA and JA-Ile during the first 8 h–48 h, and strong decreases at 10d (Fig. 4E–F). The OPDA levels significantly ($P < 0.01$) decreased at 8h in the presence of Cd alone, and then remained stable (Fig. 4D). The two combined treatments did not modify significantly JA concentrations (Fig. 4E). Only a slight but significant ($P < 0.05$) increase in JA-Ile occurred under 100Cd plus 50As treatment at 8h (Fig. 4F). On the contrary, OPDA changed during the culture with a significant decrease after 8 h–48 h of exposure to combined treatments (Fig. 4D).

In *cpm2* roots and shoots, JA was not detectable, independently of the heavy metal and/or metalloid exposure and treatment duration. However, racemic forms of JA-Ile and OPDA, non-enzymatically produced, were detected in *cpm2*. In fact, in the mutant roots, non-exposed to the toxic compounds, a significant ($P < 0.001$) increase of OPDA levels was observed at 10d (Fig. 5A). Arsenic alone significantly ($P < 0.001$) increased OPDA starting from 48h (Fig. 5A). Cadmium alone did not modify OPDA level in *cpm2* roots, and the two combined treatments significantly ($P < 0.01$) increased it at 10d (Fig. 5A). Very low levels of JA-Ile were observed in *cpm2* roots, and Cd and As, alone or combined, did not influence such values during the culture (Fig. 5B). Finally, in *cpm2* shoots OPDA and JA-Ile were non-affected by the exposure to the toxic compounds during the culture (Fig. 5C–D).

3.5. OsASA2 and OsYUCCA2 expression are affected by the toxic compounds and jasmonates

Taken together, the above results show that the root system, more than the shoots, was affected by the pollutants. For this reason, subsequent analyses were focused on the roots only.

To verify the effects of JAs on *OsASA2* and *OsYUCCA2* expression after Cd and/or As exposure, a transcription analysis was carried out in roots of wt seedlings exposed or not to the toxic compounds, and to JAMe. The transcript levels of the two genes were also evaluated in *cpm2* roots after exposure to Cd and/or As. Fig. 6 shows *OsASA2* and *OsYUCCA2* expression levels normalized with *OsGAPDH*. Comparable results were obtained by using *OsUBQ10* as reference gene (Fig. Supplementary Information 3 A–C).

The exposure to As and Cd alone induced a significant ($P < 0.001$) over-expression of *OsYUCCA2* in the wt roots. Cadmium alone strongly increased *OsASA2* expression (Fig. 6A), whereas the expression levels of both genes in combined treatments were similar to the Control (Fig. 6A).

The JAMe treatment, instead, strongly ($P < 0.001$) increased *OsASA2* and *OsYUCCA2* expression in comparison with the Control (Fig. 6B). Interestingly, when the toxic compounds were combined with JAMe no significant variation in gene expression was observed, except for As, that induced a significant ($P < 0.01$) over-expression of *OsASA2* when combined with JAMe, even if many folds lower than with JAMe alone (Fig. 6B).

The RT-qPCR analysis in the mutant roots showed that *YUCCA2* expression was strongly affected by As and Cd alone and by 100Cd plus 50As (Fig. 6C). *OsASA2* expression also significantly ($P < 0.01$)

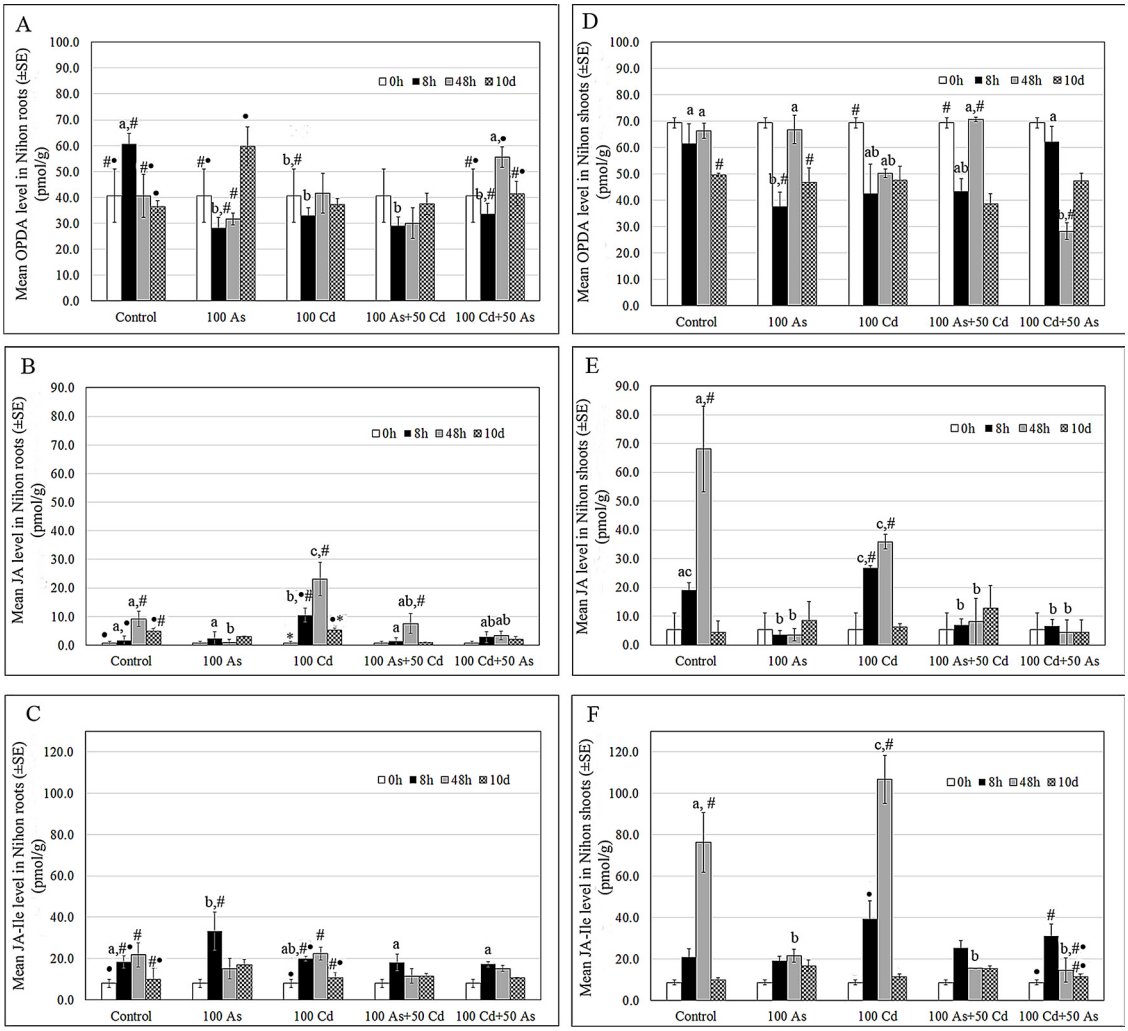

Fig. 4. Endogenous jasmonates contents in wt (Nihon). A-F, OPDA, JA, JA-Ile levels in roots (A,B,C) and shoots (D,E,F) of Nihon non-exposed (Control) or exposed to 100 μM $Na_2HAsO_4{\cdot}7H_2O$ (100As), 100 μM $CdSO_4$ (100Cd), 100 μM $Na_2HAsO_4{\cdot}7H_2O$ + 50 μM $CdSO_4$ (100As + 50Cd) and 100 μM $CdSO_4$ + 50 μM $Na_2HAsO_4{\cdot}7H_2O$ (100Cd + 50As) for 10 days.
Different letters show statistic differences among treatments at the same time point. Different symbols show statistic differences within the same treatment at different times. Significant differences at least at $P < 0.05$ level are shown. Mean of 3 biological replicates.

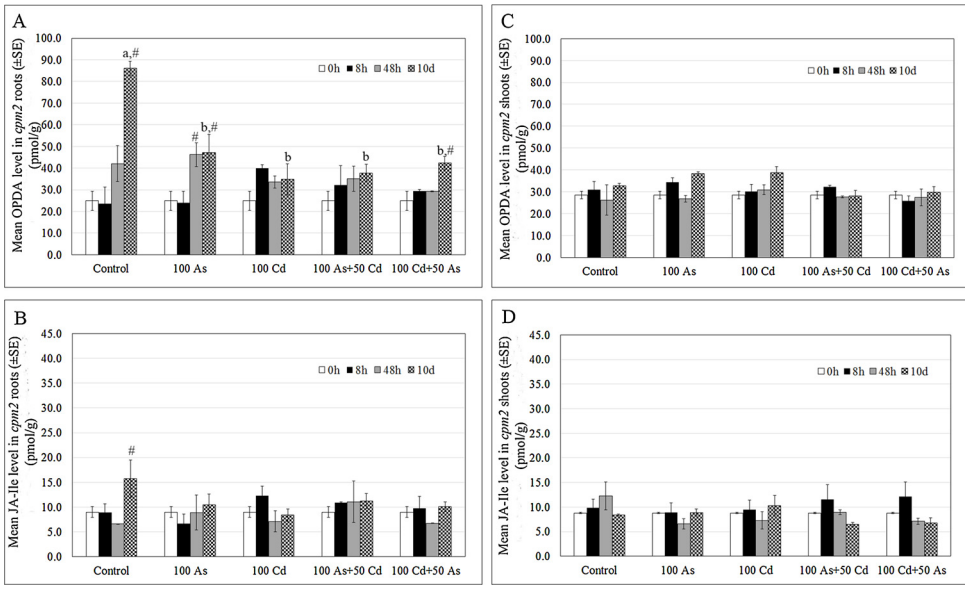

Fig. 5. Endogenous jasmonates contents in *cpm2*. A-D, OPDA and JA-Ile levels in roots (A-B) and shoots (C-D) of *cpm2* non-exposed (Control) or exposed to 100 μM $Na_2HAsO_4{\cdot}7H_2O$ (100As), 100 μM $CdSO_4$ (100Cd), 100 μM $Na_2HAsO_4{\cdot}7H_2O$ + 50 μM $CdSO_4$ (100As + 50Cd) and 100 μM $CdSO_4$ + 50 μM $Na_2HAsO_4{\cdot}7H_2O$ (100Cd + 50As) for 10 days.
Different letters show statistic differences among treatments at the same time point. Different symbols show statistic differences within the same treatment at different times. Significant differences at least at $P < 0.05$ level are shown. Mean of 3 biological replicates.

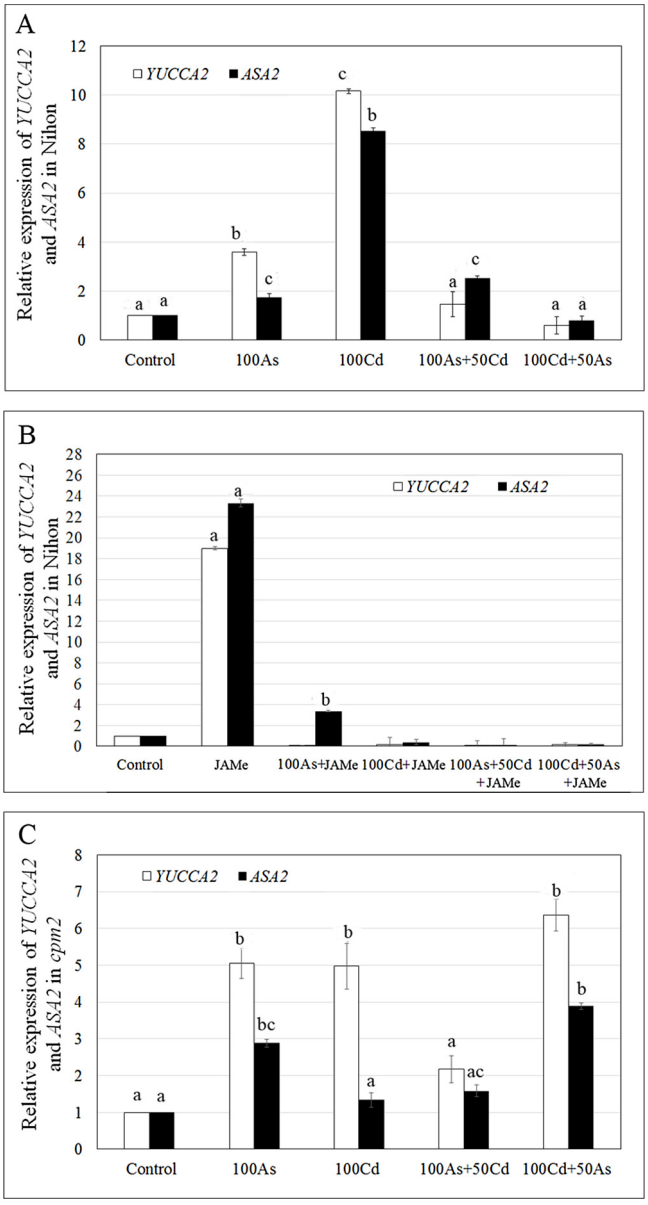

Fig. 6. Relative expression of *OsASA2* and *OsYUCCA2* genes (RT-qPCR analysis) in wt (Nihon) and *cpm2* roots. A-B, *OsASA2* and *OsYUCCA2* expression in Nihon non exposed (Control) or exposed to 100 µM $Na_2HAsO_4{\cdot}7H_2O$ (100As), 100 µM $CdSO_4$ (100Cd), 100 µM $Na_2HAsO_4{\cdot}7H_2O$ plus 50 µM $CdSO_4$ (100As + 50Cd) and 100 µM $CdSO_4$ plus 50 $Na_2HAsO_4{\cdot}7H_2O$ µM (100Cd + 50As) (A) and/or to 1 µM jasmonic acid methyl ester (JAMe) (B) for 10 days. C, *OsASA2* and *OsYUCCA2* expression in *cpm2* non exposed (Control) and exposed to 100As, 100Cd, 100As + 50Cd and 100Cd + 50As for 10 days.
The expression levels of the two genes in the Control were set to 1. Different letters show statistic differences for the same gene among treatments. Significant differences at least at $P < 0.05$ level are shown. Mean of 3 biological replicates.

increased in the presence of As alone and 100Cd plus 50As (Fig. 6C).

3.6. Auxin distribution in the rice root system is altered by Cd and As and JAMe

To evaluate whether JAs affected auxin distribution in the roots treated with Cd and/or As, a histochemical analysis was carried out using *OsDR5::GUS* seedlings exposed or not to JAMe, and to Cd and/or As (Fig. 7). The embryonic ARs exhibited a *DR5::GUS* signal similar to the Control for every treatment (data not shown), whereas this was not the case for LRPs and LRs (Fig. 7 A–M).

According to Ronzan and co-workers (2018), the *DR5* signal in LRPs/LRs was classified as “Regular” when present in the root cap and provascular cells or in the basal region of the LRP, as “Diffused” if spread to the entire root meristem and to the root cap, as “Reduced” when present only in the columella cells and the provascular cells, or as “Absent” when not observed all over the root tip.

In the Control, most of the LRPs showed *DR5* expression in the basal part (Fig. 7A), in most of LRs in the root cap and slightly in provascular cells (Fig. 7B). However, in about 20% of LRs, the *DR5* signal was more extended to provascular cells (Fig. 7C). The exposure to JAMe alone resulted into a reduction in the intensity of the GUS signal in the LRPs and the LRs (Fig. 7D). In accordance, the evaluation of the mean percentage of LRPs/LRs with Regular, Diffused, Reduced or Absent GUS signal, showed that JAMe strongly ($P < 0.001$) increased the percentage of roots with Reduced signal in comparison to the Control (Fig. 7N). Also, As alone reduced *DR5* expression in LRs, while expression was prevalently Regular in LRPs (Fig. 7E–F). Because LRPs were more abundant than LRs, the combination of the two categories resulted into an increase in the percentage of LRPs/LRs with Regular signal in comparison to the JAMe alone treatment (Fig. 7N). A decrease, or a diffusion, of the GUS signal was observed in LRPs and LRs treated with 100As plus JAMe, where the percentage of roots with Regular signal was very low, i.e., about 5% (Fig. 7N), while the percentage with a Reduced signal increased (Fig. 7G,N).

In the Cd treatment a significant ($P < 0.001$) decrease in the LRPs/LRs with a Regular signal, and a strong ($P < 0.001$) increase in those with a Diffused signal, in comparison with the Control, was observed (Fig. 7H, N). The addition of JAMe to Cd induced a significant increase of LRPs and LRs with Reduced signal ($P < 0.001$) in comparison to Cd alone (Fig. 7I-J, N). In both the combined treatments a significant ($P < 0.001$) decrease of LRPs and LRs with Regular signal and a significant ($P < 0.001$) increase in those with a Reduced signal, compared to the Control, was observed (Fig. 7K, N). The addition of JAMe to the As and Cd combined treatments strongly reduced *DR5* expression (Fig. 7L-M, N).

3.7. The absence of endogenous jasmonates increase lipid peroxidation in the roots, and mainly in As presence

To evaluate the extent of lipid peroxidation in root cells exposed to As and/or Cd, and in the absence/reduction of endogenous JAs, as occurs in the *cpm2*, the MDA levels were evaluated in the wt and *cpm2* root systems exposed for 5 and 10 days to the toxic compounds.

In wt ARs and LRPs/LRs, the MDA levels significantly ($P < 0.001$) increased in the Control at 10d of culture in comparison to 5d (Fig. 8). In the mutant roots, not treated with the pollutants, the MDA levels were higher than in the wt already at 5d, and further increased until 10d (Fig. 8). Compared to the Control, As significantly ($P < 0.001$) increased MDA levels in wt roots at 5d, whereas the other treatments only induced a significant increase later (Fig. 8). In *cpm2* mutant, the MDA levels were significantly higher ($P < 0.001$) in comparison to wt, either at 5d (Control, As alone, and combined treatments), or at 10d (all treatments), with the highest ($P < 0.001$) increase in comparison with the Control for the As single treatment at 5d (Fig. 8).

4. Discussion

Present data show that the combination of exogenous JAMe with Cd and/or As alters the rice root system affecting endogenous levels of IAA and of the biologically active JAs, auxin distribution and lipid peroxidation extent. The increase in AR length in *cpm2* mutant, exposed or not to the toxic compounds, suggests that JA acts as a stressor for AR development in rice. In accordance, Cho et al. (2007) showed that exogenous JAs suppressed a number of genes in rice roots, mostly coding for defence-related proteins or enzymes with antioxidant

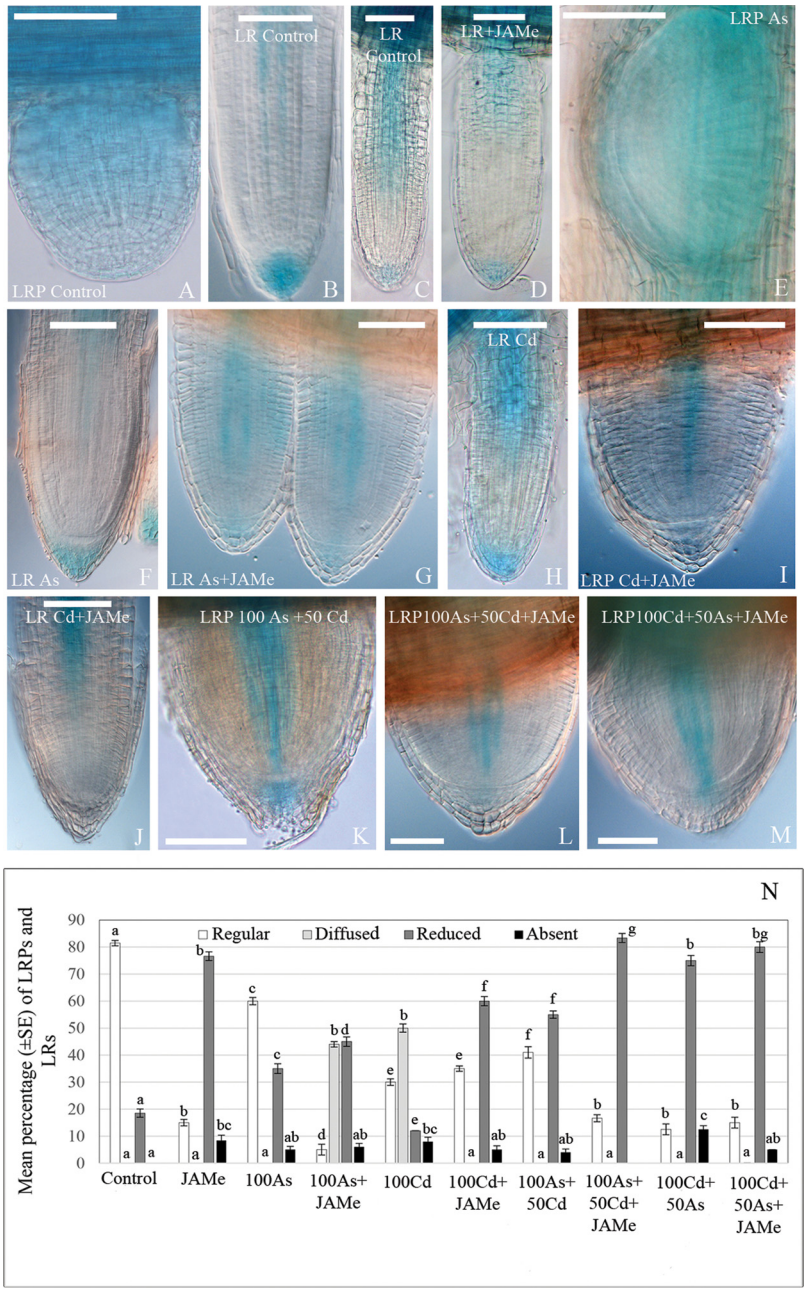

Fig. 7. Expression pattern of *DR5::GUS* in lateral root primordia (LRPs) and lateral roots (LRs) of *OsDR5::GUS* seedlings non exposed (Control, A-C) or exposed to 1 μM jasmonic acid methyl ester (JAMe, D), 100 μM $Na_2HAsO_4{\cdot}7H_2O$ (100As, E-F), 100 μM $Na_2HAsO_4{\cdot}7H_2O$ + 1 μM jasmonic acid methyl ester (100As + JAMe, G) 100 μM $CdSO_4$ (100 Cd, H), 100 μM $CdSO_4$ + 1 μM jasmonic acid methyl ester (100Cd + JAMe, I-J), 100 μM $Na_2HAsO_4{\cdot}7H_2O$ + 50 μM $CdSO_4$ (100As + 50Cd, K), 100 μM $Na_2HAsO_4{\cdot}7H_2O$ + 50 μM $CdSO_4$ + 1 μM jasmonic acid methyl ester (100As + 50Cd + JAMe, L) and 100 μM $CdSO_4$ + 50 μM $Na_2HAsO_4{\cdot}7H_2O$ + 1 μM jasmonic acid methyl ester (100Cd + 50As + JAMe, M). Bars = 100 μm.
N, Mean percentage (± SE) of LRPs/LRs with Regular, Diffused, Reduced or Absent *DR5::GUS* expression.
Different letters show statistic differences for the same signal category among treatments. Significant differences at least at $P < 0.05$ level are shown. N = 20.

activities. However, the wt AR length was reduced only when JAMe was combined with As or Cd, suggesting a common action of Cd/As and JAMe as stressors. The effect of these stressors changed with the developmental origin of the root type. In fact, the LRs increased in density when JAMe was combined with As and Cd alone. The induction of new roots may be interpreted as an adaptation response to stress conditions, e.g. for helping the root system to better explore the soil in the presence of pollutants. However, when the two pollutants were applied together, LR formation became too reduced for being reverted by JAMe application. Thus, differently from the effect on the embryonic-in-origin ARs, JAs might function as counteractors of As/Cd effects on the post-embryonic LRs, as long as the toxicity is not too high. Dhakerey and coworkers (2017) reported that the drought-stressed root system of *cpm2* develops better than in the wt. In accordance with this, present data show that the AR length in the mutant was always higher than in the wt, and unaffected by the treatments. However, the LRP/LR density was lower than the wt, but again unaffected by the treatments, suggesting that a reduction in the LR component of the root system might be a consequence of the JA biosynthesis impairment of the mutant.

We observed that As and Cd levels in roots were differently affected by the treatments. In particular, the levels of both elements were significantly reduced by the simultaneous presence of Cd and As in the medium. On the contrary, JAs presence slightly affected Cd and As levels in the roots (Fig. 2A-B). Differences in the metal/metalloid uptake in plants can depend on elements bioavailability in the growing medium. In fact, when Cd and As are present separately in the soil their bioavailability is greater than when they are combined (Liu and Zhang, 2007; Sun et al., 2008; Ghiani et al., 2014).The data observed here are in accordance with these findings, showing that Cd and As show an antagonistic effect on their uptake also in rice seedlings when applied together. This could be due to the variation of the soil pH (Ghiani et al., 2014; Punshon et al., 2017), but also to a possible association of an arsenate fraction with Cd to form cadmium arsenate, therefore resulting into a reduction of As and Cd available for uptake (Ghiani et al., 2014). Thus, we can speculate that, similarly to wheat seedlings (Liu and Zhang, 2007), also in our conditions Cd and As levels in rice roots are

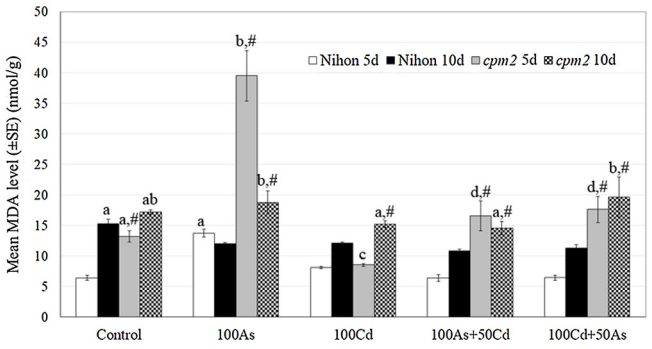

Fig. 8. MDA content in wt (Nihon) and *cpm2* roots non-exposed (Control) or exposed to 100 μM $Na_2HAsO_4{\cdot}7H_2O$ (100As), 100 μM $CdSO_4$ (100Cd), 100 μM $Na_2HAsO_4{\cdot}7H_2O$ + 50 μM $CdSO_4$ (100As + 50Cd) and 100 μM $CdSO_4$ + 50 μM $Na_2HAsO_4{\cdot}7H_2O$ (100Cd + 50As) for 5 days and 10 days.
Different letters show statistic differences for the same days/genotype among the treatments. Different symbols show statistic differences between different genotype at the same day and treatment. Significant differences at least at $P < 0.05$ level are shown. Mean of 3 technical replicates.

affected by their different bioavailability in the culture medium occurs for.

Proper auxin levels and distribution are key factors for a correct root system development. The present results show that Cd and/or As induced a strong and precocious increase in IAA levels in wt roots, matching with an increase in the expression levels of the IAA biosynthetic genes *ASA* and *YUCCA*. It is possible that the high IAA levels, induced by Cd and As in the wt ARs, contribute to reduce their capability to form LRs, because too high for a correct LR induction and/or an exact auxin distribution along the AR. The results show that the alteration of the *DR5::GUS* auxin-localization signal in the roots caused by the toxic elements can be further accentuated by exogenous JAMe, suggesting an interaction between JAMe and auxin homeostasis during root system construction mediated by Cd and/or As. However, this interaction did not reduce the toxic effects of the pollutants, as already reported to occur in rice drought stress reaction (Dhakarey et al., 2017).

Cadmium and/or As presence enhanced *OsYUCCA2* and *OsASA2* expression in *cpm2* roots, but no increase in IAA levels occurred. These contrasting effects are possibly linked to the absence of JA biosynthesis, and result into irregular LR formation. In line with this hypothesis, exogenous JAMe induced an over-expression of the same genes in the wt, but failed to do it when combined with Cd and/or As. Moreover, the enhanced expression of *OsASA2* and *OsYUCCA2* was not related to an increase in the *DR5::GUS* signal in the LRPs. Similarly, Sun and coworker (2009) reported that exogenous JAs increase the expression of ASA1 in Arabidopsis, but can also inhibit the regular auxin transport, acting primarily on PIN1. It is possible that also in rice the JAs are necessary for a fine-tuned regulation of auxin accumulation in the LRP founder cells, and that there is a counteracting action of JAs with Cd and As at least in this type of roots.

The observed effects of As and/or Cd on lipid peroxidation in wt roots demonstrate that As alone significantly increased MDA content, while there was no increase induced by Cd. In our conditions As resulted more toxic than Cd, thus inducing a higher production of reactive oxygen species in root cells which result into higher MDA levels. In *cpm2* non-exposed to toxic compounds, the MDA levels were higher than in the wt, sustaining a role for the endogenous JAs in reducing the oxidative stress in the wt, in accordance with exogenous JAs effects observed in other plants (Yan et al., 2015). Interestingly, MDA levels strongly increased in the mutant in As presence, further sustaining the high toxicity of As in rice and the protective effects of jasmonates on the cell membrane lipids. In fact, it is possible that the reduced levels of JAs, occurring in the *cpm2* mutant, result into an increase of cell membranes damage caused by As, thus making *cpm2* more sensitive to this pollutant. Nevertheless, also the physiological endogenous JAs levels of the wt did not reduce significantly lipid peroxidation induced by Cd and As, suggesting that other factors are necessary to counteract the pollutants toxicity.

5. Conclusions

Root system development affects overall plant growth and fitness, especially in stress conditions, as in heavy metal/metalloid polluted soils. The remodelling of root architecture in response to toxic elements can be used by plants as a strategy to escape from a pollutant stress or to adapt/survive to it. Altogether, present results show that JAs reduce or enhance the damages induced by As and/or Cd in rice depending on the root type, with a growth reduction in ARs and an increase in LRs. Even if with a minor role in alleviating the dangerous effects of the two pollutants when in combination, JAs interact with auxin, affecting its homeostasis, during the development of the rice root system in the presence of heavy metal/metalloid stress.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

RM (Sapienza University of Rome) designed and carried out the research. PD, FL and DRF (Sapienza University of Rome) contributed to carry out the morphological analyses. RM (Botanical Institute, Molecular Cell Biology of Karlsruhe Institute of Technology) contributed to carry out the analyses on *cpm2* seedlings. EE (Institute of Applied Geosciences) contributed to the elements analysis. CE (CREA-OFA, Rome) contributed to the MDA analysis. HB and ZJ (Leibniz Institute of Biochemistry, Halle) carried out the hormonal analysis. BC (University of Perugia) contributed to the experimental design and to the molecular analysis. AMM and FG (Sapienza University of Rome) analysed the data and wrote the manuscript. All authors read and approved the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2019.05.013.

References

Bahmani, R., Kim, D.G., Kim, J.A., Hwang, S., 2016. The density and length of root hairs are enhanced in response to cadmium and arsenic by modulating gene expressions involved in fate determination and morphogenesis of root hairs in Arabidopsis. Front. Plant Sci. 7, 1763. https://doi.org/10.3389/fpls.2016.01763.

Balcke, G., Handrick, V., Bergau, N., Fichtner, M., Henning, A., Stellmach, H., Tissier, A., Hause, B., Frolov, A., 2012. An UPLC-MS/MS method for highly sensitive high-throughput analysis of phytohormones in plant tissues. Plant Methods 8, 47. https://doi.org/10.1186/1746-4811-8-47.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., Friml, J., 2003. Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115 (5), 591–602. https://doi.org/10.1016/S0092-8674(03)00924-3.

Bhattacharya, P., Samal, A.C., Majumdar, J., Santra, S.C., 2010. Arsenic contamination in rice, wheat, pulses, and vegetables: a study in an arsenic affected area of West Bengal. India. Water Air Soil Poll. 213 (1-4), 3–13. https://doi.org/10.1007/s11270-010-0361-9.

Bruno, L., Pacenza, M., Forgione, I., Lamerton, L.R., Greco, M., Chiappetta, A., Bitonti, M.B., 2017. In *Arabidopsis thaliana* cadmium impact on the growth of primary root by altering *SCR* expression and auxin-cytokinin cross-talk. Front. Plant Sci. 8, 1323. https://doi.org/10.3389/fpls.2017.01323.

Cai, X.T., Xu, P., Zhao, P.X., Liu, R., Yu, L.H., Ziang, C.B., 2014. Arabidopsis ERF109 mediates cross-talk between jasmonic acid and auxin biosynthesis during lateral root formation. Nat. Commun. 5, 5833. https://doi.org/10.1038/ncomms6833.

Cho, K., Agrawal, G.K., Shibato, J., Jung, Y.H., Kim, Y.K., Nahm, B.H., Jwa, N.S., Tamogami, S., Han, O., Kohda, K., Iwahashi, H., Rakwal, R., 2007. Survey of differentially expressed proteins and genes in jasmonic acid treated rice seedling shoot and root at the proteomics and transcriptomics levels. J. Proteome Res. 6 (9), 3581–3603. https://doi.org/10.1021/pr070358v.

Della Rovere, F., Fattorini, L., D'Angeli, S., Veloccia, A., Falasca, G., Altamura, M.M., 2013. Auxin and cytokinin control formation of the quiescent centre in the adventitious root apex of Arabidopsis. Ann. Bot. 112 (7), 1395–1407. https://doi.org/10.1093/aob/mct215.

Demiral, T., Türkan, I., 2005. Comparative lipid peroxidation, antioxidant defence systems and proline content in roots of two rice cultivars differing in salt tolerance. Environ. Exp. Bot. 53 (3), 247–257. https://doi.org/10.1016/j.envexpbot.2004.03.017.

Dhakarey, R., Raorane, M.L., Treumann, A., Peethambaran, P.K., Schendel, R.R., Sahi, V.P., Hause, B., Bunzel, M., Henry, A., Kohli, A., Riemann, M., 2017. Physiological and proteomic analysis of the rice mutant *cpm2* suggests a negative regulatory role of jasmonic acid in drought tolerance. Front. Plant Sci. 8, 1903. https://doi.org/10.3389/fpls.2017.01903.

Fattorini, L., Hause, B., Gutierrez, L., Veloccia, A., Della Rovere, F., Piacentini, D., Falasca, G., Altamura, M.M., 2018. Jasmonate promotes auxin-induced adventitious rooting in dark-grown Arabidopsis thaliana seedlings and stem thin cell layers by a cross-talk with ethylene signalling and a modulation of xylogenesis. BMC Plant Biol. 18, 182. https://doi.org/10.1186/s12870-018-1392-4.

Fattorini, L., Ronzan, M., Piacentini, D., Della Rovere, F., De Virgilio, C., Sofo, A., Falasca, G., Altamura, M.M., 2017a. Cadmium and arsenic affect quiescent centre formation and maintenance in *Arabidopsis thaliana* post-embryonic roots disrupting auxin biosynthesis and transport. Environ. Exp. Bot. 144, 37–48. https://doi.org/10.1016/j.envexpbot.2017.10.005.

Fattorini, L., Veloccia, A., Della Rovere, F., D'Angeli, S., Falasca, G., Altamura, M.M., 2017b. Indole-3-butyric acid promotes adventitious rooting in *Arabidopsis thaliana* thin cell layers by conversion into indole-3-acetic acid and stimulation of anthranilate synthase activity. BMC Plant Biol. 17 (1), 121. https://doi.org/10.1186/s12870-017-1071-x.

Ghiani, A., Fumagalli, P., Van, T.N., Gentili, R., Citterio, S., 2014. The combined toxic and genotoxic effects of Cd and As to plant bioindicator Trifolium repens. PloS One 9 (6), e99239. https://doi.org/10.1371/journal.pone.0099239.

Hentrich, M., Böttcher, C., Düchting, P., Cheng, Y., Zhao, Y., Berkowitz, O., Masle, J., Medina, J., Pollmann, S., 2013. The jasmonic acid signaling pathway is linked to auxin homeostasis through the modulation of *YUCCA8* and *YUCCA9* gene expression. Plant J. 74 (4), 626–637. https://doi.org/10.1111/tpj.12152.

Hodges, D.M., DeLong, J.M., Forney, C.F., Prange, R.K., 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207 (4), 604–611. https://doi.org/10.1007/s004250050.

Hu, Y.F., Zhou, G., Na, X.F., Yang, L., Nan, W.B., Liu, X., Zhang, Y.Q., Li, J.L., Bi, Y.R., 2013. Cadmium interferes with maintenance of auxin homeostasis in *Arabidopsis* seedlings. J. Plant Physiol. 170 (11), 965–975. https://doi.org/10.1016/j.jplph.2013.02.008.

Kanna, M., Tamaoki, M., Kubo, A., 2003. Isolation of an ozone-sensitive and jasmonate semi- insensitive Arabidopsis mutant (*oji1*). Plant Cell Physiol. 44 (12), 1301–1310. https://doi.org/10.1093/pcp/pcg157.

Keramat, B., Kalantari, K.M., Arvin, M.J., 2010. Effects of methyl jasmonate treatment on alleviation of cadmium damages in soybean. J. Plant Nutr. 33 (7), 1016–1025. https://doi.org/10.1080/01904161003728685.

Kosolsaksakul, P., Farmer, J.G., Oliver, I.W., Graham, M.C., 2014. Geochemical associations and availability of cadmium (Cd) in a paddy field system, northwestern Thailand. Environ. Pollut 187, 153–161. https://doi.org/10.1016/j.envpol.2014.01.

Liptáková, L., Huttová, J., Mistrík, I., Tamás, L., 2013. Enhanced lipoxygenase activity is involved in the stress response but not in the harmful lipid peroxidation and cell death of short-term cadmium-treated barley root tip. Plant Physiol. 170 (10), 646–652. https://doi.org/10.1016/j.jplph.

Liu, X.L., Zhang, S.Z., 2007. Intraspecific differences in effects of co-contamination of cadmium and arsenate on early seedling growth and metal uptake by wheat. J. Environ. Sci. (China) 19, 1221–1227.

Liu, Z., Zhang, S., Sun, N., Liu, H., Zhao, Y., Liang, Y., Zhang, L., Han, Y., 2015. Functional diversity of jasmonates in rice. Rice 8 (1), 5. https://doi.org/10.1186/s12284-015-0042-9.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plantarum 15 (3), 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Patel, K.S., Sahu, B.L., Ramteke, S., Bontempi, E., 2016. Contamination of paddy soil and rice with arsenic. JEP 7, 689–698. https://doi.org/10.4236/jep.2016.75061.

Piotrowska, A., Bajguz, A., Godlewska-Żyłkiewicz, B., Czerpak, R., Kamińska, M., 2009. Jasmonic acid as modulator of lead toxicity in aquatic plant *Wolffia arrhiza* (*Lemnaceae*). Environ. Exp. Bot. 66 (3), 507–513. https://doi.org/10.1016/j.envexpbot.2009.03.019.

Punshon, T., Jackson, B.P., Meharg, A.A., Warczack, T., Scheckel, K., Guerinot, M.L., 2017. Understanding arseni dynamics in agronomic systems to predict and prevent uptake by crop plants. Sci. Total Environ. 581-582, 2019–2220. https://doi.org/10.1016/j.scitotenv.2016.12.111.

Rao, M.V., Davis, K.R., 2001. The physiology of ozone induced cell death. Planta 213 (5), 682–690. https://doi.org/10.1007/s004250100618.

Rebouillat, J., Dievart, A., Verdeil, J.L., Escoute, J., Giese, G., Breitler, J.C., Gantet, P., Espeout, S., Guiderdoni, E., Périn, C., 2009. Molecular genetics of rice root development. Rice 2, 15–34. https://doi.org/10.1007/s12284-008-9016-5.

Riemann, M., Haga, K., Shimizu, T., Okada, K., Ando, S., Mochizuki, S., Nishizawa, Y., Yamanouchi, U., Nick, P., Yano, M., Minami, E., Takano, M., Yamane, H., Iino, M., 2013. Identification of rice *Allene Oxide Cyclase* mutants and the function of Jasmonate for defense against *Magnaporthe oryza*. Plant J. 74 (2), 226–238. https://doi.org/10.1111/tpj.12115.

Ronzan, M., Piacentini, D., Fattorini, L., Rovere, Della, F, Eiche, E, Riemann, Altamura, M.M., Falasca, G., 2018. Cadmium and arsenic affect root development in *Oryza sativa* L. negatively interacting with auxin. Environ. Exp. Bot. 151, 64–75. https://doi.org/10.1016/j.envexpbot.2018.04.008.

Singh, I., Shah, K., 2014. Exogenous application of methyl jasmonate lowers the effect of cadmium-induced oxidative injury in rice seedlings. Phytochemistry 108, 57–66. https://doi.org/10.1016/j.phytochem.2014.09.007.

Sofo, A., Vitti, A., Nuzzaci, M., Tataranni, G., Scopa, A., Vangronsveld, J., Remans, T., Falasca, G., Altamura, M.M., Degola, F., Sanità di Toppi, L., 2013. Correlation between hormonal homeostasis and morphogenic responses in *Arabidopsis thaliana* seedlings growing in a Cd/Cu/Zn multi-pollution context. Physiol. Plantarum 149 (4), 487–498. https://doi.org/10.1111/ppl.12050.

Sun, Y., Li, Z., Guo, B., Chu, G., Wei, C., 2008. Arsenic mitigates cadmium toxicity in rice seedlings. Environ. Exp. Bot. 64, 264–270. https://doi.org/10.1016/j.envexpbot.2008.05.009.

Sun, J., Xu, Y., Ye, S., Jiang, H., Chen, Q., Liu, F., Zhou, W., Chen, R., Li, X., Tietz, O., Wu, X., Cohen, J.D., Palme, K., Li, C., 2009. *Arabidopsis ASA1* is important for jasmonate-mediated regulation of auxin biosynthesis and transport during lateral root formation. Plant Cell 21 (5), 1495–1511. https://doi.org/10.1105/tpc.108.064303.

Taulavuori, E., Hellström, E.K., Taulavuori, K., Laine, K., 2001. Comparison of two methods used to analyse lipid peroxidation from *Vaccinium myrtillus* (L.) during snow removal, reacclimation and cold acclimation. J. Exp. Bot. 52 (365), 2375–2380. https://doi.org/10.1093/jexbot/52.365.2375.

Uraguchi, S., Fujiwara, T., 2013. Rice breaks ground for cadmium-free cereals. Curr. Opin. Plant Biol. 16 (3), 328–334. https://doi.org/10.1016/j.pbi.2013.03.012.

Ulmasov, T., Murfett, J., Hagen, G., Guilfoyle, T.J., 1997. Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell 9, 1963–1971. https://doi.org/10.1105/tpc.9.11.1963.

Veloccia, A., Fattorini, L., Della Rovere, F., Sofo, A., D'Angeli, S., Betti, C., Falasca, G., Altamura, M.M., 2016. Ethylene and auxin interaction in the control of adventitious rooting in Arabidopsis thaliana. J. Exp. Bot. 67 (22), 6445–6458. https://doi.org/10.1093/jxb/erw415.

Verma, S., Dubey, R.S., 2003. Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. Plant Sci. 164 (4), 645–655. https://doi.org/10.1016/S0168-9452(03)00022-0.

Wang, R., Wang, J., Zhao, L., Yang, S., Song, Y., 2015. Impact of heavy metal stresses on the growth and auxin homeostasis of *Arabidopsis* seedlings. BioMetals 28 (1), 123–132. https://doi.org/10.1007/s10534-014-9808-6.

Weigel, D., Glazebrook, J., 2002. Arabidopsis: a Laboratory Manual. Cold Spring Harbor Laboratory Press, USA.

Yan, Z., Zhang, W., Chen, J., Li, X., 2015. Methyl jasmonate alleviates cadmium toxicity in *Solanum nigrum* by regulating metal uptake and antioxidative capacity. Biol. Plantarum 59 (2), 373–381. https://doi.org/10.1007/s10535-015-0491-4.

Yan, Z., Tam, N.F.Y., 2013. Effects of lead stress on anti-oxidative enzymes and stress-related hormones in seedlings of *Excoecaria agallocha* Linn. Plant Soil 367 (1-2), 327–338. https://doi.org/10.1007/s11104-012-1467-1.

Yuan, H.M., Huang, X., 2016. Inhibition of root meristem growth by cadmium involves nitric oxide-mediated repression of auxin accumulation and signalling in Arabidopsis. Plant Cell Environ. 39 (1), 120–135. https://doi.org/10.1111/pce.12597.

Zanella, L., Fattorini, L., Brunetti, P., Roccotiello, E., Cornara, L., D'Angeli, S., Della Rovere, F., Cardarelli, M., Barbieri, M., Sanità di Toppi, L., Degola, F., Lindberg, S., Altamura, M.M., Falasca, G., 2016. Overexpression of *AtPCS1* in tobacco increases arsenic and arsenic plus cadmium accumulation and detoxification. Planta 243 (3), 605–622. https://doi.org/10.1007/s00425-015-2428-8.

Ziegler, J., Quwegwer, J., Schubert, M., Erickson, J.L., Schattat, M.H., Bürstenbinder, K., Grubb, C.D., Abel, S., 2014. Simultaneous analysis of apolar phytohormones and 1-aminocyclopropan-1- carboxylic acid by high performance liquid chromatography /electrospray negative ion tandem mass spectrometry via 9-fluorenylmethoxycarbonyl chloride derivatization. J. Chrom. A 1362, 102–109. https://doi.org/10.1016/j.chroma.2014.08.029.