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A Protective role for Nitric oxide and Salicylic acid for Arsenite Phytotoxicity in Rice (*Oryza sativa* L.)

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Abstract

Nitric oxide (NO) and salicylic acid (SA) are important signaling molecules in plant system. In the present study both NO and SA showed a protective role against arsenite (As^{III}) stress in rice plants when supplied exogenously. The application of NO and SA alleviated the negative impact of As^{III} on plant growth. Nitric oxide supplementation to As^{III} treated plants greatly decreased arsenic (As) accumulation in the roots as well as shoots/ roots translocation factor. Arsenite exposure in plants decreased the endogenous levels of NO and SA. Exogenous supplementation of SA not only enhanced endogenous level of SA but also the level of NO through enhanced nitrate reductase (NR) activity, whether As^{III} was present or not. Exogenously supplied NO decreased the NR activity and level of endogenous NO. Arsenic accumulation was positively correlated with the expression level of *OsLsi1*, a transporter responsible for As^{III} uptake. The endogenous level of NO and SA were positively correlated to each other either when As^{III} was present or not. This close relationship indicates that NO and SA work in harmony to modulate the signaling response in As^{III} stressed plants.

Key words: Arsenic, Rice, Nitric oxide, Salicylic acid, Transporters

Introduction

Arsenic (As) poses a serious threat to human beings, it is associated with several health risks such as skin lesions, cancer, and cardiovascular and renal diseases (Ratnaike 2003). Contaminated water is the principal source of As exposure. Rice is particularly efficient in As accumulation and toxic concentrations of As in rice grains has been reported (Ma et al. 2008). As rice is a traded commodity it thus, serves as an entry route for As in the food chain for regions where there is no As contamination (Mondal et al. 2010). Arsenic exposure to plants causes oxidative stress that damages tissue through enhanced production of reactive oxygen species (ROS). Although the exact mechanism is not known, there are increasing evidences to suggest that As leads to a disturbed cell redox state (Dixit et al. 2015). To cope with increased ROS in cells, plant system is protected by various radical scavengers, such as antioxidant enzymes as well as non enzymatic compounds like glutathione, ascorbate, carotenoids and α tocopherol (Mittler 2002; Gupta & Ahmad, 2014; Kumar et al. 2015).

Nitric oxide (NO) is a short lived gaseous signaling molecule, which have a variety of functions in plants, including abiotic stress abatement (Arasimowicz & Floryszak-Wieczorek, 2007; Neill et al. 2008). Two mechanisms have been postulated for NO-mediated stress mitigation. The first mechanism is that NO is a free radical, therefore, it can directly scavenge ROS (Lamattina et al. 2003). Second, it can serve as antioxidant inducer by triggering antioxidant gene expression or activating antioxidant enzymes (Grün et al. 2006; Groβ et al. 2013) by post translational modification of these antioxidant enzymes (Grennan 2007; Tanou et al. 2009). Application of exogenous NO donor (sodium nitroprusside, SNP) has been shown to confer resistance to various abiotic stresses such as salt (Tanou et al. 2009) and heavy metals (Zhang et al. 2011, Singh et al., 2016). Nitric oxide has also been shown to

improve internal iron (Fe) availability by forming Fe-nitrosyl complexes (Graziano et al. 2002).

Salicylic acid (SA) is a phenolic compound, it serves as a growth regulator and has a crucial role in various physiological processes such as germination, flowering and heat production in thermogenic plants (Rivas-San Vicente and Plasencia 2011). Salicylic acid mediated defense signaling has been widely studied in plants during the last decade, largely against biotic stresses (Yang et al. 2004; Chen et al. 1993). Various reports indicated that exogenously applied SA mitigated the Hg and Cd mediated toxicity in plants (Zhou et al. 2009: Metwally et al. 2003). Rice shoots has extremely high level of SA (5-30 μg g^{-1} fresh weight) in comparison to other plants so endogenous level of SA in shoots is largely insensitive to exogenous SA but rice roots have low level of endogenous SA that makes them sensitive to exogenous application of SA (Yang et al. 2004; Chen et al. 1997).

The relationship between NO and SA signaling has been studied under biotic stress conditions. Nitric oxide treatment was shown to induce substantial increase in the levels of total SA, while NO activity have been also shown to be dependent endogenous level of SA (Song and Goodman 2001). Salicylic acid may induces NO synthesis *via* calcium and casein kinase 2 pathway (Zottini et al. 2007). Thus, there appears a complementary relationship between NO and SA.

In rice roots, arsenite (As^{III}) is known to be transported through silicic (Si) acid transporter, OsLsi1. While another transporter OsLsi2 mediates efflux of As^{III} and Si towards the xylem (Ma et al. 2008). Recent reports have showed that the mechanism of Fe uptake correlates with the As accumulation and affects As transport in plants (Tiwari et al. 2014).

There have been a large number of transporters identified in plants that have the ability to transport iron. Natural Resistance Associated Macrophage Protein (NRAMPs), primarily identified as Fe transporters, also retain transport ability for other heavy metals.

OsNRAMP5 is polarly localized at the distal side of both exodermis and endodermis cells, and uptakes Fe as well as manganese (Mn) and cadmium (Cd) (Ishimaru et al. 2006). Recently, overexpression of *OsNRAMP1* in *Arabidopsis thaliana* has been reported to be involved in transport of As and Cd (Tiwari et al. 2014).

Graminaceous plants solubilize soil Fe by secreting Fe(III) chelators called mugenic acid (MAs) from their roots. The resulting Fe(III)-MA complexes are then absorbed into the roots by Yellow Stripe Like (YSL) transporters (Bashir et al. 2010; Kobayashi and Nishizawa 2012). However, rice also possesses the ability to transport Fe(II) through the transporters OsIRT1 and OsIRT2. Once the Fe is located in the plant a number of transporters are involeved in transport of the Fe from the roots to the shoots. OsFRDL1, a citrate effluxer, localized on the roots pericycle cells is required for efficient Fe translocation to shoots (Yokosho et al. 2009). OsYSL2 is responsible for long-distance transport of nicotianamine-chelated Fe and Mn into sink tissues including leaves and grains (Koike et al. 2004). A number of key regulators of Fe transport have been identified. For example, OsIRO2 positively regulates various genes responsible for Fe transport (*OsNAS1*, *OsNAS2*, *OsNAAT1*, *OsDMAS1*, *TOM1* and *OsYSL15*). OsIRO2 also affects the expression of some Fe deficiency-inducible transcription factors, which might be involved in the indirect regulation of *OsIRO2*-downstream genes (Ogo et al. 2007).

In the present study, the role of NO and SA was evaluated in mitigating As^{III} toxicity in hydroponically grown rice. The dependency of NO and SA on each other during As^{III} stress and their effects on As and Fe transporters and mineral nutrition were investigated. Oxidative stress related parameters were analyzed to explore the stress mitigation mechanisms.

MATERIAL AND METHODS

Growth conditions and experimental design

Seeds of *Oryza sativa* (cv. Sarjoo52) collected from Masina Research Center, Pvt. Ltd., Bihar (India) were surface sterilized using 10% hydrogen peroxide for 30 s and washed with double distilled water. Seeds were germinated in moist pre-sterilized blotting sheets on a tray in seed germinator for 4 d at 25^{o}C at 65% relative humidity. The 4 d old seedlings were transferred to perforated cups with 10 seedlings/cup. The cups were placed in trays holding 3 L of full-strength Hewitt nutrient medium prepared in Milli-Q water. The seedlings were grown for 10 d under 210 µ mol cm^{-2} s^{-1} (16/8 h day/night) white florescent light in a culture room maintained at 24–26 ^{0}C. After 10 d, As^{III} (25µM) as $NaAsO_2$, SA (40µM) and SNP (30µM; NO donor) were supplemented in the nutrient medium. The rice seedlings grown in 25µM of As^{III} were abbreviated as As^{III}, 40µM of SA as SA and 30µM SNP as NO. Similarly, 25µM As^{III} with 40µM SA as As^{III}+SA and 25µM As^{III} with 30µM SNP as As^{III}+NO and seedlings grown in Hewitt nutrient medium served as control. The nutrient media was changed after every 48 h. The treated seedlings were harvested for analysis after 7 d of exposure to the treatments. All the chemicals were purchased from Sigma Aldrich (USA) or as mentioned separately.

For germination studies, seeds were surface sterilized as described aboved and 50 seeds transferred to a Petri plate. Seeds were soaked with the above mentioned treatments in Milli-Q water for 5 d and germination rate was observed.

Biochemical analysis

For chlorophyll estimation, 100 mg fresh leaves were crushed in 5 ml 80% ice cold acetone and centrifuged at 10,000 g for 10 min. Chlorophyll content in supernatant was estimated as described by the method of Arnon (1949). For thiobarbituric acid reactive substances (TBARS) and hydrogen peroxide estimation, 300 mg of roots or shoots were crushed in 3 ml

of 0.2% trichloroaceitic acid (TCA) (w/v), centrifuged at 10,000 g for 10 min, the supernatant was collected for further estimation. TBARS and hydrogen peroxide were estimated as described by Heath and Packer (1968) and Velikova et al. (2000), respectively. For analysis of enzyme activities, leaves or roots (300 mg each) were ground using liquid nitrogen in a chilled mortar and pestle and extracted with 3 ml of ice-cold 100 mM potassium phosphate buffer (pH 7.5) containing 1% (w/v) poly-vinylpyrrolidone (PVP). The homogenate was centrifuged at 10,000 g for 15 min and the supernatant was used for enzyme assays. The activity of superoxide dismutase (SOD) was measured using the method as described by Beauchamp and Fridovish et al.(1971), ascorbate peroxidase (APX) by Nakano and Asada (1981), guiacol peroxidase (GPX) by Kato and Shimizu (1987), CAT by Scandalios et al. (1983) and NR by Hageman and Reed (1980). Enzymatic activities were calculated in per unit protein estimated by the method of Lowry et al.(1951).

Element analysis

The elemental composition of the plants material was determined following method of Mallick et al. (2013). Plants were washed three times in 1 mM phosphate buffer (4^oC; pH 5.6). Roots and shoots were separated and oven dried at 70 ^0C. Dried plant tissues (leaf and root, 100 mg each) were digested using HNO_3: $HClO_4$ (3:1). Digested samples were filtered (Whatman No 42) and the volume was made up to 10 ml using Milli-Q water. Arsenic was analyzed by AAS (GBS Avanta ∑, Australia) supported with a hydride generator (MDS 2000) using NaH_2BO_4 and NaOH (3 M) and HCl (3 M) and other elements were analyzed by AAS (GBC Avanta ∑, Australia). The values are presented in µg per g dry weight.

Superoxide and hydrogen peroxide Staining

Superoxide and hydrogen peroxide were determined in the leaves by staining using nitro blue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB), respectively, as described previously by Thordal-Christensen et al. (1997) and Orozco-Cárdenas et al. (1999). Rice leaves were excised at the base with a razor blade and NBT (1 mg ml^{-1}) or DAB (0.5 mg ml^{-1}) solutions were supplied through the cut ends for 8 h. Leaves were then decolorized in boiling ethanol (95%) for 15 min. At least 3 leaves were used for each treatment.

Nitric Oxide Detection

For NO detection, roots of approximately equal thickness were incubated for 30 min at 25^{o}C in the dark with 10 mM DAF-FM-DA (Calbiochem; excitation at 495 nm, emission at 515 nm) prepared in 10 mM Tris-HCl (pH 7.4) buffer, as described by Sandalio et al. (2008). For negative control, roots were incubated with 1 mM cPTIO, a NO scavenger. Then roots were washed 3 times for 10 min each with the same buffer and DAF-FM-T, fluorescence was visualized by confocal microscopy (Carl Zeiss LSM510 Meta, Germany). Fluorescence intensity was estimated by measuring the average pixel intensity. For each treatment, triplicate analysis was performed.

Salicylic Acid Estimation

Presence of SA in the sample was analyzed by HPLC (Dionex Ultimate 3000, USA by using Charomeleon 6.8) using a UV detector at 210 nm following the method of Pan et al. (2010). The mobile phase was programmed with linear gradient of A (0.1% of formic acid in methanol) and B (0.1% of formic acid in water) programmed as 0-20 min; 30-100% A, 20-22 min; 100% A and then 22-25 min; 100-30% of A. Flow rate was maintained at 0.3 ml min^{-1}, the retention time for SA was recorded at 22.4 min.

Gene Expression Analysis Using Quantitative RT-PCR

100 mg roots sample was crushed in liquid N_2 and RNA were isolated by using RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instraction. Quality and quantity of RNA was assessed by spectrophotometer (Nano Drop, USA). Approximately 5 µg of RNase free DNase-treated total RNA isolated from roots of rice plants exposed to various treatments and control was reverse-transcribed using SuperScriptII (Fermentas, USA), following the manufacturer's recommendation. The synthesized cDNA was diluted 1:5 in RNase free water and subjected to quantitative RT-PCR (qRT-PCR) analysis. The qRT-PCR was performed using an ABI 7500 instrument (ABI Biosystems, USA) using gene specific primers (Supplemental Information Table S1). Each qPCR reaction contained 5 µl of SYBR Green Supermix (ABI Biosystems, USA), 1 µl of the diluted cDNA reaction mixture (corresponding to 5 ng of starting amount of RNA) and 10 pM of each primer in a total reaction volume of 10 µl. qPCR reactions were performed under the following conditions: 10 min at 95℃ and 40 cycles of the one step thermal cycling of 3 s at 95 °C, 30 s at 60℃ in a 96-well reaction plate. The rice *Actin1* gene was used as an internal control to estimate the relative transcript levels of the target gene. Specificity of amplicons generated in qPCR reactions was verified by melting curve analysis. Each qPCR reaction was performed in triplicate (technical replicates) for each biological replicate (three for each treatment). Relative gene expression was calculated using $-^{\Delta\Delta}$CT method (Livak and Schmittgen 2001).

Statistical analysis

Analysis of variance (ANOVA), Duncan's multiple range test (DMRT) and Pearson's correlation analysis were performed to determine the significant difference between treatments at the 95% confidence level.

RESULTS

Morphological changes

In the absence of As^{III}, SA or NO has no significant effect on seed germination. Arsenite treatment drastically reduced the seed germination potential. Nitric oxide or SA treatment of the seeds decreased the negative impact that As^{III} on seed germination potential (Supplemental Information Fig. S1). Nitric oxide and SA treatment enhanced the shoot, root length and total chlorophyll content in comparison to control. A more prominent growth response was observed in SA treated plants than NO (Table -1 and Supplemental Information Fig. S2 and S3). Exposure to As^{III} decreased the shoot and root length by 27% and 47%, respectively, compared to the control. Arsenite treatment also had a similar affect on total biomass. The chlorophyll content was decreased by 44% in the As^{III} exposed plants. Root and leaf growth was decreased under As^{III} treatment (Supplemental Information Fig. S4 and S5). Nitric oxide and SA supplementation along with As^{III}, partially restored over all plant growth, particularly shoot length. Growth of root, leaf and total chlorophyll content were completely restored upto control levels. In absence of As^{III}, SA was more responsive for growth enhancement, while in presence of As^{III}, NO performed better.

Oxidative stress and antioxidant enzymes

Arsenite exposure increased hydrogen peroxide and TBARS concentrations in roots by ca. 4 and 2.5 fold and shoots by ca. 2 and 2.5 fold compared to the controls (Fig. 1A, B and Supplemental Information Fig S6 A, B). Salicylic acid treatment significantly increased the level of hydrogen peroxide in the roots compared to the controls. Supplementation of SA or NO along with As^{III} resulted in significantly decreased the production of hydogen peroxide and TBARS in comparison to As^{III} alone treated plants (Fig. 1A, B and Supplemental Information Fig S6 A, B). Histochemical staining also showed higher level of superoxide

radicals in As^{III} stressed plants than As^{III} and NO treated or As^{III} and SA treated plants (Supplemental Information Fig. S7).

In the As^{III} treated plants, the activity of antioxidant enzymes SOD, CAT, GPX, APX and GR were significantly increased in both the roots and shoots compared to the control plants. Salicylic acid treatment decreased the CAT activity (40%) in the roots while in the shoots it remained unaltered in comparison to the control. Nitric oxide decreased CAT and APX activity in both the roots and the shoots significantly compared to the control. Nitric oxide or SA supplementation to As^{III} treated plants decreased the activity of all the antioxidant enzymes in roots and shoots compared to the As^{III} treatment. However, GR activity was enhanced in roots at As^{III}+SA treated plants in comparison to As^{III} alone. (Fig. 1C-G and Fig S6C-G).

Endogenous levels of NO, SA and NR activity

The endogenous level of NO in roots did not change significantly by exogenous application of NO but exogenous application of SA enhanced the endogenous level of SA. Arsenite stress, however, caused a significant reduction in the concentration of NO which was decreased to half of the control. Nitric oxide and SA supplementation along with As^{III} enhanced the level of endogenous NO by 35% and 77%, respectively compared to As^{III} alone exposed roots, although the levels were still lower than control (Fig.2A and Fig.3). The endogenous level of SA showed a significant increase by exogenous application of both NO and SA (56 and 250% respectively) in roots. In As^{III} exposed roots, endogenous SA level was decreased by 63% than control roots. Nitric oxide supplementation along with As^{III} did not change endogenous SA level while SA supplementation enhanced the endogenous SA level by 324% than As^{III} stressed roots (Fig. 2B).

NR plays a crucial role in NO synthesis in plant systems. In NO treated plants, NR activity decreased by 6 fold while SA treatment enhanced the activity by around 9 fold compared to the controls. In As^{III} stressed roots, NR activity enhanced by ca. 2 fold compared to controls. Salicylic acid supplementation further enhanced the NR activity by ca. 2 fold while NO supplementation decreased the NR activity ca. 5 fold compared to As^{III} treated roots (Fig. 2C).

Accumulation of arsenic and other elements

Arsenite exposed plants accumulated significantly higher amount of As. Most of the As (90%) was confined to roots, approximately 10% was transported to shoots (Table 2 A, B and C). Nitric oxide supplementation along with As^{III} significantly decreased As accumulation in roots (35%) and shoots (61%) compared to plants treated with just As. Salicylic acid treatment to AsIII stressed plants, decreased the As accumulation in the shoots (27%) while it showed no significant impact on roots As accumulation than As^{III} alone treated plants. Nitric oxide treatment also reduced the shoots/roots translocation factor (TF; 0.06) by ~50% in comparison to As^{III} alone treated plants (TF; 0.1).

In the As^{III} treated plant roots, Fe accumulation was enhanced 92%, however, the level of Fe in shoots remained unaltered compared to control. Nitric oxide or SA alone did not altered Fe accumulation significantly in the roots while in the shoots, Fe accumulation was enhanced by NO (15%) and SA (18%) compared to the controls. Nitric oxide supplementation to As^{III} treated plants caused a significant reduction (21%) in the level of Fe in the roots, while increased Fe (21%) in shoots in compared to the plants treated with As^{III} only. Thus, NO supplementation to As^{III} treated plants enhanced the shoots/roots TF than As^{III} treated plants (Table 2C). Whereas, SA supplementation to As^{III} treated plants did not

affect the level of Fe in roots significantly, while in the shoots it enhanced the Fe accumulation as well as increased the shoots/roots TF in comparison to As^{III} alone treatment.

Nitric oxide and SA treatment alone did not significantly alter the level of Ca and Zn in shoots and roots. Arsenite also showed no significant effect on Ca and Zn accumulation both in roots and shoots, except the Ca accumulation was lowered in the roots of As^{III} treated plants compared to the control plants. Accumulation of Mn was enhanced significantly in the roots by NO and SA treatments but there was no impact on Mn accumulation in shoots. As^{III} alone or in combination with NO or SA have no significant impact compared to the controls on Mn accumulation.

Impact of NO and SA on Fe and As transporters

In NO treated plants, *OsLsi1* expression level was lowered by 20%, while the SA treated plants had enhanced expression (~2 fold) of *OsLsi1* compared to the control plants. In As^{III} exposed plants, *OsLsi1* expression was higher than control plants. NO supplemenatation to As^{III} treated plants decreased the expression of *OsLsi1*, whereas, SA supplementation in combination with As^{III} significantly enhanced the expression of *OsLsi1* compared to the plants treated with just As^{III}.

In NO treated plants the expression level of *OsLsi2* was enhanced by more than 2 fold while SA treatment have no significant impact on *OsLsi2* compared to the control plants. In As^{III} treated plants, *OsLsi2* expression level was also more than 5 fold greater than the control. Nitric oxide and SA supplementation to As^{III} treated plants decreased the *OsLsi2* expression when compared to the plants just treated with As^{III} , although more reduction was observed in SA treated plants (Fig. 4A, B).

Both NO and SA treatments enhanced the expression of all the Fe transporters studied, except *OsFRDL1* which was not enhanced in response to NO. OsIRO2, a positive

transcription regulator, was unaltered by NO and SA treatments. Arsenite treatment alone significantly enhanced the expression of *OsIRT1* and *OsYSL2* (11 and 10 folds, respectively) and inhibited the expression of *OsFRDL1* (77%) in comparison to the control. When NO was applied along side As^{III}, the expression levels of *OsIRT1, OsIRO2 and OsYSL2* were decreased, whereas, *OsFRDL1* and *OsNRAMP5* remained unaltered compared to the plants just treated with As^{III} . When SA was used in conjunction to the As^{III} treated plants there was a reduction in the expression of *OsIRT1*, *OsYSL2* and *OsIRO2* expression levels but *OsFRDL1* was enhanced in comparison to As^{III} only treated plants (Fig. 4C-G).

Discussion

The present study analyze the effect of SA and NO on rice plants exposed to a toxic concentration of As^{III}. Under As^{III} stress, NO and SA levels sharply declined. Plant growth was also significantly hampered. Nitric oxide and SA show a protective role against As^{III} toxicity in rice plants and enhanced the plant growth. Exogenously supplied NO decreased the As accumulation in roots and its transport to shoots. Arsenite treatment inhibits seed germination and growth, shoots, roots length and total chlorophyll content (Dixit et al. 2015a,b; Kumar et al. 2014a,b, 2016) these were mostly restored by NO and SA application (Table 1; Supplemental Information Fig. S1, S2, S3, S4 and S5). The roots are the first organ which come in contact with As^{III}, it was observed that As^{III} inhbited the growth of roots and root hairs. However, NO and SA supplementation completely restored the root length and root hair growth (Table 1 and Supplemental Information Fig. S2, S4).

Nitric oxide treatment not only significantly reduces As accumulation in roots, but also restricts its translocation from roots to shoots. This is the first report on As^{III} and NO interactions in plants and may be the crucial factor for NO-mediated protection against As^{III} stress. Both NO and SA are reported to activate ABC transporters that are responsible for

heavy metal sequestration to vacuole and restricting its entry into shoots (Grun et al. 2006; Eichhorn et al. 2006). However, in our study, SA did not affect the accumulation of As in roots but decreased its accumulation in shoots (Singh et al. 2015). This might be due to the activation of ABC transporters in the roots hence sequestering more As in root vacuole while restricting its entry to the shoots. Nitric oxide treatment, however, drastically decreased As accumulation in the roots as well as its translocation to shoots. *OsLsi1*expression which is responsible for internalisation of As^{III} also showed a positive correlation with As accumulation in the roots ($R=0.98$, $p\leq0.05$) while *OsLsi2* expression, that is responsible for roots to shoots transport, was also positively correlated with As accumulation in the shoots (Supplemental Information Table S2).

The present study showed that As^{III} treatment resulted in oxidative stress in terms of enhanced TBARS, hydrogen peroxide and superoxide radicals. Enhanced production of ROS and peroxidation of membrane lipids (i.e. increase in the level of TBARS) by As exposure has previously been reported in rice (Singh et al. 2015). It was observed that antioxidant enzymes such as SOD, CAT, APX GPX and GR were enhanced in both roots and shoots under As^{III} stress, potentially could be to counteract the oxidative stress caused by As^{III}. Nitric oxide or SA supplementation with As^{III} caused a substantive reduction in the level of TBARS and hydrogen peroxide, which indicates the ameliorating effect of NO and SA on As^{III} induced oxidative stress (Singh et al. 2009). Furthermore NO and SA supplementation with As^{III}, decreased the activity of antioxidant enzymes compared to As^{III} alone exposed plants. It again confirms the protective role of NO and SA against As^{III} induced oxidative stress. Protective role of SA and NO supplementation has been previously observed under Cd stress in *Medicago sativa* and under As stress in rice respectively (Zhou et al. 2009; Singh et al. 2009).

Arsenite stress significantly decreased NO-dependent fluorescence compared to control roots (Fig. 3A, 6). Singh et al. (2009) also reported a significant decrease in NO dependent fluorescence in rice root under As stress. A similar decrease in NO dependent fluorescence in shoot of pea plant was observed under Cd stress (Rodriguez-Serrano et al. 2009). In contrast, Besson-Bard et al. (2008) demonstrated an increase in NO dependent florescence in both roots and shoots of *A. thalina* under Cd stress. During plant responses to heavy metal stress, NO may increase or decrease and act as an inducer or inhibitor of stress tolerance, depending on plant species and experimental setup (Arasimowicz-Jelonek et al. 2011). In the present study, endogenous NO did not increase by exogenous NO but exogenous application of SA enhanced the endogenous level of NO. Salicylic acid mediated enhancement of endogenous NO was reported in *A. thaliana* (Zottini et al. 2007). It appears that there is an optimal level of endogenous NO in rice roots that could not be further elevated. However, when the endogenous level of NO was decreased, e.g. under As^{III} stress, exogenous supplementation of NO or SA caused an increase in endogenous NO concentrations in the roots.

Arsenite exposure in rice significantly decreased the endogenous SA level. This is a contrasting behaviour than biotic stress where endogenous SA level are enhanced (Vlot et al. 2009). Enhancement of endogenous SA by application of exogenous SA has been reported which is termed as “SA dependent amplification circuit” (Xiao et al. 2003). Nitric oxide is well known to induce SA synthesis (Klessig et al. 2000; Huang et al. 2004). Genomic studies have shown that NO induces phenylalanine ammonia lyase (*PAL*) gene at the transcriptome level (Huang et al. 2004; Delledonne et al. 1998) these genes play a crucial role in SA biosynthesis via cinnamate pathway. However, under As^{III} stress there was no increase in SA level by exogenous supplementation of NO, this indicates that As^{III} disturbed the SA biosynthesis pathway.

A positive correlation between SA and NO was found, with As^{III} ($R=0.89$, $p\leq0.05$) or without As^{III} ($R=0.88$, $p\leq0.05$), which means NO and SA work synergistically (Supplemental Information Table S2). Song and Goodman (2001) also concluded similar hypothesis that NO is fully dependent on the function of SA while NO is required for full functioning of SA in tobacco under biotic stress. Exogenous NO supplementation resulted in a drastic decrease in NR activity. This might be due to the feedback inhibition of NR activity in the presence of high level of NO. Though, under As^{III} stress, NR activity was enhanced, but the level of NO was much lower than controls. This might be due to the consumption of surplus NO (due to enhanced NR activity) in the neutralization of As^{III} induced ROS. In SA treated plants NR activity was many folds higher than control and also level of NO was correspondingly higher, indicating that SA treatment induces NO synthesis through NR.

Nitric oxide treatment with or without As^{III} stress enhanced Fe accumulation in the shoots, while As^{III} alone treatment enhanced the Fe accumulation only in the roots but not in the shoots. Fe deficiency results in chlorosis (Vasconcelos and Grusak 2014). In the present study, As^{III} stressed leaves becomes chlorotic, though there was no change in the concentration of Fe in shoots in comparison to control. Thus, the reduction in chlorophyll content may be a result of its inhibited synthesis or As^{III} induced degradation (Rahman et al. 2007). Another possibility is that As^{III} stress may have an effect on availability of Fe, such as due to reduction in endogenous NO level. Iron homeostasis strongly depends on the iron storage protein ferritins which are ubiquitous iron storage proteins. Nitric oxide has been reported to influence the ferritins level (Murgia et al., 2002; Grun et al., 2006). There is *in vitro* evidence for the first mechanism that NO can remove iron from horse spleen ferritin (Graziano and Lamattina, 2005). So, NO is believed to increase the internal Fe availability and revert the chlorotic symptoms. Nitric oxide has been reported to increase internal Fe availability and reverts chlorotic symptoms (Graziano et al. 2002).

It has been reported previously that OsIRO2 serves as positive regulator of genes involved in iron uptake and utilization (Ogo et al. 2007), was found to negative correlated with shoots Fe accumulation irrespective of presence of As^{III} showing its role in Fe deficiency. *OsNRAMP5*, that is responsible for Mn uptake along with Fe (Ishimaru et al. 2006), in presence of As^{III} Mn accumulation in shoots was negatively correlated ($R= -0.95$, $p\leq0.05$) suggesting its role in Mn transportation (Supplemental Information Table S2). The transcripts of Fe uptake transporter *OsIRT1*, was negatively correlated with the Fe accumulation in shoots in presence of As^{III}, *OsIRT1* is known to strongly induced with Fe deficiency (Kobayashi and Nishizawa 2012). *OsYLS2* was negatively correlated ($R= -0.98$, $p\leq0.05$) with shoots Fe level in presence of As^{III}, suggesting its role as long distance Fe transporter (Kobayashi and Nishizawa 2012).

Conclusion

NO and SA both showed a protective role against As^{III} stress. Arsenite stresse lead to oxidative stress burst and consequently activity of antioxidant enzymes was increased. Exogenous application of NO and SA alleviated AsIII mediated oxidative stress. Nitric oxide decreased the As accumulation in roots probably through the down regulation *OsLsi1* transporter while NO and SA both decreased the As accumulation in shoots potentially through the down regulation of *OsLsi2*. Nitric oxide application enhanced the Fe accumulation in shoots and overcame the As^{III} mediated chlorosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version.

Additional information

Competing financial interests

The authors declare no competing financial interests.

Authors Contribution

RDT, PKT, VP, designed experiments and reviewed manuscript. APS, GD performed experimental work and prepared figures. AK performed statistical analysis. SD1 and NK operated confocal microscope and AAS respectively. DC, SM, SD2, PKS, GJN, and OPD reviewed manuscript. All authors have read and approved the manuscript.

SD1: Sameer Dixit

SD2: Sanjay Dwivedi

References

1. Arasimowicz M, Floryszak-Wieczorek J (2007) Nitric oxide as a bioactive signalling molecule in plant stress responses. Plant Science 172: 876-887.
2. Arasimowicz-Jelonek M, Floryszak-Wieczorek J, Gwóźdź EA (2011) The message of nitric oxide in cadmium challenged plants. Plant Sciences 181: 612-620.
3. Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant Physiology 24:1.
4. Bashir K, Ishimaru Y, Nishizawa NK (2010) Iron uptake and loading into rice grains. Rice 3: 122-130.
5. Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry: 44 276-287.
6. Besson-Bard A, Pugin A, Wendehenne D (2008) New insights into nitric oxide signaling in plants. Annual Review of Plant Biololgy 59: 21-39.
7. Chen Z, Iyer S, Caplan A, Klessig DF, Fan B (1997) Differential accumulation of salicylic acid and salicylic acid-sensitive catalase in different rice tissues. Plant Physiology 114: 193-201.
8. Chen Z, Silva H, Klessig DF (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science 262: 1883-1886.
9. Delledonne M, Xia Y, Dixon RA, Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. Nature 394: 585-588.
10. Dixit G, Singh AP, Kumar A, Dwivedi S, Deeba F, Kumar S, Suman S, Adhikari B, Shukla Y, Trivedi PK, Pandey V, Tripathi RD (2015a) Sulfur alleviates arsenic toxicity by reducing its accumulation and modulating proteome, amino acids and thiol metabolism in rice leaves. Scientific Reports 5: 16205.

11. Dixit G, Singh AP, Kumar A, Singh PK, Kumar S, Dwivedi S, Trivedi PK, Pandey V, Norton GJ, Dhankher OP, Tripathi RD (2015b) Sulfur mediated reduction of arsenic toxicity involves efficient thiol metabolism and the antioxidant defense system in rice. Journal of Hazardous Materials 298: 241-251.
12. Eichhorn H, Klinghammer M, Becht P, Tenhaken R (2006) Isolation of a novel ABC-transporter gene from soybean induced by salicylic acid. Journal of Experimental Botany 57: 2193-2201.
13. Graziano M, Beligni MV, Lamattina L (2002) Nitric oxide improves internal iron availability in plants. Plant Physiology 130:1852-1859.
14. Graziano M, Lamattina L (2005). Nitric oxide and iron in plants: an emerging and converging story. Trends in Plant Science, 10(1), 4-8.
15. Grennan, A K (2007) Protein S-nitrosylation: potential targets and roles in signal transduction. Plant Physiology 144: 1237-1239.
16. Groß F, Durner J, Gaupels F (2013) Nitric oxide, antioxidants and prooxidants in plant defence responses. Frontiers in Plant Science 4.
17. Grün S, Lindermayr C, Sell S, Durner J (2006) Nitric oxide and gene regulation in plants. Journal of Experimental Botany 57: 507-516.
18. Gupta M, Ahmad M A (2014) Arsenate induced differential response in rice genotypes. Ecotoxicology and environmental safety, 107: 46-54.
19. Hageman RH, Reed AJ (1980) Nitrate reductase from higher plants. Methods in Enzymology 69**:** 270-280.
20. Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Archieves of Biochemistry and Biophysics 125:189-198.

21. Huang X, Stettmaier K, Michel C, Hutzler P, Mueller MJ, Durner J (2004) Nitric oxide is induced by wounding and influences jasmonic acid signaling in *Arabidopsis thaliana*. Planta 218: 938-946.

22. Ishimaru Y, Suzuki M, Tsukamoto T, Suzuki K, Nakazono M, Kobayashi T, Wada Y, Watanabe S, Matsuhashi S, Takahashi M, Nakanishi H (2006) Rice plants take up iron as an Fe^{3+}-phytosiderophore and as Fe^{2+}. Plant Journal *45*: 335-346.

23. Kato M, Shimizu S (1987) Chlorophyll metabolism in higher plants. VII. Chlorophyll degradation in senescing tobacco leaves; phenolic-dependent peroxidative degradation. Canadian Journal of Botany 65: 729-735.

24. Klessig DF, Durner J, Noad R, Navarre DA, Wendehenne D, Kumar D, Zhou JM, Shah J, Zhang S, Kachroo P, Trifa Y (2000) Nitric oxide and salicylic acid signaling in plant defense. Proceedings of National Academy of Sciences USA 97: 8849-8855.

25. Kobayashi T, Nishizawa NK (2012) Iron uptake, translocation, and regulation in higher plants. Annual Review of Plant Biology 63: 131-152.

26. Koike S, Inoue H, Mizuno D, Takahashi M, Nakanishi H, Mori S, Nishizawa NK (2004) OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. Plant Journal 39: 415-424.

27. Kumar A, Dwivedi S, Singh RP, Chakrabarty D, Mallick S, Trivedi PK, Adhikari B, Tripathi RD (2014a) Evaluation of amino acid profile in contrasting arsenic accumulating rice genotypes under arsenic stress. Biologia Plantarum 58: 733-742.

28. Kumar A, Singh RP, Singh PK, Awasthi S, Chakrabarty D, Trivedi PK, Tripathi RD (2014b) Selenium ameliorates arsenic induced oxidative stress through modulation of antioxidant enzymes and thiols in rice (*Oryza sativa* L.). Ecotoxicology, 23:1153-1163.

29. Kumar A, Dixit G, Singh AP, Dwivedi S, Srivastava S, Mishra K, Tripathi RD (2016). Selenate mitigates arsenite toxicity in rice (*Oryza sativa* L.) by reducing arsenic uptake

and ameliorates amino acid content and thiol metabolism. Ecotoxicology and Environmental Safety, 133, 350-359

30. Kumar S, Dubey RS, Tripathi RD, Chakrabarty D, Trivedi PK (2015) Omics and biotechnology of arsenic stress and detoxification in plants: Current updates and prospective. Environ International 74: 221-230.
31. Lamattina L, García-Mata C, Graziano M, Pagnussat G (2003) Nitric oxide: the versatility of an extensive signal molecule. Annual Review of Plant Biology 54: 109-136.
32. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $^{2-\Delta\Delta CT}$ method. Methods 25: 402-408.
33. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193: 265-275.
34. Ma JF, Yamaji N, Mitani N, Xu XY, Su YH, McGrath SP, Zhao FJ (2008) Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. Proceedings of National Academy of Sciences USA 105: 9931-9935.
35. Mallick S, Kumar N, Singh AP, Sinam G, Yadav RN, Sinha S (2013) Role of sulfate in detoxification of arsenate-induced toxicity in *Zea mays* L.(SRHM 445): nutrient status and antioxidants. Journal of Plant Interaction 8:140-154.
36. Metwally A, Finkemeier I, Georgi M, Dietz KJ (2003) Salicylic acid alleviates the cadmium toxicity in barley seedlings. Plant Physiology 132: 272-281.
37. Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends in Plant Sciences 7: 405-410.
38. Mondal D, Banerjee M, Kundu M, Banerjee N, Bhattacharya U, Giri AK, Ganguli B, Roy SS, Polya DA (2010) Comparison of drinking water, raw rice and cooking of rice as arsenic exposure routes in three contrasting areas of West Bengal, India. Environmental Geochemistry and Health 32: 463-477.

39. Murgia I, Delledonne M, Soave C. (2002). Nitric oxide mediates iron-induced ferritin accumulation in *Arabidopsis*. The Plant Journal, 30(5), 521-528.

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

41. Neill S, Barros R, Bright J, Desikan R, Hancock J, Harrison J, Morris P, Ribeiro D, Wilson I (2008) Nitric oxide, stomatal closure, and abiotic stress. Journal of Experimental Botany 59: 165-176.

42. Ogo Y, Nakanishi Itai R, Nakanishi H, Kobayashi T, Takahashi M, Mori S, Nishizawa, NK (2007) The rice bHLH protein OsIRO2 is an essential regulator of the genes involved in Fe uptake under Fe-deficient conditions. Plant Journal 51: 366-377.

43. Orozco-Cardenas M, Ryan CA (1999) Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. Proceedings of National Academy of Sciences USA 96: 6553-6557.

44. Pan X, Welti R, Wang X (2010) Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography–mass spectrometry. Nature Protocols 5: 986-992.

45. Rahman MA, Hasegawa H, Rahman MM, Rahman MA, Miah MAM (2007) Accumulation of arsenic in tissues of rice plant (Oryza sativa L.) and its distribution in fractions of rice grain. Chemosphere 69: 942-948.

46. Ratnaike RN (2003) Acute and chronic arsenic toxicity. Postgraduate Medical Journal **79:** 391-396

47. Rivas-San Vicente M, Plasencia J (2011) Salicylic acid beyond defence: its role in plant growth and development. Journal of Experimental Botany 62: 3321-3338.

48. Rodríguez-Serrano M, Romero-Puertas MC, Pazmiño DM, Testillano PS, Risueño MC, Luis A, Sandalio LM (2009) Cellular response of pea plants to cadmium toxicity: cross

talk between reactive oxygen species, nitric oxide, and calcium. Plant Physiology 150: 229-243.

49. Sandalio LM, Rodríguez-Serrano M, Romero-Puertas MC, Luis A (2008) Imaging of reactive oxygen species and nitric oxide *in vivo* in plant tissues. Methods in Enzymology 440: 397-409.
50. Scandalios JG, Tsaftaris AS, Chandlee JM, Skadsen RW (1983) Expression of the developmentally regulated catalase (Cat) genes in maize. Developmental Genetics 4: 281-293.
51. Singh AP, Dixit G, Mishra S, Dwivedi S, Tiwari M, Mallick S, Pandey V, Trivedi PK, Chakrabarty D, Tripathi RD (2015) Salicylic acid modulates arsenic toxicity by reducing its root to shoot translocation in rice (*Oryza sativa* L.). Frontoiers in Plant Science 6: 340.
52. Singh AP, Dixit G, Kumar A, Mishra S, Singh PK, Dwivedi S, Trivedi PK, Chakraboty D, Mallick S, Pandey V, Dhankher OP, Tripathi RD (2016). Nitric oxide alleviated arsenic toxicity by modulation of antioxidants and thiol metabolism in rice (*Oryza sativa* L.). Frontiers in Plant Science, 6.
53. Singh HP, Kaur S, Batish DR, Sharma VP, Sharma N, Kohli RK (2009) Nitric oxide alleviates arsenic toxicity by reducing oxidative damage in the roots of *Oryza sativa* (rice). Nitric Oxide- Biology and Chemistry 20: 289-297.
54. Song F, Goodman RM (2001) Activity of nitric oxide is dependent on, but is partially required for function of, salicylic acid in the signaling pathway in tobacco systemic acquired resistance. Molecular Plant-Microbe Interaction 14: 1458-1462.
55. Tanou G, Job C, Rajjou L, Arc E, Belghazi M, Diamantidis G, Molassiotis A, Job D (2009) Proteomics reveals the overlapping roles of hydrogen peroxide and nitric oxide in the acclimation of citrus plants to salinity. Plant Journal 60: 795-804.

56. Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley—powdery mildew interaction. Plant Journal 11: 1187-1194.

57. Tiwari M, Sharma D, Dwivedi S, Singh M, Tripathi RD, Trivedi PK (2014) Expression in *Arabidopsis* and cellular localization reveal involvement of rice NRAMP, OsNRAMP1, in arsenic transport and tolerance. Plant Cell Environ 37: 140-152.

58. Vasconcelos MW, Grusak MA (2014) Morpho-physiological parameters affecting iron deficiency chlorosis in soybean (*Glycine max* L.). Plant and Soil 374: 161-172.

59. Velikova V, Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. Plant Science 151: 59-66.

60. Vlot AC, Dempsey DMA, Klessig DF (2009) Salicylic acid, a multifaceted hormone to combat disease. Annual Review of Phytopathology 47: 177-206.

61. Xiao S, Brown S, Patrick E, Brearley C, Turner JG (2003) Enhanced transcription of the Arabidopsis disease resistance genes RPW8. 1 and RPW8. 2 via a salicylic acid–dependent amplification circuit is required for hypersensitive cell death. Plant Cell 15: 33-45.

62. Yang Y, Qi M, Mei C (2004) Endogenous salicylic acid protects rice plants from oxidative damage caused by aging as well as biotic and abiotic stress. Plant Journal 40: 909-919.

63. Yokosho K, Yamaji N, Ueno D, Mitani N, Ma JF (2009) OsFRDL1 is a citrate transporter required for efficient translocation of iron in rice. Plant Physiology 149: 297-305.

64. Zhang Z, Wang H, Wang X, Bi Y (2011) Nitric oxide enhances aluminum tolerance by affecting cell wall polysaccharides in rice roots. Plant Cell Research 30: 1701-1711.

65. Zhou ZS, Guo K, Elbaz AA, Yang ZM (2009) Salicylic acid alleviates mercury toxicity by preventing oxidative stress in roots of *Medicago sativa*. Environmental and Experimental Botany 65: 27-34.

66. Zottini M, Costa A, De Michele R, Ruzzene M, Carimi F, Schiavo FL (2007) Salicylic acid activates nitric oxide synthesis in *Arabidopsis*. Journal of Experimental Botany 58: 1397-1405.

Figure legends

Fig. 1: Effect of different combinations of NO, SA and As^{III} on (A) H_2O_2, (B) TBARS, (C) SOD, (D) CAT, (E) APX, (F) GPX and (G) GR activity in roots of *Oryza sativa* after 7 d treatment. P value marked with same alphabets are not significantly different (DMRT, $p<0.05$). All the values are mean of three replicates ±SD.

Fig. 2: Effect of different combinations of NO, SA and As^{III} on (A) endogenous NO dependent fluorescence, (B) endogenous level of SA and (C) nitrate reductase activity in roots of *Oryza sativa* after 7 d treatment. P value marked with same alphabets are not significantly different (DMRT, $p<0.05$). All the values are the mean of three replicates ±SD.

Fig. 3: Imaging of NO production in *Oryza sativa* by CLSM. Images are showing the NO-dependent DAF-FM 2DA fluorescence (green; excitation at 495 nm, emission at 515 nm) after 7 d treatment with different combinations of NO, SA and As^{III}. Negative control is treated with 10Mm cPTIO, a NO scavenger and prior to staining with DAF-FM2DA.

Fig. 4: Quantitative real-time PCR analysis to study the expression gene pattern. Y-axis represents relative fold change expression of mRNA level in different combinations of NO, SA and As^{III} on (A) OsLsi1, (B) OsNRAMP5, (C) OsIRT1, (D) OsIRO2 , (E) OsFRDL1 and (F) OsYSL2 transporters roots of *Oryza sativa* after 7 d treatment. Effect P value marked with same alphabets are not significantly different (DMRT, $p<0.05$). All the values are the mean of three replicates ±SD.

Table 1. Effect on root, shoot, biomass and total chlorophyll content of *Oryza sativa* after 7d treatment with different combinations of NO, SA and As^{III}. P value marked with same alphabet is not significantly different (DMRT, $p<0.05$). All the values are mean of three replicates ±SD.

Treatments	Root (cm)	Shoot (cm)	Biomass (g)	Total Chlorophyll (mg g^{-1})
Control	7.20^{fg}±0.1	23.08^{c}±1.3	0.242^{c}±0.02	2.69^{bc}±0.05
NO	7.58^{gh}±0.4	28.66^{de}±1.6	0.385^{e}±0.01	2.85^{c}±0.03
SA	8.18^{h}±1.1	30.51^{e}±1.8	0.507^{f}±0.03	3.45^{e}±0.06
As^{III}	3.75^{a}±0.2	16.83^{a}±1.0	0.164^{a}±0.01	1.56^{a}±0.03
As^{III}+NO	5.09^{bc}±0.4	25.83^{d}±1.7	0.262^{c}±0.02	2.71^{bc}±0.07
As^{III}+SA	4.54^{ab}±0.4	19.25^{ab}±1.7	0.205^{b}±0.01	2.81^{c}±0.09

Table 2: Effect on various metal accumulations (μg g^{-1}) in roots (A), shoot (B) and shoot/root Translocation Factor (C) of *Oryza sativa* after 7d treatment with different combinations of NO, SA and As^{III}. P values marked with same alphabets are not significantly different (DMRT, p‹0.05). All the values are mean of three replicates ±SD.

Table: 2A

Treatments	**Fe**	**As**	**Ca**	**Zn**	**Mn**
Control	869.4^{ab}±28.2	-	518.4^{b}±28.4	513.4^{a}±24.1	387.5^{a}±27.4
NO	830.1^{a}±54.5	-	536.8^{b}±33.4	546.3^{a}±25.1	451.2^{b}±33.6
SA	939.5^{b}±33.4	-	503.8^{b}±41.1	532.2^{a}±29.1	479.8^{b}±24.2
As^{III}	1666.5^{d}±40.0	576.9^{b}±36.6	449.5^{a}±27.6	543.7^{a}±27.4	395.2^{a}±28.2
As^{III} and NO	1312.8^{c}±64.8	370.5^{a}±21.4	521.2^{b}±31.5	531.3^{a}±33.2	389.0^{a}±19.6
As^{III} and SA	1622.6^{d}±49.1	576.1^{b}±42.3	509.5^{b}±12.5	598.38^{a}±25.1	383.3^{a}±12.4

Table: 2B

Treatments	**Fe**	**As**	**Ca**	**Zn**	**Mn**
Control	257.76^{ab}±17.1	-	4813.7^{a}±92.4	841.4^{a}±74.3	1473.1^{a}±72.2
NO	296.08^{c}±14.3	-	4801.7^{a}±118.3	859.2^{a}±77.8	1562.1^{a}±84.6
SA	304.64^{c}±26.9	-	4802.6^{a}±108.3	844.5^{a}±34.5	1533.6^{a}±91.1
As^{III}	237.08^{a}±14.2	57.19^{c}±2.9	4825.4^{a}±89.1	839.5^{a}±61.1	1469.3^{a}±64.9
As^{III} and NO	286.56^{bc}±15.2	22.14^{a}±1.4	4794.5^{a}±106.5	878.5^{a}±34.5	1460.4^{a}±116.3
As^{III} and SA	292.28^{bc}±23.1	41.25^{b}±3.4	4684.9^{a}±42.5	864.1^{a}±59.1	1530.4^{a}±62.7

Table: 2C:

Treatments	**Fe**	**As**	**Ca**	**Zn**	**Mn**
Control	0.30	-	9.29	1.64	3.80
NO	0.36	-	8.20	1.57	3.46
SA	0.32	-	9.53	1.59	3.20
As^{III}	0.14	0.10	10.74	1.54	3.72
As^{III} and NO	0.22	0.06	9.20	1.65	4.75
As^{III} and SA	0.18	0.07	9.20	1.44	3.99

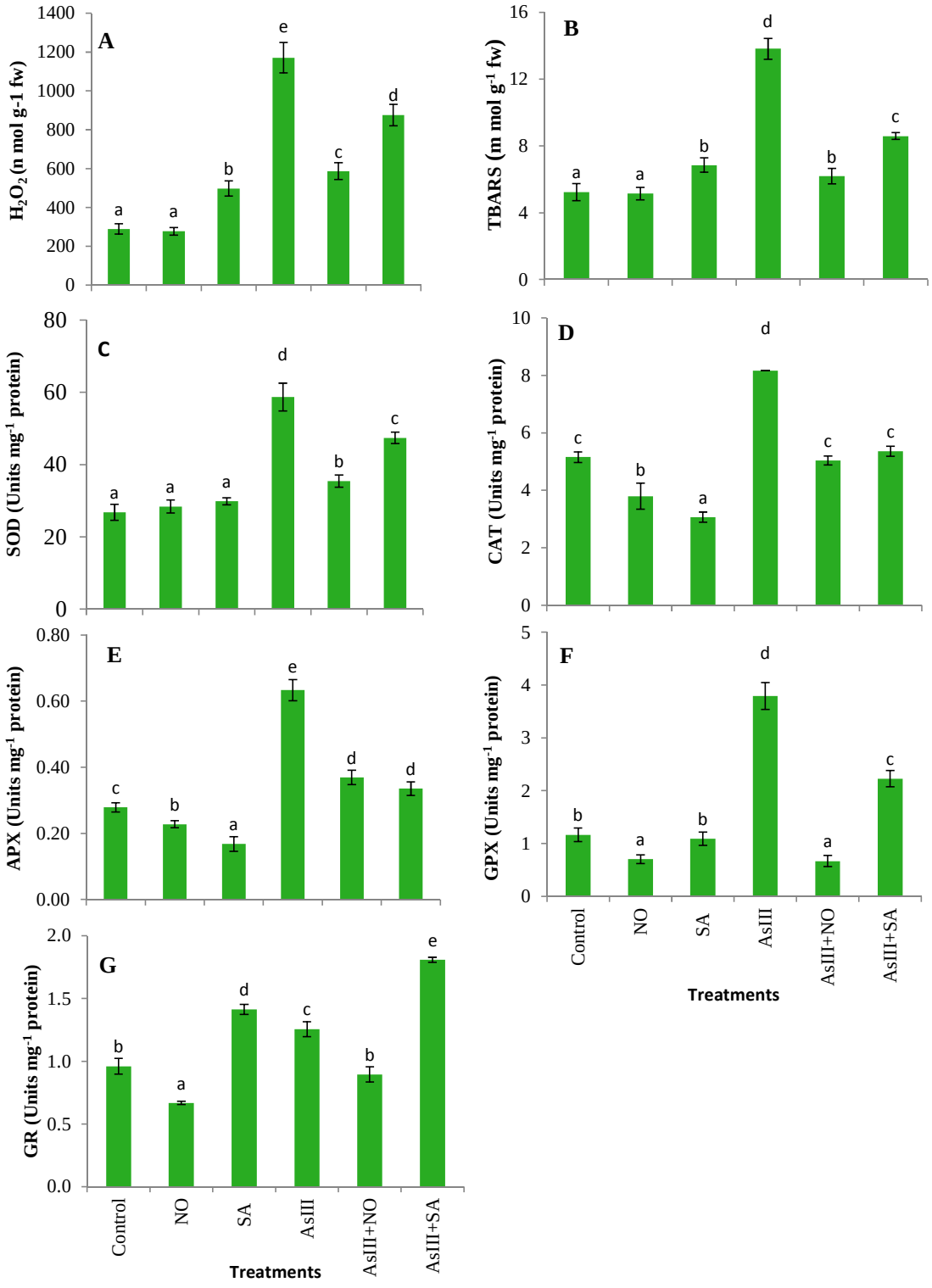
A
H_2O_2 (n mol g-1 fw)
1400
1200
1000
800
600
400
200
0
a a b e c d
B
TBARS (m mol g^{-1} fw)
16
12
8
4
0
a a b d b c
C
SOD (Units mg^{-1} protein)
80
60
40
20
0
a a a d b c
D
CAT (Units mg^{-1} protein)
10
8
6
4
2
0
c b a d c c
E
APX (Units mg^{-1} protein)
0.80
0.60
0.40
0.20
0.00
c b a e d d
F
GPX (Units mg^{-1} protein)
5
4
3
2
1
0
b a b d a c
G
GR (Units mg^{-1} protein)
2.0
1.5
1.0
0.5
0.0
b a d c b e
Control
NO
SA
AsIII
AsIII+NO
AsIII+SA
Treatments

Fig. 1.

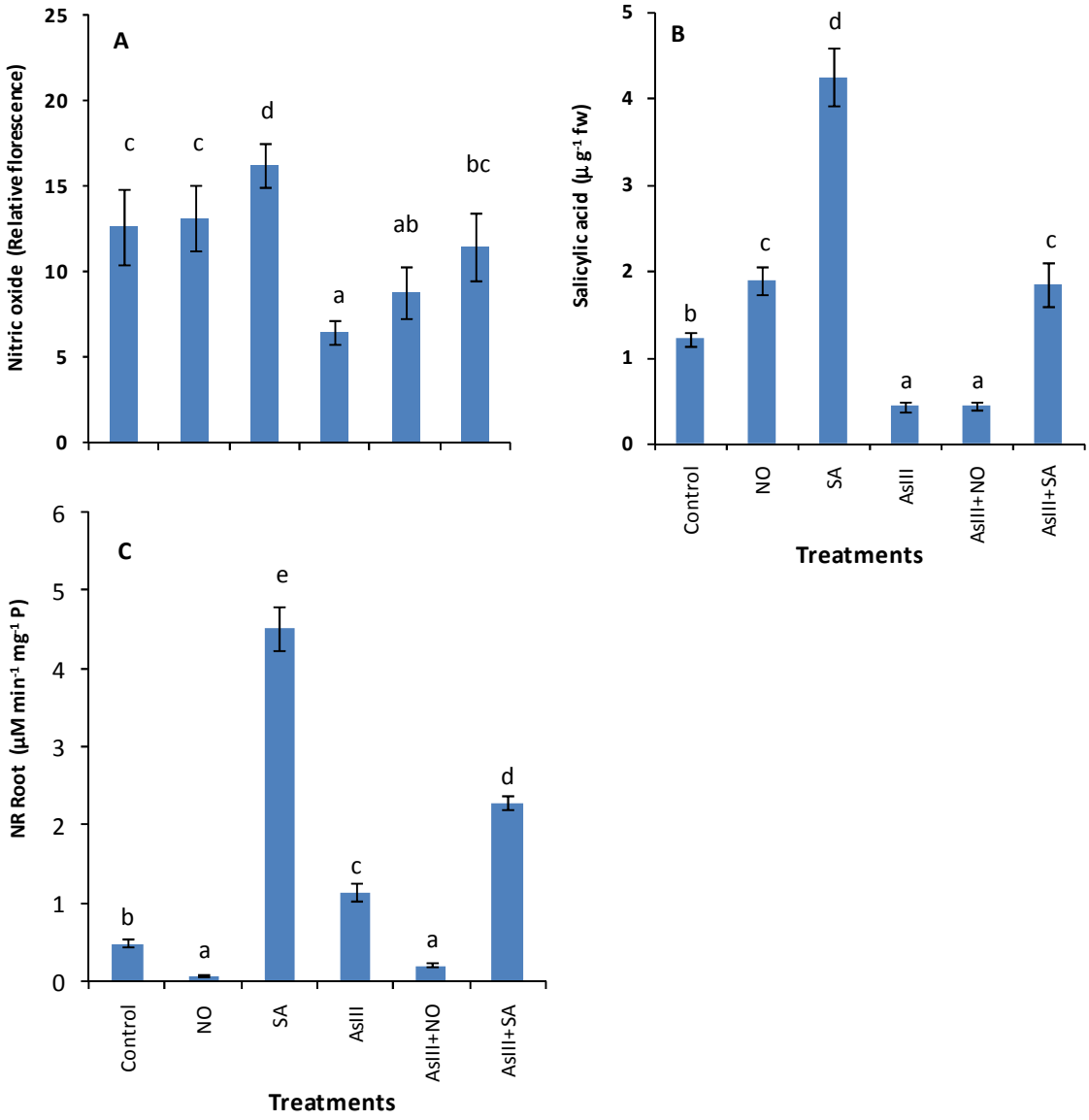
A
Nitric oxide (Relative florescence)
25
20
15
10
5
0
c
c
d
a
ab
bc
B
Salicylic acid (µ g-1 fw)
5
4
3
2
1
0
b
c
d
a
a
c
Control
NO
SA
AsIII
AsIII+NO
AsIII+SA
Treatments
C
NR Root (µM min-1 mg-1 P)
6
5
4
3
2
1
0
b
a
e
c
a
d
Control
NO
SA
AsIII
AsIII+NO
AsIII+SA
Treatments

Fig. 2.

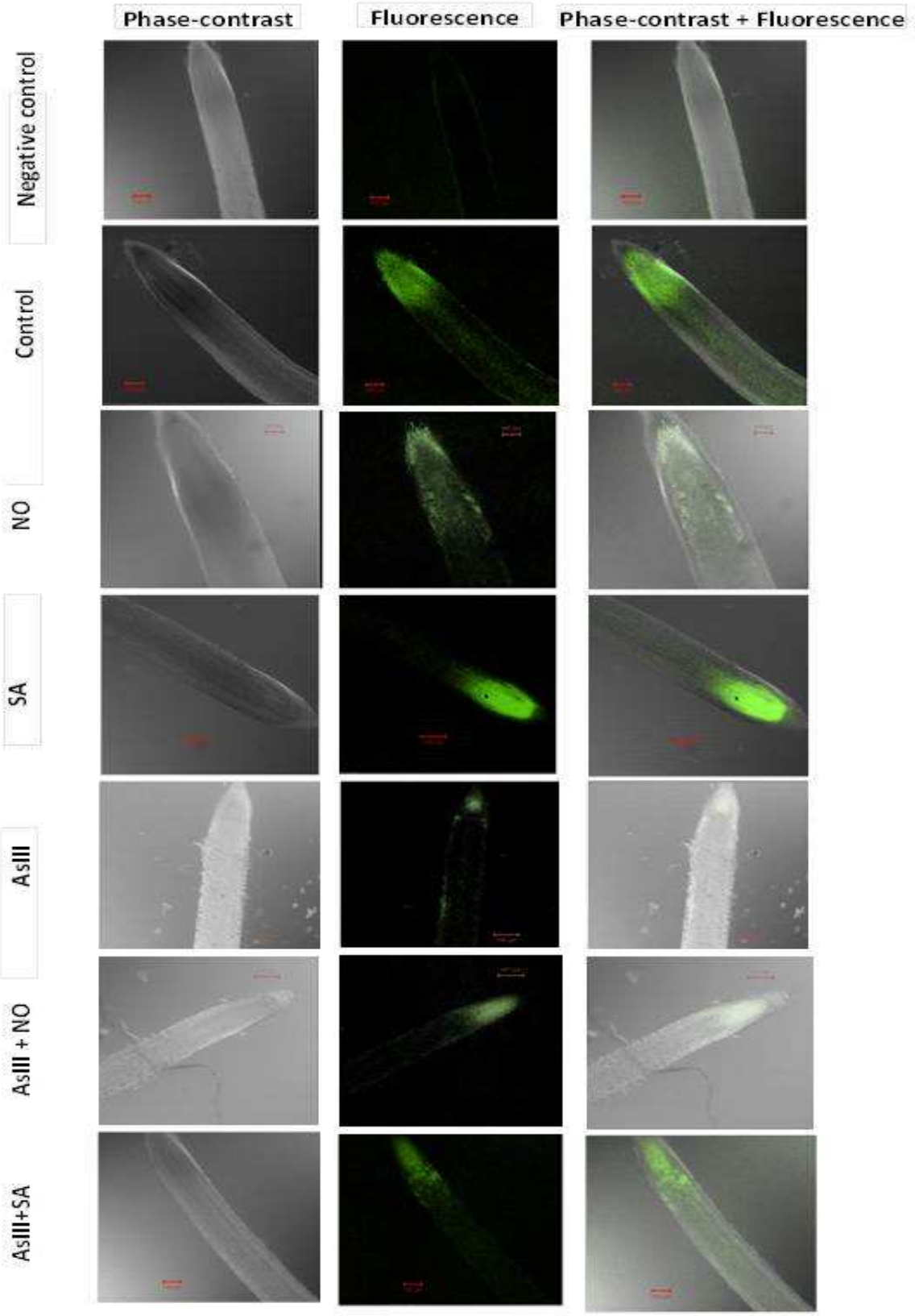
Phase-contrast
Fluorescence
Phase-contrast + Fluorescence
Negative control
Control
NO
SA
AsIII
AsIII + NO
AsIII+SA

Fig. 3.

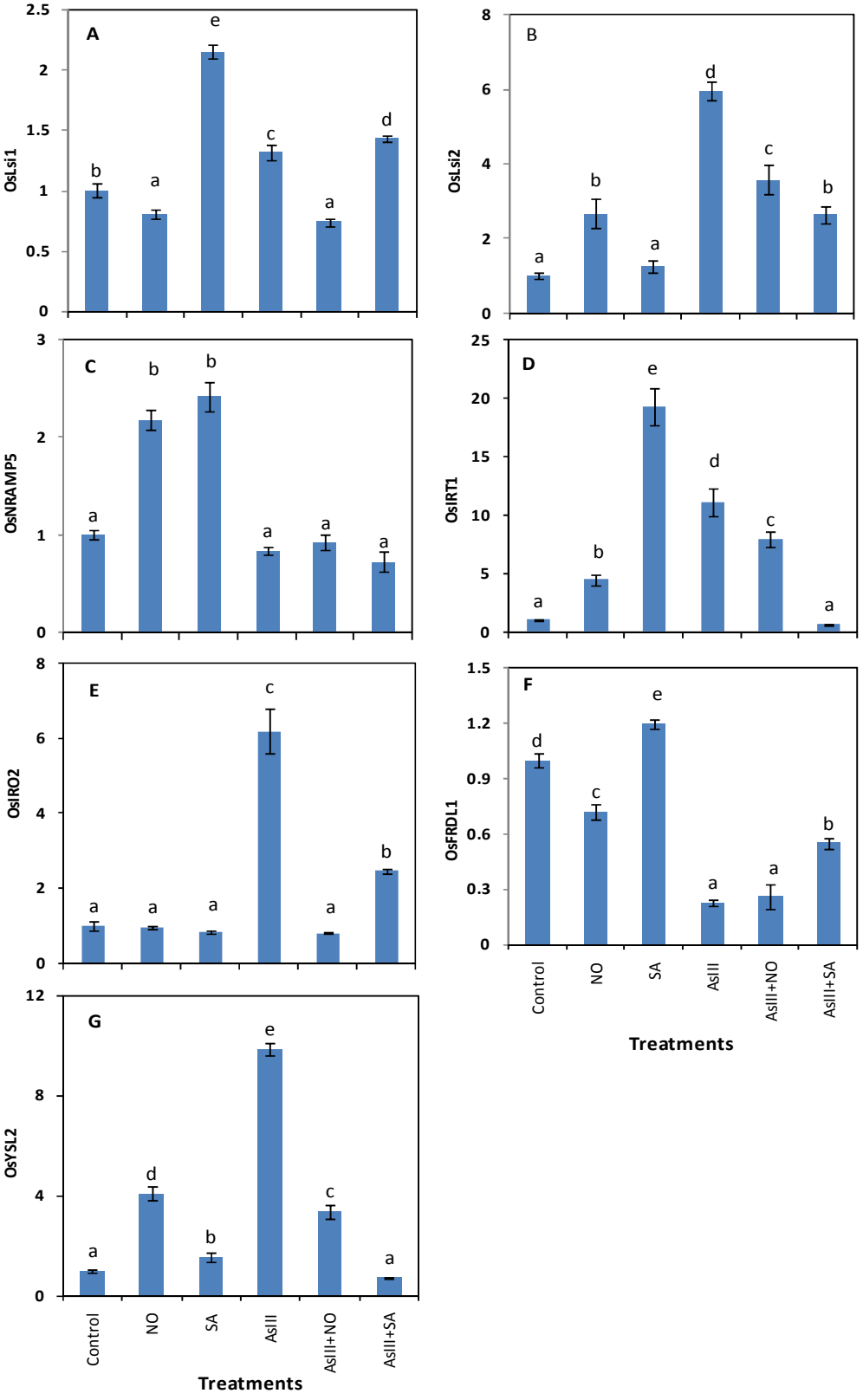
A
OsLsi1
B
OsLsi2
C
OsNRAMP5
D
OsIRT1
E
OsIRO2
F
OsFRDL1
G
OsYSL2
Control
NO
SA
AsIII
AsIII+NO
AsIII+SA
Treatments

Fig. 4.

Highlights

1. Nitric oxide (NO) decreased the arsenic (As) accumulation in both root and shoot
2. Salicylic acid (SA) decreased the root to shoot translocation of As.
3. NO and SA mitigated the As-mediated oxidative stress.
4. NO and SA were worked in a mutually coordination manner irrespective of As presence.