RESEARCH ARTICLE

Azadirachta indica and *Ocimum sanctum* leaf extracts alleviate arsenic toxicity by reducing arsenic uptake and improving antioxidant system in rice seedlings

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Abstract In the present study the potentials of aqueous extracts of the two plants, neem (*Azadirachta indica*) and Tulsi (*Ocimum sanctum*) were examined in alleviating arsenic toxicity in rice (*Oryza sativa* L.) plants grown in hydroponics. Seedlings of rice grown for 8 days in nutrient solution containing 50 μ M sodium arsenite showed decline in growth, reduced biomass, altered membrane permeability and increased production of superoxide anion ($O_2^{\cdot-}$), H_2O_2 and hydroxyl radicals ($\cdot OH$). Increased lipid peroxidation marked by elevated TBARS (thiobarbituric acid reactive substances) level, increased protein carbonylation, altered levels of ascorbate, glutathione and increased activities of enzymes SOD (superoxide dismutase), CAT (catalase), APX (ascorbate peroxidase) and GPX (glutathione peroxidase) were noted in the seedlings on As treatment. Exogenously added leaf aqueous extracts of *Azadirachta indica* (0.75 mg mL⁻¹, w/v) and *Ocimum sanctum* (0.87 mg mL⁻¹, w/v) in the growth medium considerably alleviated As toxicity effects in the seedlings, marked by reduced As uptake, restoration of membrane integrity, reduced production of ROS, lowering oxidative damage and restoring the levels of ascorbate, glutathione and activity levels of antioxidative enzymes. Arsenic uptake in the seedlings declined by 72.5% in roots and 72.8% in shoots, when *A. indica* extract was present in the As treatment medium whereas with *O. sanctum* extract, the uptake declined by 67.2% in roots and 70.01% in shoots. Results suggest that both *A. indica* and *O. sanctum* aqueous extracts have potentials to alleviate arsenic toxicity in rice

plants and that *A. indica* can serve as better As toxicity alleviator compared to *O. sanctum*.

Keywords *Azadirachta indica* · *Ocimum sanctum* · Antioxidants · Arsenic · Toxicity · Oxidative stress

Introduction

Increased industrial activities along with release of untreated effluents from the industries have resulted in accumulation of waste products, including various metals and metalloids, in the environment. Among different pollutants, the toxic metalloid arsenic (As) is a major pollutant and a potent carcinogen. In recent years, As has become an environmental pollutant of serious concern in many parts of the world (Sharma et al. 2012, 2014). It occurs naturally in different minerals and its availability in soil, water and air depends upon its release and mobilization from the sources (Roy and Saha 2002). Increased use of As in production of pigments, herbicides, wood preservatives along with various natural sources including volcanic eruptions lead to increased level of As in the environment (Tchounwou et al. 2012). Agricultural soils in many parts of the world like in India, China, Bangladesh, and USA are contaminated with up to three fold more As than basal level (Farooq et al. 2016). Drinking water and irrigation water in many countries around the world, more especially in Asia, South America are contaminated with high amounts of As. Though a non-essential element, As is readily taken up by plants along with the normal nutrient uptake and can accumulate inside the tissues to the toxic levels (Brackhage et al. 2014).

Excess As in the soil or in the growth medium causes retarded seed germination, decreases plant growth,

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ultimately leading to decline in crop productivity (Hettick et al. 2015). With high concentration of As in plant tissues, increased generation of various ROS (reactive oxygen species) such as O_2^- (superoxide), H_2O_2 and $\cdot OH$ (hydroxyl radical) takes place leading to a state of oxidative stress in the cells (Mishra et al. 2011). Enhanced lipid peroxidation, DNA damage, protein oxidation and impairment of redox homeostasis in the cells are observed in As exposed plants due to oxidative stress (Mishra et al. 2011; Sharma et al. 2014). To cope up with the ROS induced damage, antioxidative defense system exists in the tissues which comprises of ascorbate and glutathione as non enzymic antioxidants as well as the antioxidative enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (GPX, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.7), monodehydroascorbatereductase (MDHAR, EC 1.6.5.4) and dehydroascorbatereductase (DHAR, EC 1.8.5.1). These antioxidants work in a specific and coordinated way to maintain a redox homeostasis inside the plants (Sharma et al. 2012).

Azadirachta indica or ‘Neem’ which is also called as Indian lilac belongs to family Meliaceae and it is an evergreen or deciduous tree prevalent in tropical regions. Neem is being used in Ayurveda, Unani and homeopathy along with the modern medicine and it contains more than 100 bioactive ingredients which possess a wide range of medicinal, antibacterial, insecticidal and various other types of biological activities (Dohroo et al. 2016). The polyphenolic compounds inactivate the lipid free radicals and also prevent their formation by inhibiting hydroperoxides decomposition (Nahak and Sahu 2010). The polyphenols also chelate metal ions and hence reduce bioavailability of metals in plants (Harikrishnan et al. 2010). *Ocimum sanctum* or ‘Tulsi’ on the other hand has been used traditionally for many years due to its potent medicinal and antioxidative properties. The leaves of Tulsi possess a wide range of pharmacologically active volatile oils such as eugenol, carvacrol, euginal, linalool, caryophyllene, limatrol and many others (Pattanayak et al. 2010). In addition, a variety of triterpenoids, saponins, flavonoids and tannins are also present. Leaves also contain some water soluble flavanoids such as orientin and vicenin (Pattanayak et al. 2010).

Rice serves as important constituent in diets for the global population. Arsenic is taken up from the soil by the roots of rice plants in the form of either arsenite (AsIII) or arsenate (AsV) (Sharma et al. 2014). Both shoots and grains of rice can accumulate very high concentrations of As compared to other cereals and consuming As-contaminated rice grains may lead to severe health hazards like cancer of lung, skin, liver as well as respiratory and cardiovascular impairments (Guillod-Magnin et al. 2018).

This necessitates a detailed study of As transport and the mechanisms of As induced toxicity in rice plants and to explore the methods for mitigating As uptake, its accumulation and toxicity effects in rice plants. To mitigate toxicity effects of the metals Mn, Ni, Cr, Al in plants, many chemicals like nitric oxide, salicylic acid, Mg, Ca and extracts of different parts of plants with high antioxidant potentials such as *Terminalia arjuna*, *Phyllanthus emblica*, etc. have been used with successful results (Srivastava and Dubey 2012; Pandey et al. 2013; Rajpoot et al. 2016; Pandey et al. 2019a). These chemicals or herbal extracts reduce the uptake of metals in the tissues and lower the degree of oxidative stress induced by the metals in the plants and thus help in mitigating metal toxicity effects in plants. As the plants *A. indica* and *O. sanctum* have proven antioxidant potential, in the present study we have used aqueous leaf extracts of these plants to examine possible alleviation of arsenic toxicity in rice plants. The studies were therefore conducted to investigate effects of As on growth parameters, ROS production, oxidative damage, level of antioxidants, ascorbate and glutathione and the activities of key antioxidative enzymes in rice seedlings grown in hydroponics. The aqueous extracts of *A. indica* and *O. sanctum* were examined for their role in alleviating As toxicity in the seedlings.

Materials and methods

Extracts from *Azadirachta indica* and *Ocimum sanctum* leaves

Fresh leaves from the tree of *A. indica* and from the shrub of *O. sanctum* were collected from within the campus of Banaras Hindu University, India. Leaves were dried in sun and crushed to powder. Five gram leaf powder of *A. indica*, mixed with 100 mL water was placed on a shaker for 72 h. After centrifugation at $12,000 \times g$ for 15 min, supernatant was collected and evaporated to dryness. The dried residue weighing 0.750 mg dissolved in 1 mL water served as stock solution of 0.75 mg mL^{-1} (w/v). Similarly *O. sanctum* extract was prepared by weighing 0.875 mg residue in 1 mL double distilled water and a stock solution of 0.875 mg mL^{-1} (w/v) was prepared. When 5 g dried leaf powder of *A. indica* was extracted in water, 0.750 mg dried residue was obtained, whereas with extraction of 5 g dried leaf powder of *O. sanctum*, 0.875 mg residue was obtained.

Rice plants and treatments

Rice (*Oryza sativa* L.) seeds of cultivar Malviya-36, obtained from agriculture farm of Banaras Hindu University were used. Seeds were surface sterilized with 0.1%

sodium hypochlorite, soaked in water for 48 h and were then spread in Petri plates on moist filter paper and germinated for 5 days in an incubator (York Scientific Industries, New Delhi, India) maintained at 28 ± 1 °C with 80% relative humidity. Germinated seeds were then placed in 200 mL Yoshida nutrient solution in plastic pots (Yoshida et al. 1976) which served as control, whereas nutrient solutions containing either 3 mL *A. indica* aqueous extract or 3.5 mL *O. sanctum* aqueous extract also served as controls in order to examine any direct effects of extract in the absence of As. Nutrient solution containing 50 μ M sodium arsenite (NaAsO_2) served as As treatment solution, As treatment solution containing 3 mL *A. indica* (Ai) extract served as As + Ai treatment solution, and nutrient solution containing 50 μ M NaAsO_2 and 3.5 mL *O. sanctum* (Os) extract served as As + Os treatment solution. Preliminary experiments were performed and the doses 3 mL of 0.75 mg mL⁻¹ stock of *A. indica* extract in 200 mL nutrient solution = 2.25 mg *A. indica* extract in 200 mL solution and 3.5 mL of 0.875 mg mL⁻¹ stock of *O. sanctum* extract in 200 mL nutrient solution = 3.0625 mg *O. sanctum* extract in 200 mL nutrient solution were determined which elicited similar effects. In a greenhouse maintained at 28 ± 1 °C under 80% relative humidity and 12 h light/dark cycle with 190–200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance, pots were placed for growth of the seedlings. Seedlings were taken out at 4 and 8 days of growth for various analyses and biochemical determinations.

Growth of seedlings and uptake of arsenic

At 4th and 8th day of growth, length and fresh biomass of roots and shoots of the seedlings were determined in triplicate using 10 samples in each set. Dry weights were determined after drying in oven at 60 °C for 72 h. Relative water content (RWC) was determined following the method of Weatherley (1950). Concentration of arsenic in oven dried powdered root/shoot samples was determined according to Allen et al. (1986) employing ICP-OES (Inductive coupled plasma-optical emission spectrometer, Optima 7000 DV, Perkin Elmer, USA) and was expressed as $\mu\text{moles g}^{-1}$ tissue dry weight. The analytical standard used for ICP-OES analysis was TraceCERT®, 1 mg/L Arsenic in nitric acid (Merck). The ICP measurement data may be taken as estimates for the concentration of As in the samples, as we did not use standard reference material (SRM) processed at the same time as the experimental samples.

Measurement of superoxide anion production, H_2O_2 and their histochemical localizations

Production of $\text{O}_2^{\cdot-}$ due to autoxidation of epinephrine was measured spectrophotometrically according to Mishra and Fridovich (1972) using freshly cut 3–5 mm pieces of root/shoot samples. Using 1.2 mM epinephrine (prepared in 0.1 N HCl) reaction was initiated and production of $\text{O}_2^{\cdot-}$ was recorded at 480 nm using spectrophotometer (Spectronic 20D+, Thermo Scientific, USA). H_2O_2 was measured following Jana and Choudhuri (1981) using titanium sulfate. Absorption of colour developed was recorded spectrophotometrically at 410 nm and H_2O_2 level was calculated using extinction coefficient as $0.28 \mu\text{M}^{-1} \text{cm}^{-1}$. Nitrobluetetrazolium (NBT) was used to detect histochemical localization of $\text{O}_2^{\cdot-}$ in leaves following Frahry and Schopfer (2001). Dark blue insoluble formazon deposits were visible on leaf surface as a result of reaction of $\text{O}_2^{\cdot-}$ with NBT, which were visualized using microscope. H_2O_2 was histochemically detected in leaves according to Thordal et al. (1997) employing DAB (3,3'-diaminobenzidine, Amresco, Solon, OH, USA). Reddish brown spots appeared on leaves due to reaction of DAB with H_2O_2 and peroxidase, which were visualized using microscope.

Determination of $\cdot\text{OH}$ radicals and lipid peroxidation

Formation of $\cdot\text{OH}$ in fresh root/shoot samples was quantified spectrophotometrically according to Halliwell and Gutteridge (1984) using a deoxyribose assay. The absorbance was recorded at 532 nm. The level of lipid peroxidation products was determined in root/shoot samples using 2-thiobarbituric acid (TBA) according to Heath and Packer (1968) and as modified according to Rajpoot et al. (2016) and expressed in terms of nmol TBARS (thiobarbituric acid reactive substances) g^{-1} fresh weight using extinction coefficient of $155 \text{ mM}^{-1} \text{cm}^{-1}$. Histochemical localization of lipid peroxides was detected according to Pompella et al. (1987) using Schiff's reagent. Pink coloured spots showing aldehyde formation were observed under microscope.

Alteration in integrity of plasma membrane

At various levels of treatments, alteration in integrity of root plasma membrane was assessed histochemically using Evan's blue uptake according to Schützendübel et al. (2001). Dark blue stained roots, due to uptake of the dye Evan's blue, were examined under microscope. Spectrophotometric assay was also used to quantify the extent of Evan's blue dye taken up by the roots as described by

Pandey et al. (2019a). The uptake of the dye was calculated in terms of its fold uptake by the roots.

Determination of non-enzymic antioxidants

The levels of reduced (ascorbate, AsA) and oxidized form of ascorbate (dehydroascorbate, DHA) as well as of total ascorbate (AsA + DHA) were determined following the method of Law et al. (1983). Initially total ascorbate and AsA were determined. The DHA level was determined by subtracting AsA from total ascorbate. The levels of different forms of glutathione (reduced glutathione GSH and oxidized glutathione GSSG) and of total glutathione (GSH + GSSG) were determined according to Griffith (1980) using sulphosalicylic acid, DTNB (5,5'-dithiobis-2-nitrobenzoic acid), glutathione reductase, NADPH and BSA (bovine serum albumin).

Activities of antioxidative enzymes

The activity of SOD was assayed following the method of Mishra and Fridovich (1972) using nitroblue tetrazolium (NBT) reduction method. Reduction of NBT was recorded by measuring absorbance at 560 nm. Catalase was assayed according to Beers and Sizer (1952). Decomposition of H_2O_2 was monitored at 240 nm using UV–Vis spectrophotometer. Catalase activity was expressed as $\mu\text{mol H}_2\text{O}_2$ oxidized $\text{min}^{-1} \text{mg}^{-1}$ protein. APX activity was assayed following Nakano and Asada (1981). Oxidation of ascorbic acid was monitored at 290 nm using UV visible spectrophotometer. GPX activity was assayed following Egley et al. (1983) using guaiacol and H_2O_2 . Enzyme activity was expressed in terms of $\text{mmol H}_2\text{O}_2$ reduced $\text{min}^{-1} \text{mg}^{-1}$ protein. Assay of MDHAR was done using ascorbate oxidase and NADH. NADH oxidation was monitored at 340 nm using spectrophotometer and activity was expressed as $\text{nmol NADH oxidized min}^{-1} \text{mg}^{-1}$ protein. Activity of DHAR was assayed following the method of Doulis et al. (1997). Reduction of DHA was monitored at 265 nm using UV–Vis spectrophotometer.

Non-protein thiol, protein-thiol, carbonylation of proteins and proteolysis

Total thiol content, which included protein thiol and non-protein thiol, was estimated following Dekok and Kuiper (1986) using DTNB. Total thiol content was calculated using extinction coefficient as $13,600 \text{ M}^{-1} \text{cm}^{-1}$. The content of non-protein thiol was subtracted from total thiol to get the content of protein thiol. Contents were expressed in terms of $\text{nmol thiols g}^{-1}$ tissue fresh weight. Method of Levine et al. (1994) as modified by Pandey et al. (2013) was followed to determine the extent of protein

carbonylation in rice seedlings using DNPH reagent. Absorbance was recorded at 370 nm using UV–Vis spectrophotometer. Extinction coefficient of $22,000 \text{ M}^{-1} \text{cm}^{-1}$ was used to calculate the content of protein carbonyls and was expressed in terms of nmol mg^{-1} protein in the tissues.

Determination of protein

In all preparations, protein concentration was determined using Bradford (1976) reagent using BSA (bovine serum albumin, Sigma) as standard.

Statistical treatment of the data

Experiments for each analysis were conducted in triplicate. The values represent mean of three independent determinations $\pm$ standard deviation (SD). One way ANOVA and Tukey's multiple range test were used to analyze the differences between control and treatment values. The typical mean with the highest absolute value and other means not statistically different from the highest value have been represented as letter 'a' and lesser mean values progressively have been represented as letters 'b' and 'c'.

Results

Arsenic effects on growth of rice seedlings and role of *A. indica* and *O. sanctum* extracts

Rice seedlings were raised in hydroponics for 8 days under control and various treatments. Plant height including root/shoot length, fresh weight and relative water content (RWC) were recorded at day-4 and day-8 of treatment. A significant reduction in root/shoot length as well as fresh biomass and relative water content was apparent in 50 μM sodium arsenite treated seedlings compared to the seedlings raised in Yoshida nutrient solution (controls) (Fig. 1). Addition of either extract to the nutrient solution did not affect either root or shoot length. By day 8, As treatment caused a 35% reduction in root length and a 42% reduction in shoot length (Fig. 1a). Further, 50 μM As + *O. sanctum* treated seedlings showed 46.8% and 60.1% restoration in root and shoot growth respectively in comparison to As treated seedlings. Similarly, treatment with 50 μM As for 8 days caused 53.6% and 22.7% reductions in the fresh weights of roots and shoots respectively whereas when *A. indica* extract was present in 50 μM As containing growth medium, 62.7% restoration in root weight and 23.5% restoration in shoot weight were observed compared to As treated seedlings. Similarly, 50 μM As + *O. sanctum* treatment caused 58.8% and 20.5% restoration in root and

Fig. 1 Effect of arsenic (As), As + *Azadirachta indica* extract (Ai) and As + *Ocimum sanctum* (Os) extract treatments in the growth medium on **a** length, **b** fresh weight and **c** relative water content of roots and shoots of rice seedlings at 4 and 8 days of treatment. Seedlings were grown in hydroponics for 8 days under control, control + *A. indica* extract (control + Ai), control + *O. sanctum* (control + Os), 50 μ M As (As) and 50 μ M As + *A. indica* extract (As + Ai) and 50 μ M As + *O. sanctum* extract (As + Os). Values are mean $\pm$ SD based on three independent determinations and bars indicate standard deviations. The typical mean with the highest absolute value and other means not statistically different from the highest value have been represented as letter 'a' and lesser mean values progressively have been represented as letters 'b' and 'c'

shoot growth respectively in comparison to As treated seedlings (Fig. 1b). Relative water content declined by 20.5% and 11.5% in roots and shoots respectively due to 50 μ M As treatment compared to controls and a restoration of 17.02% and 9.6% respectively in RWC was noted in As + *A. indica* treated seedlings compared to As treatment. Further 50 μ M As + *O. sanctum* treatment caused 17.02% and 9.1% restoration in root and shoot RWC respectively in comparison to As treated seedlings (Fig. 1c). The ICP-OES analysis revealed that with 8 days of 50 μ M As treatment, roots of the seedlings had 300.52 μ mol As g^{-1} dry wt., whereas shoots had As level of 251.78 μ mol g^{-1} dry wt. (Table 1). Arsenic uptake by growing rice seedlings substantially declined with the addition of *A. indica* and *O. sanctum* extract in the growth medium. With *A. indica* extract in the medium, 72.5% decline in As uptake was noted in roots and 72.8% decline in shoots, whereas with the presence of *O. sanctum* extract,

67.2% decline in As uptake was recorded in roots and 70.01% decline in shoots as compared to As uptake level observed in 50 μ M As treated seedlings. No As could be detected in control roots and shoots in ICP-OES analysis. The detection level of the instrument was 3.6 μ g L^{-1} (ppb).

Effect of *A. indica* and *O. sanctum* extracts on As induced generation of reactive oxygen species

In rice seedlings grown in presence of As, increased formation of $O_2^{\cdot-}$ was noted in comparison to controls (Fig. 2). With As treatment for 8 days, 66.6% increased $O_2^{\cdot-}$ generation was noted in roots and 44.3% in shoots. A significant decline of As induced $O_2^{\cdot-}$ production could be noted with *A. indica* or *O. sanctum* extracts in the medium (Fig. 2a). Production of $O_2^{\cdot-}$ was histochemically observed in leaves using the dye NBT. Blue colored formazon spots were clearly evident as a result of NBT reaction with $O_2^{\cdot-}$.

Table 1 Arsenic content in roots and shoots of rice seedlings at 8 days of growth under various treatments

Samples	Treatments	As content ($\mu\text{mol g}^{-1}$ dry weight)
Root	Control	ND
	Control + Ai	ND
	Control + Os	ND
	As	$300.52 \pm 2.229^{***}$
	As + Ai	$80.27 \pm 1.137^{***}$
	As + Os	$96.21 \pm 2.012^{***}$
Shoot	Control	ND
	Control + Ai	ND
	Control + Os	ND
	As	$251.78 \pm 3.009^{***}$
	As + Ai	$68.4 \pm 2.088^{***}$
	As + Os	$75.5 \pm 1.023^{***}$

Seedlings were raised in Yoshida nutrient solution (control), nutrient solution containing *A. indica* extract (Control + Ai), nutrient solution containing *O. sanctum* extract (Control + Os), nutrient solution containing 50 μM As_2O_3 (As), nutrient solution containing 50 μM As_2O_3 and *A. indica* extract (As + Ai) and nutrient solution containing 50 μM As_2O_3 and *O. sanctum* extract (As + Os). ND depicts not detected. Values are mean $\pm$ SD based on three independent determinations and asterisks (***) represent significant differences compared to control at $p \leq 0.001$ according to Tukey's multiple range test

The intensity of dark blue stained spots increased in leaves due to As treatment, showing enhanced $\text{O}_2^{\cdot-}$ production on As treatment. In leaves of rice seedlings treated with As + *A. indica* extract or As + *O. sanctum* extract, less intense patches were observed in comparison to As treated seedlings indicating suppression of As induced $\text{O}_2^{\cdot-}$ generation in rice leaves due to the extracts of *A. indica* or *O. sanctum* (Fig. 2b).

With As treatment, seedlings showed higher level of H_2O_2 production. In 8 days As treated seedlings, H_2O_2 level increased by 85.7% in roots and 91.5% in shoots, while the production of H_2O_2 declined by 41.02% in roots and 34.7% in shoots when *A. indica* extract was included in As containing medium (Fig. 3a). Similarly, with *O. sanctum* extract in As treatment medium, 41.5% decline in H_2O_2 content in roots and 38.3% decline in shoots were noted as against H_2O_2 content in As-stressed seedlings. When localization of H_2O_2 was examined histochemically with DAB staining, untreated control seedlings showed lesser accumulation of H_2O_2 marked by very few spots while more intense stained spots were seen in seedlings treated with As (Fig. 3b). When leaves from seedlings grown in As + *A. indica* or As + *O. sanctum* containing medium were examined after DAB staining, the intensity of spots were less intense in comparison with the intensity in As treated seedlings which suggests a protective role of

A. indica and *O. sanctum* extracts by lowering H_2O_2 production under As treatment (Fig. 3b).

The production of another ROS, $\text{OH}^{\cdot}$ was also higher in the seedlings on As treatment. The level of $\text{OH}^{\cdot}$ was 55.5% higher in roots and 61.9% higher in shoots of As-treated seedlings as against the levels in untreated controls (Fig. 4). Arsenic induced production of $\text{OH}^{\cdot}$ declined in both roots and shoots when *A. indica* or *O. sanctum* extracts were present in As treatment medium. This indicates a marked role of *A. indica* and *O. sanctum* extracts in lowering As induced $\text{OH}^{\cdot}$ production in the seedlings (Fig. 4).

Arsenic induced lipid peroxidation and roles of the extracts

In As-treated seedlings, the content of lipid peroxides was higher than the content in controls (Fig. 5a). TBARS level, indicative of lipid peroxides, increased by 52.4% in shoots and 69.5% in roots in the seedlings due to 50 μM As treatment for 8 days. Decrease in the production of TBARS was observed by 31.5% in roots and 20.7% in shoots in seedlings treated with As + *A. indica* extract compared to As treated seedlings. Similarly, with *O. sanctum* treatment, lesser level of lipid peroxides was noted in roots and shoots than the level in As-treated seedlings (Fig. 5a). This is suggestive of protective roles of the extracts of *A. indica* or *O. sanctum* to the seedlings by lowering lipid peroxidation under As toxicity. With Schiff's reagent, lipid peroxidation was also examined histochemically. Peroxidation of lipids causes aldehyde production which reacts with Schiff's reagent and forms bright red products. Lipid peroxides formation increased marked by intense bright red colored spots in As treated rice seedlings in comparison with controls (Fig. 5b). Whereas, in seedlings grown with As + *A. indica* or As + *O. sanctum* extract in the medium, lesser lipid peroxidation was noted than As-grown seedlings, suggesting that the plant extracts lower lipid peroxidation in As-exposed seedlings.

Arsenic induced membrane damage in root tips

Evan's blue dye uptake method was used to assess membrane damage in roots under various treatments. A higher uptake of the dye was observed in the roots of As-treated seedlings, in comparison to controls (Fig. 6). Whereas, with the presence of *A. indica* or *O. sanctum* extract in the growth medium, dye uptake by roots declined in comparison to As-treated seedlings (Fig. 6a). In histochemical observations, roots of As treated seedlings showed intense blue stain in comparison with controls, whereas roots of seedlings grown in As + *A. indica* or As + *O. sanctum* containing medium showed less intense blue stain

Fig. 2 a Effect of As, As + Ai and As + Os treatments in the growth medium on the levels of superoxide anion in roots and shoots of rice seedlings at 4 and 8 days of growth.

b Microscopic view of localization of superoxide anion (O_2^-) in situ in rice roots and leaves using NBT staining. Rice seedlings were raised in hydroponics under different treatments and all statistical analyses were done as described in Fig. 1

suggesting the protective role of *A. indica* and *O. sanctum* extracts against As induced plasma membrane damage (Fig. 6b).

Arsenic induced alterations in antioxidants in the seedlings and the role of leaf extracts

The potential antioxidants ascorbate and glutathione were examined for their level in the seedlings under different treatments. Reduced ascorbate (AsA) level as well the level of its oxidized form (DHA) was higher, however AsA/DHA ratio declined in the seedlings with As treatment (Fig. 7a). The level of AsA increased by 87.4% in roots and 20% in shoots due to 50 μM As treatment for 8 days,

whereas the level of AsA declined by 32.7% in roots and 10.55% in shoots when extract of *A. indica* was included in the As medium. Similarly, with *O. sanctum* extract in As containing medium, decline in AsA level was noted in roots and shoots. Root DHA level increased to 112.5% and shoot level to 69.5% with 8 days of As treatment (Fig. 7b). Whereas with the addition of *A. indica* or *O. sanctum* extract in As treatment medium, decline in root and shoot DHA level was seen as against DHA levels As-treated seedlings. A 39.61% decline in AsA/DHA ratio in roots and 39.7% decline in ratio in shoots was evident due to 50 μM As treatment for 8 days (Fig. 7c), whereas with the addition of *A. indica* and *O. sanctum* extracts in As

Fig. 3 a Effect of As, As + Ai and As + Os treatments in the growth medium on the levels of hydrogen peroxide (H_2O_2) in roots and shoots of rice seedlings at 4 and 8 days of growth. Seedlings were raised and statistical analyses were done as in Fig. 1.

b Histochemical detection of hydrogen peroxide in situ in rice roots and leaves by 3,3'-diaminobenzidine (DAB) uptake method. Views of DAB stained leaves as observed under microscope (a, b, c, d) and DAB stained roots (e, f, g, h). Dark brown spots represent localization of H_2O_2 (color figure online)

Fig. 4 Effect of As, As + Ai and As + Os treatments in the growth medium on the levels of hydroxyl radical in roots and shoots of rice seedlings at 4 and 8 days of growth. Rice seedlings were raised in hydroponics under control, control + *A. indica* extract

(Control + Ai), Control + *O. sanctum* extract (Control + Os), 50 μ M As (As) and 50 μ M As + *A. indica* extract (As + Ai) and 50 μ M As + *O. sanctum* extract (As + Os). Analyses of the data were similar as described in Fig. 1

Fig. 5 a Effect of As, As + Ai and As + Os treatments in the growth medium on the level of thiobarbituric acid reactive substances (TBARS, a measure of lipid peroxidation) in roots and shoots of rice seedlings at 4 and 8 days of growth.

b Histochemical detection of lipid peroxides in stained rice leaves. Pink-red stained portions represent extent of lipid peroxidation in the leaves (a, b, c, d) and roots (e, f, g, h) of stained rice seedlings (color figure online)

treatment medium, significant restoration in the ratio AsA/DHA was noted comparable to untreated controls.

With As treatment, elevated level of GSH and a decline in GSSG level was noted in the seedlings. Arsenic treated seedlings for 8 days had 81.3% higher GSH level in roots and 109.1% higher level in shoots as against untreated controls (Fig. 8a). With extract of *A. indica* in As containing medium, 33.1% decline in GSH level in roots and 45.76% decline in shoots, and with *O. sanctum* extract 32.31% decline in GSH in roots and 45.0% decline in shoots was evident as against GSH levels in seedlings grown in presence of As. Oxidized glutathione (GSSG) level declined by 32.02% in roots and 33.8% in shoots due to As treatment, whereas the level was restored by 30.2% in roots and 23.7% in shoots with extract *A. indica* in As containing medium. Similarly, seedlings grown in As + *O. sanctum* containing medium, the level of GSSG was

restored by 23.8% in roots and 30.1% in shoots as compared to As treated seedlings (Fig. 8b). An increased ratio of GSH/GSSG was noted in the seedlings after treatment with As (Fig. 8c).

Antioxidative enzyme activities and role of leaf extracts

On exposure to As, the activities of SOD, CAT, APX and GPX increased in the seedlings. Arsenic treatment for 8d caused 59.1% increased activity of SOD in roots and 50% increased activity in shoots over controls. Whereas, with the addition of *A. indica* or *O. sanctum* extract in As treatment medium, SOD activity declined in roots and shoots in comparison to the activity in As treated seedlings (Fig. 9a). On As treatment, 29.06% higher CAT activity was noticed in roots and 29.7% higher in shoots, whereas

Fig. 6 **a** Loss of plasma membrane integrity in the roots of rice seedlings at various treatments, measured in terms of Evan's blue uptake. Rice seedlings were raised in hydroponics for 8 days under different treatments as mentioned in Fig. 1. **b** Evan's blue stained root portions are seen (color figure online)

the activity declined by 18.2% in roots and 18.4% in shoots with incorporation of *A. indica* extract in As containing medium. Similarly seedlings growing in 50 μ M As + *O. sanctum* medium for 8 days showed 10.4% and 16.7% reduced CAT activities in roots and shoots respectively as against the activities in As-treated seedlings (Fig. 9b). This indicates restoration in activity levels of the enzymes SOD and CAT in the seedlings with the addition of *A. indica* and *O. sanctum* extracts in As treatment medium (Fig. 9). Similar to SOD and CAT, with As treatment, APX activity increased by 87.4% in roots and 44.8% in shoots, whereas with the addition of extracts of *A. indica* and *O. sanctum* in As treatment medium, APX activity was lowered in both roots and shoots as against the activity in As-treated seedlings (Fig. 10a). Similarly, As treatment resulted in 82.08% increased GPX activity in roots and 49.5% increased activity in shoots over controls but it declined in both roots and shoots with the addition of either of the extracts in As treatment medium (Fig. 10b).

The enzymes MDHAR and DHAR also showed elevated activity levels in the seedlings with As treatment. MDHAR activity increased by 61.8% in roots and 42.7% in shoots

with As treatment, but with the addition of *A. indica* extract in the medium, 31.9% decline in root enzyme activity and 21.5% decline in shoot enzyme activity was observed (Fig. 11a). Similarly, compared to As-treated seedlings, with *O. sanctum* treatment, decline in root and shoot MDHAR activities were observed. Like MDHAR, higher root and shoot DHAR activities were noted in As treated seedlings compared to controls (Fig. 11b), whereas with the addition of *A. indica* or *O. sanctum* extracts in As containing medium, root and shoot enzyme activities declined as compared to the activities in As treated seedlings (Fig. 11b).

Levels of thiols, protein carbonylation and effects of extracts

A marked reduction in the level of protein thiol and increase of non-protein thiol was evident in rice seedlings with As treatment (Fig. 12a). In As treated 8 days grown seedlings, protein thiol declined by 47.7% and 29.8% in roots and shoots respectively as against untreated controls, whereas this decline became less with *A. indica* or *O.*

Fig. 7 Effect of As, As + Ai and As + Os treatments in the growth medium on the levels of **a** reduced ascorbate (AsA), **b** dehydroascorbate (DHA), and **c** ratio of AsA/DHA in roots and shoots of rice seedlings at 4 and 8 days of growth. Rice seedlings were raised in

hydroponics as described in Fig. 1. Values are mean $\pm$ SD based on three independent determinations and bars indicate standard deviations

sanctum extract in the medium. This indicates a protective role of *A. indica* and *O. sanctum* extracts for protein thiols under As toxicity in rice seedlings (Fig. 12a). In As-treated

seedlings, level of non-protein thiol increased by 105% and 37% in roots and shoots respectively as against controls, whereas with *A. indica* or *O. sanctum* extracts in the

Fig. 8 Effect of As, As + Ai and As + Os treatments in the growth medium on the contents of **a** reduced glutathione (GSH), **b** oxidized glutathione (GSSG), and **c** ratio of GSH/GSSG in roots and shoots of

rice seedlings at 4 and 8 days of growth. Treatment conditions of the seedlings and statistical analyses of the data were similar as described in Fig. 1

medium, the increased level of non-protein thiols remained only up to 10 to 23% in roots and 7–9% in shoots compared to controls (Fig. 12b). Arsenic treatment caused increase in protein carbonylation in the seedlings (Fig. 12c). With 50 μ M As treatment in the medium, the level of protein

carbonyls was higher by 53% and 27% in roots and shoots respectively in comparison to the level in untreated seedlings. Whereas, when rice seedlings were grown in presence of As + *A. indica* extract in the medium, the As induced elevated level of protein carbonyls was lower by

Fig. 9 Effect of As, As + Ai and As + Os treatments in the growth medium on the activities of **a** superoxide dismutase (SOD) and **b** catalase (CAT) in roots and shoots of rice seedlings at 4 and 8 days

33% and 18% respectively in roots and shoots. Similarly, with *O. sanctum* extract, As induced elevated level of protein carbonyls declined by 30% in roots and 18% in shoots. This indicates protective roles of *A. indica* as well as *O. sanctum* extracts against As induced protein carbonylation in the seedlings (Fig. 12c).

Discussion

Our results show that As treatment to rice seedlings causes marked decline in growth with increase in production of ROS, increased As uptake by roots, increased oxidative stress in the tissues marked by enhanced peroxidation of lipids, carbonylation of proteins, increased membrane permeability, decreased protein thiols, altered AsA/DHA and GSH/GSSG ratios and increased activities of the enzymes SOD, CAT, APX, GPX, MDHAR and DHAR. Arsenic toxicity in crop plants including rice has been well established (Mishra et al. 2011; Hettick et al. 2015). Arsenic reduces growth of plants by interfering with key metabolic processes responsible for proper plant growth.

of growth. Treatment conditions of the seedlings and statistical analyses of the data were similar as described in Fig. 1

Arsenic toxicity in plants is associated with overproduction of ROS, which in turn causes oxidative stress in the tissues (Sharma et al. 2012). When aqueous extracts of either *A. indica* or *O. sanctum* were added in As containing growth medium, in our studies, a marked reduction in As uptake, comparatively lesser production of ROS and substantial restoration in growth of seedlings was observed compared to As level, ROS production and growth parameters in As-treated seedlings. With *A. indica* extract, As uptake was more reduced compared to *O. sanctum* extract, suggesting that among these two herbal extracts *A. indica* better alleviates As toxicity in rice plants by reducing bioavailability of As. *A. indica* extract contains a large number of glycosides, tannins, phenolic compounds, saponins, proteins, amino acids, triterpenoids and steroids (Prasanth and Krishnaih 2014), therefore it is possible that the phenolic compounds might form complexes with As leading to its decreased uptake by the roots. This could be one of the reasons for the reduced uptake of As by the roots thereby leading to its reduced bioavailability in the tissues due to the presence of *A. indica* aqueous extract in the medium. The phenolics present in extract might be involved in

Fig. 10 Effect of As, As + Ai and As + Os treatments in the growth medium on the activities of **a** ascorbate peroxidase (APX) and **b** guaiacol peroxidase (GPX) in roots and shoots of rice seedlings at 4 and 8 days of growth. Rice seedlings were raised in hydroponics under different treatments and analyses of the data were done as described in Fig. 1

scavenging free radicals generated under As toxicity and could provide antioxidative defense against As induced oxidative stress. Similarly, a wide range of natural products like eugenol, eugenol, carvacrol, limatrol, saponins, flavanoids, triterpenoids, tannins, rosameric acid, propanoic acid, apigenin, and some water soluble flavanoids such as orientin and vicenin are present in leaves of *O. sanctum* (Hakkim et al. 2007). Due to the presence of these compounds, *O. sanctum* aqueous extract has strong chelating as well as free radical scavenging potential (Hakkim et al. 2007). These properties of *O. sanctum* appear to be responsible for reducing As uptake and providing antioxidative defense in the seedlings thereby lowering As bioavailability and its toxicity in the seedlings. A wide range of natural compounds present in *A. indica* and *O. sanctum* extract may form complexes with As leading to its decreased absorption by roots. The antioxidative properties of the phenolic compounds and flavanoids present in *A. indica* and *O. sanctum* extract could be responsible for its

direct protective and antioxidative action within the tissues. Phenolic compounds are associated with the antioxidant capacity of plants and can directly inactivate free radicals and prevent decomposition of hydroperoxides into free radicals (Maisuthisakul et al. 2007).

In our studies, ROS production increased in the seedlings with As treatment, whereas when extracts of *A. indica* and *O. sanctum* were present in As containing medium, reduced formation of ROS was evident than the ROS level in As alone treated seedlings. Increased ROS production has been reported in a wide range of crops grown under excess levels of different metals (Verma and Dubey 2003; Caverzan et al. 2016; Pandey and Dubey 2019; Pandey et al. 2019a). Arsenic is a potent toxic metalloid and is responsible for inducing increased generation of ROS in plants (Mishra et al. 2011; Pandey et al. 2019b). The two commonly available toxic species of arsenic present in soil are arsenate (V) and arsenite (III), which are easily taken up by growing plants and thereafter cause increased ROS

Fig. 11 Effect of As, As + Ai and As + Os treatments in the growth medium on the activities of **a** monodehydroascorbate reductase (MDHAR) and **b** dehydroascorbate reductase (DHAR) in roots and shoots of rice seedlings at 4 and 8 days of growth. Treatment conditions for the seedlings and analyses of the data were similar as described in Fig. 1

production in the tissues (Sharma et al. 2014; Pandey et al. 2019b). Electron transport activities of mitochondria, chloroplasts and plasma membranes appear to be responsible for leakage of electrons and increased formation of O_2^- in plant cells under arsenic toxicity (Sharma et al. 2014). Moreover arsenic induces anatomical and morphological changes in mitochondria and a rapid decline of mitochondrial membrane potential causes uncontrolled formation of O_2^- (Jomova et al. 2010). The electron transport chain activities of chloroplast, mitochondria, NADPH oxidase of plasma membrane, cell wall associated peroxidases are mainly involved in enhanced H_2O_2 production under stressful conditions in plants (Sharma et al. 2012). Rice and sunflower plants exposed to toxic levels of As show increased production of H_2O_2 (Mishra et al. 2011; Issam et al. 2015). In our studies, all the three ROS (O_2^- , H_2O_2 and $OH^\cdot$) examined, showed increased levels in rice plants on As exposure and the level declined in the presence of *A. indica* or *O. sanctum* extracts in the treatment medium. This suggests that both *A. indica* and *O. sanctum* extracts alleviate As toxicity effects in rice seedlings by reducing As bioavailability and lowering ROS production. Peroxidation of membrane lipids is regarded as a biochemical marker of oxidative stress in plants. Increased lipid peroxidation in membranes causes alteration in

membrane permeability. In our study, TBARS level, the byproducts of lipid peroxidation increased in As-treated seedlings, which indicate that lipid peroxidation increases in the seedlings on As treatment which further causes alteration in permeability of plasma membrane. A marked decline in TBARS level in As-treated seedlings was observed in our studies when *A. indica* or *O. sanctum* extracts were added in the medium. This suggests that *A. indica* and *O. sanctum* extracts reduce oxidative stress in the seedlings caused due to As and provide protection to the cellular biomolecules against oxidative stress. Alteration in membrane permeability in As exposed rice seedlings was also histochemically confirmed in our studies using Evan's blue uptake assay. In presence of *A. indica* and *O. sanctum* extracts in As containing medium, a reduction in the internalization of Evan's blue dye occurred inside the root cells compared to the dye internalized in As stressed seedlings, suggesting that *A. indica* and *O. sanctum* extracts protect membrane integrity under As toxicity conditions.

It has been observed that under stressful conditions such as metal toxicity, plants restrict lipid peroxidation and membrane damage by increasing the level of non-enzymic antioxidants like reduced ascorbate and glutathione (Sharma et al. 2014). Reduced ascorbate helps in reduction

Fig. 12 Effect of As, As + Ai and As + Os treatments in the growth medium on **a** protein thiol, **b** non-protein thiol and **c** carbonyl content in roots and shoots of rice seedlings at 4 and 8 days of growth. Rice seedlings were raised in hydroponics under different treatments and analyses of the data were done as described in Fig. 1

of lipid peroxide radicals by formation of α -tocopherol (Fryer 1992) and in this way plays important role in protection of cellular components against oxidative damage caused due to ROS (Gill and Tuteja 2010). In our studies, with As treatment, decline in AsA/DHA ratio was noticed in the seedlings, while the ratio increased when *A. indica* or *O. sanctum* were present in As treatment medium. This suggests that the extracts of *A. indica* and *O. sanctum* appear to have roles in maintaining needed reduced ascorbate level in As treated seedlings. Arabidopsis plants expressing high levels of ascorbate show increased oxidative stress tolerance (Wang et al. 2010). GSH helps in maintaining thiol groups of proteins in reduced state and it serves as an important redox buffer in the cells (Dalton 1995). Due to its importance in plants, high concentration

of GSH is maintained in the cells to serve as antioxidant to combat oxidative stress under stressful conditions (Sharma et al. 2012). Arsenic treatment to the seedlings, in our studies, caused elevated GSH level and decline in the level of GSSG. At 8th day of As treatment of seedlings, the GSH/GSSG ratio increased while the ratio declined when seedlings were grown in As + *A. indica* or As + *O. sanctum* containing medium. The increased ratios suggest increased production of GSH to modulate antioxidant potential to better combat As induced oxidative damage in the seedlings.

All the antioxidative enzymes examined in our studies, such as SOD, APX, CAT, GPX, MDHAR and DHAR showed their increased activities in the seedlings with As treatment, whereas with the presence of *A. indica* or *O.*

sanctum extracts in As treatment medium, a significant reduction in all the enzyme activities was noticed. In order to withstand the oxidative damage that occurs under stressful conditions, increase in activities of antioxidative enzymes has been observed in plant tissues (Verma and Dubey 2003; Pandey and Dubey 2019). The enzymes CAT, APX and GPX help in detoxification of H_2O_2 produced in plant tissues on exposure to stress. Arsenic toxicity caused increase in CAT activity in *Pteris vitata* (Tartar et al. 2009) and *Pteris ensiformis* (He et al. 2017), increased APX activity in black gram (Srivastava and Sharma 2013). Due to the presence of *A. indica* and *O. sanctum* in As containing growth medium, the reduction in activities of antioxidative enzymes in comparison to the activity in As treated rice seedlings, suggests that *A. indica* and *O. sanctum* extracts contribute in maintaining the requisite antioxidative activity status and in alleviating As toxicity in rice seedlings. Ascorbic acid plays crucial role in the cells by maintaining the redox balance due to formation of its reduced form through enzymes MDHAR and DHAR (Arrigoni 1994). Our study reveals increase in the activity of both the enzymes MDHAR as well as DHAR in rice seedlings on As treatment and a lowering in activity due to the presence of *A. indica* and *O. sanctum* in As treatment medium. Increased MDHAR activity has been reported in As (Mishra et al. 2011), Mn (Fecht-Christoffers et al. 2003), Ni (Baccouch et al. 2001) and Cu (Cuyppers et al. 2000) treated plants whereas increased activity of DHAR has been observed As treated plants (Mishra et al. 2011; Rahman et al. 2015).

Attempts have been made earlier to look for inorganic chemicals, hormones, herbal extracts which can be used to alleviate metal toxicity in plants and encouraging results have been obtained (Srivastava and Dubey 2012; Pandey et al. 2013; Rajpoot et al. 2016; Pandey et al. 2019a). These alleviators when added in the growth medium of plants, reduce uptake of metals and modulate antioxidative status of the plants so that plants can survive better under metal toxicity conditions. In excised rice leaves, it has been shown that Mn toxicity can be alleviated with application of nitric oxide by reducing the level of oxidative stress (Srivastava and Dubey 2012). Similarly, exogenously added Mg, Ca or salicylic acid in the hydroponics growth medium of rice alleviates Al toxicity by reducing uptake of Al and elevating antioxidative defense potential in plants (Pandey et al. 2013). Among herbs, *Terminalia arjuna* bark aqueous extract alleviates Ni toxicity, whereas aqueous extract from dried fruits of *Phyllanthus emblica* alleviates Cr toxicity in growing rice seedlings (Rajpoot et al. 2016; Pandey et al. 2019a). Herbal extracts due to the presence of phenolics, alkaloids, terpenoids and many antioxidant compounds serve as metal chelators and free radical scavengers and reduce

metal uptake by the plants (Hakkim et al. 2007; Rajpoot et al. 2016; Pandey et al. 2019a). In areas where water is contaminated with As species, toxicity due to As is a major constraint in rice cultivation and studies have shown that leaf aqueous extracts of *A. indica* and *O. sanctum* contain compounds with high antioxidant capacity and radical scavenging activity, this prompted us to look into the alleviating activity of these two extracts towards As toxicity in growing rice seedlings. Addition of both of these extracts retarded uptake of As by roots of rice plants and modulated the levels of AsA, GSH as well as activities of antioxidative enzymes, in order to provide better antioxidant potential to As-stressed rice seedlings. This shows potential alleviating role of these two herbal extracts in mitigating As toxicity in rice plants.

To conclude, it can be inferred that in growing rice plants As toxicity is associated with increase in uptake of As by the roots, increased formation of ROS ($O_2^{\cdot-}$, $OH^{\cdot}$ and H_2O_2), increased membrane permeability, increased oxidative stress in the tissues, altered levels of ascorbate, glutathione and elevated activities of the enzymes SOD, CAT, APX, GPX, MDHAR, DHAR so as to maintain sufficient antioxidant potential in rice seedlings in order to combat arsenic induced oxidative stress. Further, the aqueous extracts of *A. indica* and *O. sanctum*, when present in the medium appear to have potential in alleviating As toxicity in rice seedlings by reducing As bioavailability in rice roots and shoots and lowering oxidative stress in the tissues. Thus, both of these extracts can be used for As toxicity alleviation in rice. However, the details of molecules with antioxidant properties present in the extracts and the mechanism how they protect plants against the harmful effects of As need to be worked out in our future experiments with pure compounds.

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Compliance with ethical standards

Conflict of interest Authors declare that there is no conflict of interest.

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