A-5734
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RESEARCH ARTICLE Indian Journal of Agricultural Research, Volume Issue : ()

Antioxidant Responses of Arsenite-induced Oxidative Stress in Rice (*Oryza sativa* L.) and its Modulation by Eugenol (Extracted from *Ocimum sanctum*)

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10.18805/IJARe.A-5734

ABSTRACT

Background: Irrigation with arsenic-contaminated groundwater is leading to high arsenic-laden rice seeds and lower yields. In the present study, the effect of exogenous treatment of eugenol (extracted from *Ocimum sanctum* L leaf) on hydroponically grown rice seedlings was examined by investigating the antioxidant system under arsenic stress.

Methods: In the experiment 7 day old rice seedlings (IR-64) were exposed to 10,50,100 µM of arsenite separately and co-treatment with 10,50,100 µM eugenol in a hydroponic medium for 7 days. The activity of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, glutathione peroxidase, catalase and lipid peroxidation (malondialdehyde) in root and shoot tissues were determined separately by standard protocol.

Result: Under arsenic treatment oxidative stress was induced by overproduction of reactive oxygen species (ROS) and disruption of antioxidant defense system measured in terms of increased activity of antioxidant enzymes and lipid peroxidation (malondialdehyde) in root and shoot tissues separately. Eugenol-treated seedlings along with arsenic exposure substantially decreased the level of arsenic uptake in plants resulting in a substantial reduction in ROS overproduction and MDA content. SOD, CAT, GPX activities perform an influential role in arsenic stress acclimatization and eugenol treated seedlings with arsenic exposures indicated substantial changes in all variables evaluated as compared to arsenic treatment only. This study suggests that oxidative stress caused by arsenic was ameliorated by eugenol.

Key words: Arsenic, Antioxidant scavenging enzymes, Eugenol, ROS, Rice.

INTRODUCTION

Arsenic, one of the most dangerous toxicants to the global environment is causing serious health problems in South East Asia especially in West Bengal and Bangladesh with elevated concentrations of up to 3200µg/L in drinking water. (Carty *et al*., 2011) Arsenic-contaminated water irrigation of soils significantly increases the concentrations of arsenic in the soil, which adversely affects various physiological and biochemical abnormalities (Li *et al*., 2006) in plants and humans. In humans, arsenic toxicity is associated with several chronic diseases that involve skin, bladder, lung, liver and kidney cancer (Mandal *et al*., 2002; Waalkes *et al*., 2004; Mehmood *et al*., 2017). While in plants, arsenic uptake adversely affects the metabolic processes and causes several physiological and cellular disorders and hinders the growth and development in various manners (Singh *et al*., 2015). Arsenic induces oxidative stress due to excessive production of reactive oxygen species causing damage to the cell membranes, lipids, proteins and eventually cell death (Srivastava *et al*., 2007) by activating oxidative signaling pathways. Against such oxidative stress, plants develop an integrative defense system including both enzymatic and non-enzymatic antioxidants. These reactive oxygen species can be controlled by increasing the production of various enzymes such as APX, GPX, SOD, CAT, glutathione reductase and externally supplied antioxidants, especially polyphenolic compounds. Eugenol is a phenylpropanoid phenol that can serve as an antioxidant or pro-oxidant agent. It also has cytotoxic, anti-carcinogenic and anti-mutagenic effects (Bezerra *et al*., 2017) and is used to suppress the activation of nuclear factor kappa B (NF-kβ) in mouse skin with TPA-induced inflammation (Kaur *et al*. 2010). Rice is also one of the important crops in the arsenic-contaminated

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How to cite this article: Mairaj, S., Nagar, R.D., Bhardwaj, L.K., Rehman, F., Punnakal, A., Chauhan, A. and Jindal, T. (2021). Antioxidant Responses of Arsenite-induced Oxidative Stress in Rice (*Oryza sativa* L.) and its Modulation by Eugenol (Extracted from *Ocimum sanctum*). Indian Journal of Agricultural Research. DOI: 10.18805/IJARe.A-5734.

Submitted: 04-01-2021 **Accepted:** 21-06-2021 **Online:** 19-10-2021

region and rice arsenic toxicity has newly been subjected to immense adversity (Mehrag *et al*., 2004). No studies were reported at current on the role of exogenous eugenol treatment in arsenic-induced oxidative damage and antioxidant defense systems in rice seedlings to the best of knowledge.

The present study examined the role of treatment of rice seeds with herbal extract eugenol to modulate the oxidative stress in *Oryza sativa* L, induced by arsenic toxicity.

MATERIALS AND METHODS

Chemical and reagent

Analytical grade chemicals were used for the research and purchased from Sigma-Aldrich. From the leaf the Ocimum sanctum plant, eugenol was extracted and characterized by element detection, IR, NMR and other parameters.

Extraction, isolation and identification of eugenol from *Ocimum sanctum* (Tulsi)

Eugenol is extracted from the essential oil of tulsi leaves by co-distillation with indirect steam and separated from the distillate by using an organic solvent such as dichloromethane. Finally isolated by evaporation of the organic solvent and dried by anhydrous sodium sulfate desiccator, b.p. 248°C.

Eugenol was characterized by elemental analysis, IR, NMR spectra

Anal ($C_{10}H_{12}O_2$)	
Found	: C 73.28 ; H 7.26; O 19.46
Required	C 73.14 ; H 7.37 ; O 19.49
IR(KBr)	: 3516(OH), 1268,1235(C-OCH_3), 1638(C=C, alkene),1613,1516 (aromatic(C=C) and 820 (Benzene ring substitution)cm^{-1}
NMR ($CDCl_3$) (ppm)	: 4.36(s, 3H,-CH_3), 3.73 (s, 3H, -OCH_3), 6.82(d,2H, ArH), 6.72(s,1H, ArH), 6.38(bh, 1H, -OH), 5.18(m,2H, CH_2 = CH), 6.02(M,1 H, CH_2=CH-CH_2)

Hydroponic medium and treatments

Oryza Sativa L. seeds of specific genotype (IR 64) arranged from IARI, New Delhi were screened in hydroponic condition (Dave *et al*. 2013) for antioxidant enzymes and oxidative stress markers assay in shoot and root respectively during arsenite and eugenol exposure. For the experimental procedure, seeds were sterilized with 0.1 percent solution of $HgCl_2$ for about 0.5 minutes, after seeds were washed 3-4 times by distilled H_2O and soaked in double-distilled water for one day. These seeds were then transferred to Petri-dishes and kept in the culture room at 27°C in dark for proper germination for up to seven days. After that these seedlings were exposed to arsenite, eugenol separately and co-treatment (10, 50 and 100 µM) for 7 days under similar conditions. All treatments were in triplicate. After seven-day treatment, plants were harvested, rinsed by double-distilled water and used for different parameters.

Antioxidant enzymes and oxidative stress markers assay

Treated and untreated rice seedling samples were homogenized in a chilled mortal with 3ml 0.1M Na_3PO_4 buffer at seven pH having 1.0% polyvinyl polypyrrolidone, 1.0mM disodium-EDTA and 0.5M sodium chloride. The amalgamate were centrifuged at 10,000 rpm about15 minute at 4°C and the supernatant was utilized to determine APX, SOD, CAT, GPX activities. The ascorbate peroxidase was estimated according to (Nakano and Asada 1987) by assessing the ascorbate oxidation rate. The superoxide dismutase exploit was measured as per (Beauchamp - Fridovich 1971) via assessing the inhibition of the reduction of NBT-dye by superoxide dismutase anion. The GPX-activity was estimated as per (Hammer Schmidt *et al*., 1982). The catalase action was assessed according to (Aebi *et al*., 1983). The malondialdehyde content was assessed as per the modified process of (Hodges *et al*., 1999).

Statistical analysis

All investigations in triplicate were statically analyzed by analysis of variance (ANOVA) to confirm the variability of data and validity of results and Duncan's multiple range test (DMRT) was performed to determine the significant differences between treatments at 0.05.

RESULTS AND DISCUSSION

The 7- day old eugenol treated seedlings were analyzed for different parameters: antioxidant activity (APX, CAT, SOD, GPX), lipid peroxidation [malondialdehyde (MDA) content], exposed to applied concentrations (100µM, 50µM and 10µM) of arsenite, eugenol and their joint exposure were shown in Fig 1,2.

Malondialdehyde(MDA)

The MDA increased by 100%, 76%, 26% in root while by 90%, 70%, 22% in the shoot at 7 d treatment by 100µM, 50µM,10µM AsIII respectively as compared to control (R=0.964221, P=0.05). Upon eugenol treatment under the same days and same concentration as AsIII treatment, the MDA decreased only by 16%,8%,4.25% in root while in shoot 19%, 8.3%, 3.5% as compared to control. Upon joint application of eugenol with AsIII, the increased levels of MDA in root was reduced by about 46%,36%16% while in shoot 40%, 35% and 14% as compared to AsIII treated rice seedling under the same concentration only and was positively correlated with As accumulation (R=0.94617, P=0.05) as shown in Fig 1.

Effect of antioxidant

In comparison with control, the APX activity increased by 78%, 37% and 30 % in the root, while by 77%, 36%, 28% in the shoot at 7 d treatment with 100µM, 50µM, 10µM As III (R=0.937097, P=0.05). The activity of APX decreased by 26%, 21%, 19%, in the root, while in shoot 38%, 19%,15% on eugenol treatment under the same day and same

concentration as AsIII treatment as compared to control. Upon joint exposure of eugenol with AsIII, the increased root APX level was reduced by approximately 61%, 30%, 24%, while the shoot was by approximately 64%, 28%, 13% compared to AsIII treated rice seedling under the same concentration only (R=0.859663, P=0.05) (Fig 2A).

The CAT activity increased by 34%, 28%,14% in root while by 29%,23%,12% in shoot at 7 d treatment with 100µM, 50µM, 10µM AsIII respectively (R=0.93181,P=0.05), while its level decreased by 20%,11%,7.2% in root and in shoot by 13%,8%,5.3% as compared to control under the same conditions as arsenite treatment. Eugenol when jointly treated with AsIII, the increased level of CAT in root was reduced by about 19%,13%,6% while in shoot 18%,11%,6% as compared to only AsIII treated rice seedling under the same concentration only (R=0.94617, P=0.05) (Fig 2B). GPX activity increased by 41%, 29%, 21% in the root, whereas by 34%, 26%, 15% in the shoot with 100ìM, 50µM, 10µM AsIII at 7 d treatment (R=0.918705, P=0.05) while its value decreased by 17%, 5%, 1.8% in root whereas in shoot 12%, 4.6%,1.5% with 100µM, 50µM, 10µM eugenol treatment as compared to control. The root GPX activity decreased on average by about 29%, 15%, 12% while in shoots 15%, 12%, 7.6%, with the joint application of eugenol with AsIII, compared with only AsIII treated rice seedling with the same concentration only (R= 0.935489, P= 0.05) (Fig 2C). The SOD activity in the root improved by 34%, 28%, 14%, while shoot 29%, 23%, 12% with 100ìM, 50µM,10µM As III at 7d treatment compared to control (R=0.953166, P=0.05). The SOD declined by 27%, 24%, 15 % in root while in shoot 21%, 17%, 8% during eugenol treatment under the same condition as AsIII treatment, compared to control. The increased level of SOD in the root was decreased by approximately 46-53% whereas in shoots 31-35% upon joint application of eugenol with AsIII treated rice seedling under the same concentration as As III treated only and was positively associated with As accumulation (R=0.99218, P=0.05) (Fig 2D).

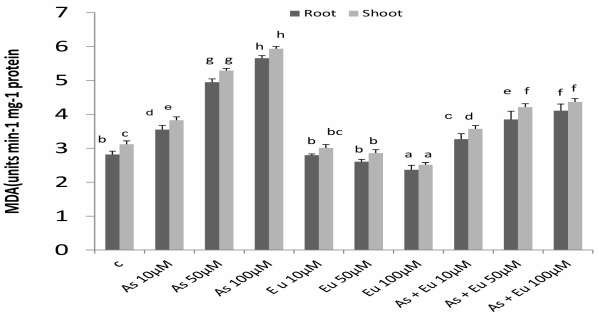

Fig 1: Changes in the level of malondialdehyde (MDA) in roots and shoots of the rice seedlings after the seventh day of treatments with (100µM,50µM,10µM eugenol, 100µM,50µM,10µM As and 100µM, 50µM,10µM As + 100µM,50µM,10µM Eugenol); All results are average of three (n = 3)±SD replicates. Significant ANOVA at P ≤0.05.

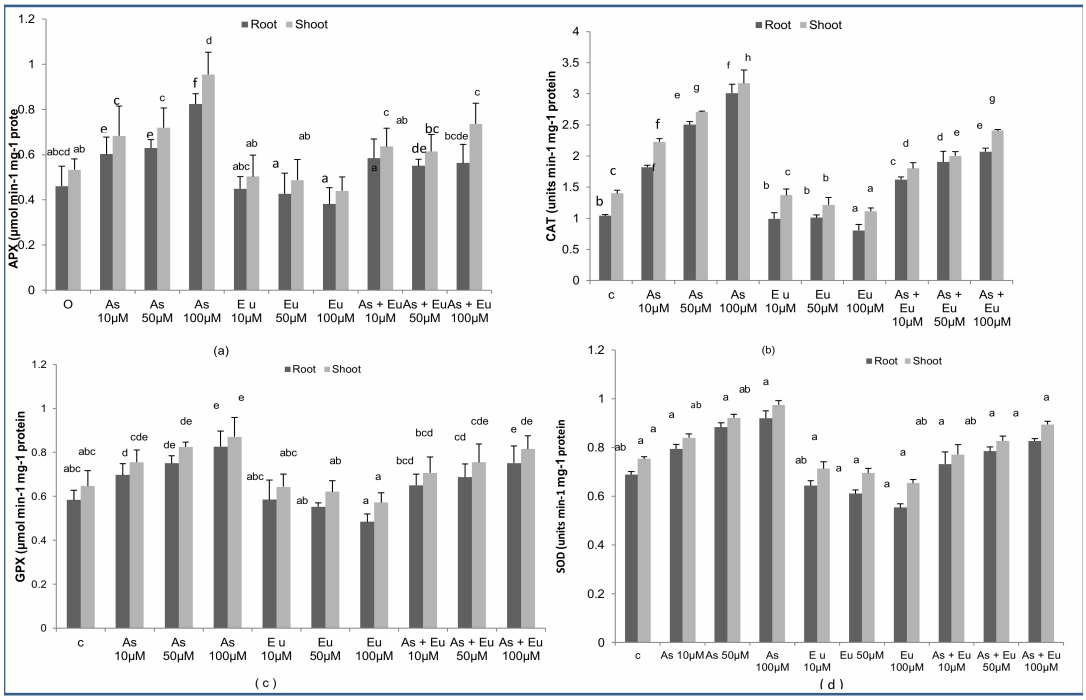

Fig 2: Changes in the activities of (A) ascorbate peroxidase (APX) (B) catalase (C) guaiacol peroxidase (GPX) (D) superoxide dismutase (SOD) in rice seedlings roots and shoots after the seventh day of treatment with (100µM, 50µM, 10µM eugenol, 100µM,50µM,10 µM As and 100µM,50µM,10µM As + 100µM,50µM,10µM eugenol; All results are average of three (n = 3)±SD replicates. Significant ANOVA at P ≤ 0.05.

Treatment of rice (*O.sativa*) seedling with eugenol, when investigated with arsenite treatment in hydroponic, showed a significant result for MDA, antioxidant enzymes as compared to only found by arsenic-treated rice seedling alone.

Substantial augmentation of APX activity on arsenic exposure can rely on greater availability of H_2O_2 due to the effective breakdown of the ASC-GSH cycle, whereas substantial reduction of the APX value when treated with eugenol may be attributable to the neutralizing adverse effects of arsenic due to complex formation and a reduction of $O_2^{\cdot-}$ to molecular oxygen (Gautam *et al*., 2019) resulting in decreased H_2O_2 levels and supported by (Dave *et al*., 2013; Mairaj *et al*., 2020; Souri *et al*., 2017). The activity of the CAT, GPX enzyme in the root and shoot was reduced on average when exposed to eugenol in arsenite treated rice seedlings, suggesting that eugenol causes a significant reduction in oxidative stress caused by toxic arsenic and well supported by (Gautam *et al*., 2019). Findings also suggest that during arsenic stress, enhanced SOD activity may be correlated with H_2O_2 production. This arsenic stress has been ameliorated by lowering the levels of H_2O_2 when treated with eugenol. In this way, eugenol plays a protective role against oxidative stress caused by arsenic. High dose exposure of eugenol in a hydroponic medium decreases the activity of the enzymes in rice plants exposed to arsenic and helpful in reducing arsenic-mediated rice plant toxicity (Gautam *et al*., 2019). In the present study, it has been noted that the MDA level increased with arsenic exposure in rice seedlings, which may be attributed to the excessive production of reactive oxygen species such as $O_2^{\cdot-}$, $^{\cdot}OH$, H_2O_2 under stressed condition by the interaction of arsenic with intracellular components (Dave *et al*., 2013; Chandrakar *et al*., 2017 b) . The similar results regarding the up-regulation of MDA activity were also noted in arsenic exposed *Zea mays* L, *Withaniasomnifera, Oryzatenuiflorum L, Glycine max L and* supported by (Hartley- Whitaker *et al*., 2001; Srivastava *et al*., 2005; Anjum *et al*., 2016).

CONCLUSION

Taking into account the findings, arsenic exposure in rice plants is presumed to adversely affect the antioxidant defense system by excessive production of reactive oxygen species causing oxidative stress in terms of enhanced H_2O_2 and may increase the activity of antioxidative enzymes and oxidative stress marker which was correlated with arsenic accumulation. Arsenite supplementation with eugenol induces a substantial reduction in the activity of APX, CAT, GPX, SOD and MDA levels in the root and shoot of the rice plant. This study suggests that arsenic-induced oxidative stress in rice seedlings has been substantially improved by treatment with eugenol.

ACKNOWLEDGEMENT

Amity University Uttar Pradesh is highly acknowledged for providing the infrastructure and facilities for the work done.

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