Alleviation of arsenic stress from rice plant (*Oryza sativa* L.) and subsequent reduction in rice grain arsenic using different amendments

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Dedicated to
my Parents

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ABSTRACT

Rice grain arsenic (As) contamination is a worldwide problem nowadays, as it is the staple crop for 50% of the world population. Irrigation of agricultural fields with As-contaminated groundwater has led to build-up elevated level of arsenic in soil, with consequent elevation of arsenic in crops and vegetables grown up on this soil. Moreover, transportation of As through rice grain from As exposed to unexposed areas and consequent dietary intakes leads to great threats for the population residing in non-endemic areas. The arsenic species present in rice grain are mainly inorganic in nature, which are toxic and carcinogenic. This study includes the alleviation of arsenic toxicity from rice plants. Alleviation of As stress from rice seedlings were segregated into two parts i.e. i) alleviation of As stress from rice plant during the course of germination and seedling stage, and ii) alleviation of As stress and reduction of rice grain As. The rice or any plant is vulnerable to any kind of biotic/abiotic stress, which may cause yield loss, and growth retardation in plants. Thus, proper remediation strategies are badly required to address this burning issue nowadays. Again, rice grain is a primary source of nutrition for half of the world, so rice grain As contamination is in the priority list of researchers, which needs an immediate solution. For this remedial study, different amendments like potassium humate (K-humate, KH), iron oxide nanoparticles (FeO-NPs), selenium nanoparticles (SeNPs), inorganic sulfate compounds (S), biochars (iron modified mango leaf biochar, FeMBC) etc. were selected to reduce the As load in rice grain both in pot-scale and field level study. For improvement of rice seed germination under As stress, K-humate, iron oxide nanoparticles, and selenium nanoparticles were utilized. Whereas, for improvement of rice plant growth and reduction of rice grain As concentration, inorganic sulfate compounds, biochars etc. were used.

Rice seed germination with K-humate has been carried out in part, in first experiment we have used 100 mg/L of K-humate for seed priming. The germination percentage in K-humate primed seeds were 75 ± 5.0% and 68.3 ± 2.9% under AsV and AsIII stress, respectively. The vigour index I (VG I) and vigour index II (VG II) recorded on 12 DAS (days after seeding) were also increased by 1.47 and 1.51 fold, respectively with K-humate supplementation under As stress. Application of K-humate not only improved seed germination, seedling growth and nutrient uptake; however,

also decreased the oxidative stress markers and antioxidant activities by minimizing As uptake and translocation in the seedlings. In second experiment, we have used varying dosage of K-humate (25, 50, 75, 100 mg/L) to alleviate As toxicity (800 ppb AsIII) from rice seedlings. Application of KH significantly improved germination percentage, vigour indices and chlorophyll content by reducing the oxidative stress, antioxidant and antioxidant enzyme activities under As stress. In vivo detection of reactive oxygen species (ROS) using DCF-2DA fluorescent dye and scanning electron microscope (SEM) study of root further depicted that KH application effectively reduced ROS formation and improved root anatomical structure under As stress, respectively.

Experiment with green synthesized FeO-NPs (100 mg/L), seed germination under 50 μm AsIII and 50 μm AsV stress was significantly improved by 9.8% and 15.4%, respectively, as compared to control. The phytotoxic effect of AsIII on seed germination, seedling growth, and chlorophyll content was more severe than AsV. The uptake and translocation of As by seedlings were decreased with FeO-NPs fertigation under As stress.

In another experiment, biosynthesized SeNPs were used to assess the efficacy of SeNP in reducing As (25 μM and 50 μM AsIII) stress in rice seedlings. SeNP application improved the plant growth by increasing the chlorophyll content and reduced oxidative damage by increasing antioxidant activity. SeNP application did not alter the root uptake of As; however, it reduced the translocation of As by 34.3-30.2%. SeNP addition significantly upregulate OsPCS1, OsPCS2b and OsABCC1 gene expression in root which stimulates PC (Phytochelatin) content and subsequent vacuolar sequestration of As in roots.

Pot experiment with different levels of sulfate dosage (0, 20, 40, 60 and 80 mg/kg) was set up in this study to explore the influence of sulfate fertilizer on rice plant growth, yield, and As accumulation in rice grain. The sulfate application significantly ($p \leq 0.05$) enhanced the chlorophyll, tiller number, grains per panicle, grain and biomass yield under As stressed condition. The sulfate application also reduced the oxidative stress and antioxidant activity in rice plants. Sulfate fertigation improved the accumulation of total sulfur (S) and reduced the uptake and translocation of As in rice plants. Arsenic concentration in rice grain was reduced by 50.1% in S80 treatment (80 mg of sulfate/kg of soil) as compared to S0 set.

In our final pot study with mango (*Mangifera indica*) leaf-derived biochars (MBC) and Fe-modofied mango leaf biochar (FeMBC) to reduce As accumulation in rice grain. The results showed that 1% FeMBC enhanced the percentage of filled grains/panicle and biomass yield by

17% and 27%, respectively, compared to the control. The application of 0.5 and 1% FeMBC significantly ($p \leq 0.05$) reduced bioavailable soil As concentration by 33% and 48%, respectively, in comparison to the control. The concentration of As in rice grains was reduced by 6 and 31% in 1% MBC and 1% FeMBC, respectively, compared to the control. The reduction in As concentration in rice grain under 1% FeMBC was more pronounced due to reduced bioavailability of As and enhanced formation of Fe-plaque. The concentrations of micronutrients (such as Fe, Zn, Se, and Mn) in brown rice were also improved after the application of both MBC and FeMBC in comparison to the control.

In field study, all the amendments (KH, FeO-NPs, SeNPs, FeMBC and sulfate) were experimented for two years in As contaminated fields of Madhusudankathi village, located in highly arsenic endemic block Gaighata of North 24 Parganas district, West Bengal. The rice was cultivated in two seasons (monsoon and post-monsoon). It was observed that post-monsoon season showed better yield and higher rice grain As than monsoon season. Among all the treatments, KH showed the better yield. Both SeNPs and KH treatments showed similar rice grain As concentration, which was lower than the other treatments. However, long-term field trial with Selenium NPs are required. Thus, KH can be used as a reagent to improve rice yield and As-safe rice grain.

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Chapter 1

Introduction

1.1. Rice: an agro-economically important crop

Rice (Oryza sativa) is a cereal crop that comes under the Poaceae family. Southeast Africa and tropical and subtropical southern Asia are the native habitat of rice. The two main subspecies of *Oryza sativa*, *japonica* and *indica*, are linked to different wild types more so than to one another, suggesting two distinct domestication regions: *indica* in India and *japonica* in China (Gross and Zhao 2014). The world's socioeconomic stability and food security have traditionally depended on rice. For over half of the world's population, rice is a staple diet, and for many in underdeveloped countries, it makes about a third of their daily caloric intake. Rice production provides livelihoods for over one billion people by serving as a key source of nourishment and wealth (Yadav and Kumar, 2019). Asia produces more than 90% of the rice consumed worldwide. Approximately 60% of the output in this area is rice. The production and quality of rice are major global concerns. For the next 25 years, rice output must rise by 25% in order to keep up with rising nutritional needs (Bitoun, 2018). From 220 million tonnes prior green revolution era to 729 million tonnes in 2017, worldwide rice production improved to feed the expanding population. Global production of milled rice in 2019–2020 was estimated to be 502.9 million tons (FAO, 2021). With 30% of the world's output, China leads the world in rice production. India comes next with 24%, Bangladesh with 7%, Indonesia with 7%, Vietnam with 5%, and Thailand with 4%.

1.2. Rice cultivation in India

In India, the production of rice plays a significant role in the economy. India is the world's biggest exporter of rice and the second-largest producer of the rice grain after China. Between 1980 and 2020–21, rice output rose from 53.6 million tons to 120 million tons. Furthermore, the most area dedicated to rice farming is in this country. In actuality, it is the main crop in the whole country and one of the main food crops. Since rice is mostly cultivated in regions with substantial yearly rainfall, it is essentially a kharif crop in India. It requires at least 25 °C as well as more than 100 cm of rainfall. In regions with limited rainfall, irrigation is also used for growing rice. Various techniques may be used to produce rice depending on the kind of location. After the fields have been plowed, fertilizer (chemical or farm yard manure) is spread, and the surface is leveled. The seeds are manually transplanted, and they are subsequently grown with appropriate irrigation. Silts, loams, and gravels are just a few of the soil types that rice may grow on. Although it may grow in both acidic and alkaline soils, clayey loam soils are ideal for this crop's cultivation. Although it may grow in both acidic and alkaline soils, clayey loam soils are ideal for this crop's

cultivation. Wet rice is rice that is grown in well-watered lowland places. Slopes in mountainous regions are divided into terraces so that rice may be grown there. The western and eastern coastal strips in India, which include all of the primary deltas, the Assam plains and the low hills and foothills surrounding them, the Terai region—which is located along the Himalayas—as well as states like West Bengal, Bihar, Northern Andhra Pradesh, and Odisha—are the main areas where this crop is grown. The deltas of the Ganges-Brahmaputra (in West Bengal), Kaveri, Krishna, Godavari, Indravati, and Mahanadi rivers in India, along with a substantial network of canal irrigation, allow farmers to grow two or even three harvests year. Due to high precipitation and temperature, rice is being cultivated during summer and monsoon seasons in eastern and coastal parts of India. However, all parts in India are suitable for rice cultivation if proper irrigation is available. Winter (Boro) and summer (Ayush) rice are long and short duration crop, respectively. Boro rice is cultivated in lowland areas, which remain flooded during rainy season. Among different seasons, Boro rice has highest productivity per hectare in India.

1.3. Arsenic in rice and its exposure to human and food chain contamination through rice

To feed the growing population of the world rice production has been gone up. However, in different parts of the world, rice production substantial threat from arsenic (As) in soil and water (Brammer and Ravenscroft, 2009). Arsenic causes substantial loss in growth and yield of the rice. Rice grain As poses substantial threat of health risk to human. Arsenic is a metalloid, which is, classified as class I carcinogen. Arsenic generally found in different inorganic (AsIII and AsV) and organic (DMA: dimethyl-arsenic and MMA: monomethyl-arsenic) species. Seas, oceans, freshwaters, and groundwaters all contain both inorganic and organic species of As. Certain regions, most notably South-East Asia have far higher levels of As in their water supplies, which results in extremely high toxicity. Inorganic As (iAs) considered more toxic than their organic counterparts. Organic species of As are non-toxic to human. Among different cereal crops, rice can accumulate more As in them. In rice grains, As is mainly found in the form of iAs and DMA species (Williams et al., 2005), MMA is found rarely at low concentration or lower than detection limit (Batista et al., 2011; Williams et al., 2005). Straight head disease may be associated with elevated levels of organic As, both DMA and MMA, in the soil and rice plants (Yan et al., 2005). The plant becomes sterile due to straighthead disease, which leaves the husk empty when the crop is harvested (Rasamivelona et al., 1995) and in extreme circumstances, rice husk can disort (Rahman et al., 2008). Straight head disease in rice has been linked to a number of variables, including high organic matter content and low soil pH, as well as agronomic techniques such

soaking rice fields continuously (Rahman et al., 2008, Williams, 2005, Wilson et al., 2001). Gaining further insight into the traits of DMA in rice plants as well as rice field might help to clarify the mystery surrounding the nature of straight head disease. To evaluate the danger to human beings, a deeper comprehension of the types of As that are present, as well as their distribution and behavior in rice fields, is essential. Both grain intake by humans and livestock that eats rice byproducts like straw need to take this danger into account. Furthermore, effective agronomic strategies to manage As in rice fields for low As rice grains and crop yield can be made possible by an enhanced understanding of As cycling in rice fields.

1.4. Uptake and translocation of arsenic in rice plant

1.4.1. Arsenic uptake

The amounts and species of As in the rhizosphere have a significant impact on rice's ability to take in As from the soil (Marin et al., 1992). When rice is grown underwater in flooded soil, arsenate (AsV) is converted to arsenite (AsIII) as AsIII has a lower sorption capacity than AsV, it is then removed from the soil minerals via desorption (Yamaguchi et al., 2014). AsIII is absorbed by a subclass of aquaporins known as nodulin 26-like intrinsic proteins, or NIPs, and subsequently passes via the silicon (Si) pathway to reach the stele (Ma et al., 2008). Some of these proteins are Si transporters, which either efflux AsIII from the roots or transfer AsIII into the xylem (Zhao et al., 2010). Based on their selectivity, the ten to thirteen genes that make up the NIP gene family in rice may be categorized into three groups: NIP I, II, and III (Ma et al., 2008). AsIII is transported by aquaporin Lsi1, which also causes silicic acid inflow. In the exodermis and endodermis cells, it is expressed on the plasma membrane's outer side, which is where Casparian strips occur. AsIII can be absorbed by silicic acid transporters due to its comparable molecular diameter, however it is not often taken by aquaporins (Ma et al., 2008). AsIII is also transported by Lsi2, another silicic acid transporter that is found on the inside of the plasma membranes of exodermis and endodermis cells. Lsi2 releases AsIII into the stele and cortex (Ma et al., 2008). AsIII interacts with protein sulfhydryl groups (-SH) in the cytosol, impacting a variety of metabolic processes (Kumar et al., 2015). AsIII can be detoxified inside the cell by reacting with phytochelatins (PCs) to form AsIII–PC complexes, which are then eventually contained in vacuoles (Briat, 2010). It is believed that a C-type ATP-binding cassette (ABC) transporter mediates the complex's transit across the tonoplast; as a result, it may be crucial for plants to have As resistance (Briat, 2010; Song et al., 2010). The most common phytoavailable form of As in aerobic soils is AsV, which also strongly

binds to mineral soil constituents like iron (hydr)oxides, similar to phosphate (Takahashi et al., 2004). AsV is a phosphate analog that is acquired up by the high-affinity phosphate uptake system and is thought to be transported into xylem vessels by phosphate transporter (PHT) (Zhao et al., 2010). DMA and MMA are taken up by the root at a much slower pace than inorganic As due to the reduced affinity of transporters for organic As (oAs) (Raab et al., 2007). While the exact transport mechanisms of DMA are yet unknown, silicic acid transporter Lsi1 also plays a role in MMA uptake (Carey et al., 2011). Recent research has called into question the long-held belief that plants may methylate As to generate different types of organic As (Wu et al., 2002). It appears that plants like rice are unable to methylate As; instead, they absorb methylated As from the soil (Lomax et al., 2012). Rice takes up different amounts of As from the soil in the following order: As(III) >As(V) >DMA>MMA (Raab et al., 2007).

1.4.2. Arsenic translocation

Different inorganic As (iAs) and organic As (oAs) can be translocated to shoot from root via xylem. Seyfferth et al. (2011) analysed xylem sap of rice plant samples and observed that iAs species were dominant in xylem and its adjacent cells. The AsV was transported into shoot and incorporated into the cell via PHT. AsV was then reduced to AsIII or other forms of As inside the cell (Carey et al., 2011). AsIII was further complexed with phytochelatins and sequestered in the vacuole. Phytochelatins are metal binding proteins which are synthesized through glutathione (GSH). Induction of metal(loid) stress in plant upregulate the phytochelatin production. Phytochelatin mediated sequestration of AsIII was mainly occurs in root as compared to stem and leaf. This strategy ultimately reduced the As translocation from root to shoot and finally into the grains. After sequestration and efflux from root, the remaining AsIII is translocated to shoot via xylem (Carey et al., 2011). In shoot, AsIII is absorbed through Lsi6 aquaporins in leaf (Punshon et al., 2017). However, a small portion of oAs species enters into the root of plant from soil as compare to iAs species. The oAs species fractions in soil is very less, although, oAs species are translocated more rapidly than their iAs couterparts (Li et al., 2009; Raab et al., 2007). Due to the absence of phytochelatin mediated complexation and sequestration of DMA, it has a good rate of translocation than AsIII (Raab et al., 2007). Similar to AsIII, oAs species (DMA and MMA) are translocated to leaf via xylem and taken up by through aquaporins (Zhao et al., 2010). The amounts and species of As accumulated om grains are significantly correlate with the species, concentration of that particular species present in soil, uptake rate, As reduction capacity and xylem flow.

1.5. Rice grain arsenic

Panicles were the intermediate part in between stem and grain. About 90% of AsIII and 55% of DMA was translocated through phloem into grain (Carey et al., 2010). The above data suggests that iAs are predominantly translocated through phloem, while xylem and phloem used for translocation DMA into grain. A large fraction of DMA and MMA were translocated from flag leaf to grain. AsV is transformed into AsIII with in the flag leaf and AsIII displays no translocation (Williams et al., 2005; Norton et al., 2009). Thus, accumulation of As in rice grain was carried out through phloem (Carey et al., 2010). However, the transporter responsible for translocation from phloem into the rice grain are yet unknown (Punshon et al., 2017).

1.6. Human exposure to rice grain arsenic

Rice (*Oryza sativa*) is consider as an essential source of nutrition for half of the world population. Since rice grown in anaerobic, condition which need significant amount of water. In As contaminated areas, extensive use of As contaminated water for irrigation causes an increase in soil As concentration which eventually taken up by rice plant and accumulates in rice grain. The range of total soil As contents was below the 20000 µg/kg suggested upper limit for agricultural soil by the European Community. Human absorption of the total quantity of As in uncooked rice is hindered by its dispersal through the grain, husk, root, and straw. However, the results unequivocally showed that there is a relationship between the quantity of As present in paddy plants and the degree of As pollution in irrigation water and soil (Rahman et al., 2007). Rahman et al. (2007) discovered that root As accumulation was 28 and 75 times higher than stem and rice, respectively, regardless of the specific variety of rice. This measure of increased As accumulation in roots was found to be consistent with the results of Abedin et al. (2002). Elevated levels of As in rice straw can be hazardous to animals that consume the polluted straw, perhaps posing an indirect risk to human health through contaminated livestock and dairy products (Das et al., 2021a). Rice grain can accumulate up to 2000 µg/kg of As which is substantially greater than WHO-acceptable limit (Chowdhury; et al., 2020a; Meharg et al., 2003). Ohno et al. (2007) discovered that rice was the food that contributed the most to daily As consumption. They observed that cooked rice provided 56% of the overall As exposure, while drinking water contributed 13% on average. Because groundwater is usually utilized for drinking, it is the main exposure channel for any pollutants found in the water body. Arsenic exposure is not excluded by this pattern. Not only is this tainted water used for drinking, but it also has a big impact on agricultural land

(Brammer, 2009). Arsenic is known to be present in rice grown in As-contaminated rural West Bengal districts, and this exposure is becoming more and more of an issue (Mondal and Polya, 2008; Williams et al., 2007). The International Agency for Research on Cancer (IARC) has classified As as a human cancer-causing agent in Group-I, which is the group with the highest risks to human health (Zavala and Duxbury, 2008). Meharg et al. (2006) stated that every nation has its own set of laws governing the use of the "As Low As Reasonably Practical" (ALARP) concept in relation to the statistical incidence of As-induced cancer and the maximum tolerated/acceptable concentrations (MTCs) of As in rice. Dedicated to rice cultivation alone, 59 lakh acres make Bengal one of India's most productive states in terms of rice output (Signes et al., 2007). However, the bulk of West Bengal's agriculture fields have been contaminated due to significant As deposits in irrigated soil brought on by the use of As-affected groundwater for agricultural reasons (Roychowdhury et al., 2002, 2005).

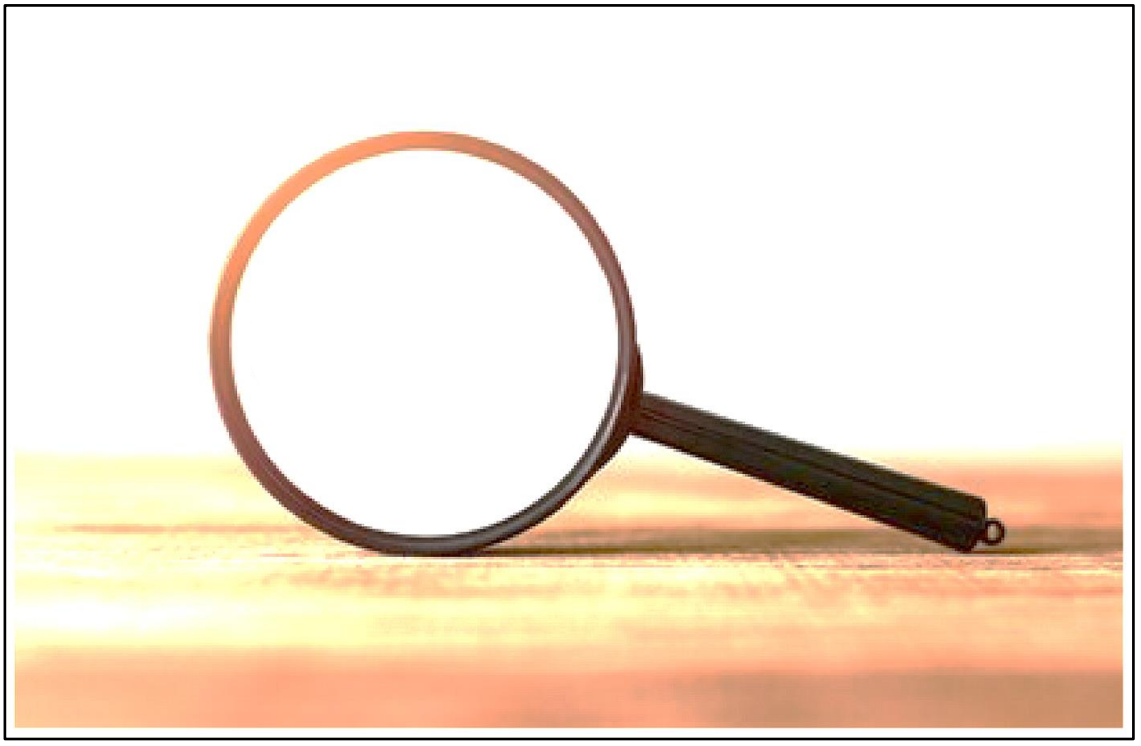
West Bengal covers 43% severely As-impacted zones out of all its As-contaminated land. These include the districts with high rates of rice production, such as Murshidabad, North 24 Parganas, Maldah, Nadia, and South 24 Parganas (Santra et al., 2013). Rice growing is a popular practice in the Hooghly and Bardhaman districts, which is among the few locations with moderate As pollution. As a kharif crop, rice requires a lot of rain or rainwater to develop properly. For agricultural purposes, groundwater is mostly utilized in a nation like India with moderate rainfall. As contamination of groundwater, which is used to create the anaerbic condition for rice cultivation, makes As(III), the most common and most hazardous species under anaerobic conditions, readily bioavailable, which improves the buildup of this toxic metalloid in the crop.

Except from drinking water, rice is shown to be one of the main food chain pathways via which humans are exposed to As. The species found there include DMA and iAs (Ohno et al. 2007; Signes et al., 2008; Williams et al., 2005). As per the findings of Jackson et al. (2012), the levels of iAs in sports drinks, infants, children meals, and rice and rice products were higher than the permissible limit of 10 µg/L for drinking water. According to Zavala and Duxbury (2008), the recommended range for the usual limit of As in rice is 82–202 µg/kg, which is far lower than the WHO's recommended safe threshold of 1000 µg/kg (specific to unexposed regions). The Food and Drug Administration (FDA) recently suggested that newborn rice cereal have an As content of no more than 100 µg/kg. It has been observed that most Asian rice is much higher in As than the FDA limit for neonates, but still falls within the WHO-approved limit. It is possible that some

agricultural fields in these countries are highly polluted with As due to higher As oncentrations in many studies. Though most marketplace studies and field investigations to find As in rice were carried out using raw rice, there is no evidence about the As-content of parboiled rice.

Chapter 2

Objectives



The objectives of this study are as follows:

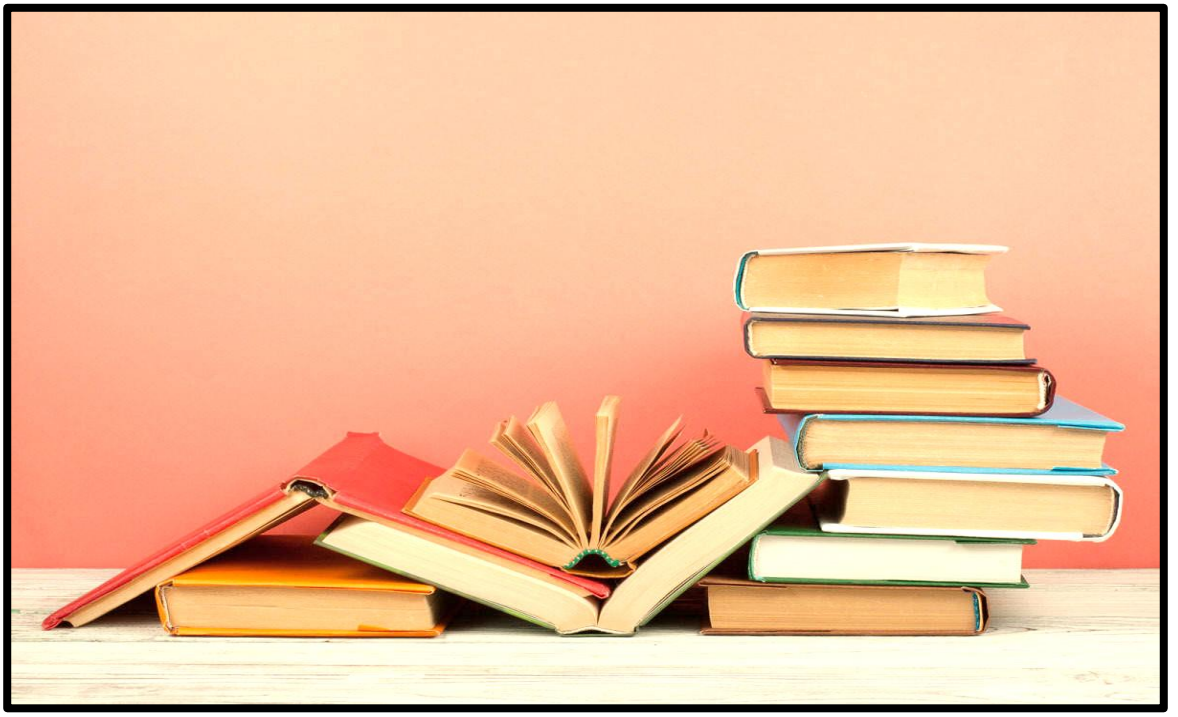
(1) Alleviation of arsenic stress and improvement of rice seedling germination and growth with potassium humate, iron oxide nanoparticles, and selenium nanoparticles.

(2) Pot study with different amendments [sulfate (Na_2SO_4), and iron modified mango leaf biochar (FeMBC)] to improve rice plant growth and reduce rice grain arsenic.

(3) Field-scale trial of these amendments in arsenic-contaminated rice fields for comparative assessment of their effectiveness and impacts on enhancement of plant yield and rice grain arsenic-reduction.

Chapter 3

Literature review



3.1. Sources of arsenic in rice plants

Arsenic is the 20th most available element in the crust of earth, which occurs naturally within the environment and found in almost 300 minerals of different type (Drahota and Filippi, 2009). The As in rice plants can be come from both geogenic and anthropogenic sources. Presence of elevated levels of As in soil and groundwater is a matter of concern.

The majority of rice-growing areas in Asia, notably in China, India, and Bangladesh, are contaminated by As (Upadhyay et al., 2020). Even groundwater used for irrigation purposes is also contaminated with As (Ravenscroft et al., 2009). Approximately, 80% of As-contaminated land in Bangladesh is used for agriculture (Biswas et al., 2020). Arsenic contamination in soil is caused by a variety of geogenic and anthropogenic sources, in addition to irrigation with As-contaminated groundwater. In the case of naturally existing As, weathering is the primary process that results from meteorological and hydrological events (Bhattacharya et al., 1997). Arsenic makes up around 4.0 × 1016 kg of the Earth's crust (Bhattacharya et al., 2002). Many alluvial and deltaic sediments, and volcanic rocks, contain significant quantities of As, which can be mobilized during weathering (Herath et al., 2016). Arsenic originating naturally in alluvial sediments and transported into groundwater is a frequent occurrence in Bangladesh, India and other parts of world (Das et al., 2021b; Raessler, 2018). Arsenic is derived from the aquifers of Australia through erosion of arsenic-rich stibnite (Sb_2S_3) mineralization in the hinterland (O'Shea et al., 2007). Microbial activity or reductive dissolution may be the cause of natural As contamination in rice paddies. Since rice is typically produced in anaerobic settings, which create reducing environments in the soil, As has a great affinity for manganese and iron hydroxides. The mobilization of As from the soil matrix is facilitated by flooding the rice cultivation areas. Microbial activity or reductive dissolution may be the cause of natural As contamination in rice paddies. One of the main causes of naturally occurring As contamination in rice crop fields is the reduction of FeO(OH), which can release As in the reducing environment (Welch et al., 2000). The estimated worldwide threshold value for total soil As concentrations is 5-10 mg/kg, with certain soil types being more prone to naturally occurring greater quantities of As (Smedley and Kinniburgh, 2002). In rice fields, soil with amounts of As more than 10 mg/kg is deemed polluted (Dittmar et al., 2007, Norra et al., 2005).

Apart from geogenic source of As, anthropogenic sources of As in the environment include mining, As-based pesticide and herbicide application, wood preservation (chromated copper

arsenate [CCA] chemicals), coal burning, and other activities (Biswas et al., 2020). The primary human-caused factors endangering rice production include industrial pollutants and runoff, as well as past land usage. The primary human-caused factors endangering rice production are runoff and pollution from industry. Until the 1980s, the widespread use of pesticides in the United States resulted in high levels of As in soil (Sarkar et al., 2005). The majority of As pollution in soil in Brazil is due to mining of naturally occurring As-rich minerals (Ciminelli et al., 2017). Fields where arsenic-based pesticides and herbicides have been used in the past are frequently the sites of straight head disease observations.

3.2. Biogeochemical processes of arsenic in paddy soils

The most frequent cause of As pollution in fields of rice is irrigation using groundwater polluted with As. Over the last twenty years, scientists have made significant progress in understanding how biogeochemical processes affect human exposure to soil As. The soil's physical, biological, and chemical activities may be the cause of environmental circulation of As. Understanding the As biogeochemical cycle is essential for determining possible exposure pathways, projecting the metal's destiny, and creating effective remediation plans to lower As absorption and buildup in plants. The portion of soil As available for plant uptake through root is called as bioavailable As. The availability of As is commanded by several factors in soil such as total As, pH, soil redox, organic matter (Norton et al., 2013). Although research has indicated a favorable link between total soil As and total As in rice grains, the total concentration of As in the soil does not necessarily correspond to the amount of As that is bioavailable. The relationship between soil and rice grain As may be better explained by the characteristics of the soil; the kind of soil can have a significant impact on the amount of As released into the pore water that is accessible for absorption (Khan et al., 2010, Wan et al., 2019). In paddy soils, biotic variables like microbial activity and mineralogy, as well as abiotic ones like pH and redox potential (EH), affect As speciation and movement (Punshon et al., 2017). The addition of microbial inoculants, mineral nutrients, and metallic oxides to As-polluted soils has been shown to significantly affect As speciation, mobility, and bioavailability in paddy soils, which in turn has been shown to influence As accumulation in rice grains, according to previous research observations (Chen et al., 2021, Mlangeni et al., 2020). Current developments in the use of soil amendments to lower As content in rice grains, as well as the effects of these variables on As speciation and movement.

3.2.1. Redox potential

The species, mobility, bioavailability, and absorption of As present can all be determined by the soil's redox potential. Rice is mostly grown in flooded paddy fields with reducing environments. One of the main forces behind the biogeochemical cycling of As in rice soil is the redox state of the soil. Flooded soils affect the As biogeochemical cycle, mainly by changing the redox processes. In reducing conditions particularly in anaerobic environments, AsIII dominates over other As species, contributing about 87-94% of the As content in the pore water. The movement and speciation of As species are influenced by the EH. Soil AsIII mobility increases overall when anaerobic circumstances cause a reduction in soil EH (Gorny et al., 2015). When compared to other grain crops, Williams et al. (2007) showed that the primary factor causing the accumulation of As in rice is the redox condition of the soil. Arsenic's solubility increases with decreasing soil reducing potential; this is mostly due to the dissolution of As from minerals containing Fe and Mn. Arsenate (AsV) is more common in aerobic soil, but it is significantly less mobile because it adsorbes to minerals that are rich in Fe (Masscheleyn et al., 1991; Xu et al., 2008). Hashimoto and Kanke (2018) have looked at the speciation and solubility of As in a redox rice soil with varying sulfate concentrations. Their findings showed that an increase in the rate at which AsV was reduced to AsIII was caused by a decrease in soil EH, which in turn raised AsIII levels.

3.2.2. pH

One of the main factors affecting the biogeochemical cycling of As in rice fields is the availability of protons, which is evaluated by pH. The solubility and type of As species found in the soil are also determined by the pH. Both high and low pH affect rice's ability to absorb and accumulate As. The desorption of As from sorption sites is facilitated by decreasing soil pH, which leads to an increase in the soil solution. The species of As in the soil solution might vary depending on the pH of the soil. Yamaguchi et al. (2011) found that AsIII was the most common species in the solution at lower pH values and that it was also more likely to be bioavailable than AsV. AsIII has a greater affinity for adsorption to soils than AsV at greater pH (>7) levels, which may lead to AsV being the predominant species that is bioavailable for absorption (Dixit and Hering, 2003). According to Bhattacharya et al. (2010), there may be a positive or negative relationship between rice plant As contents and the pH of the soil. This discrepancy might be due to the result of different soil properties or circumstances.

3.2.3. Organic matter

The mobility of As in soil can be affected either way by the chemical makeup of organic soil debris. The methylation ability and soil As availability can be impacted by the amount of organic matter in the soil. Microorganisms in the soil get their nourishment from organic materials. Arsenic within the soil may become more methylated as a result of increased activity from microbes. Increasing the amount of organic material in the soil can promote microbial development and favorable reducing conditions that lead to the reductive dissolution of Fe-oxyhydroxides and a rise in As mobility (Ma et al., 2014). As a result of the positive relationship between organic matter and As mobility, rice plants may have more As in grain (Bhattacharya et al., 2010; Norton et al., 2013). However, a number of studies have found a strong inverse relationship between As accumulation in grain and organic matter (Kar et al., 2013; Rahaman et al., 2011). The elevated activity of microbes during flooding also diminishes the soil's redox potential, since microbes continue to exhaust oxygen while breaking down organic materials (Yang et al., 2018). The total pool of available As can be increased by further reducing the redox potential, which can also liberate previously inaccessible and usually strongly bound As. Different rice-cultivation management techniques can have an impact on organic matter, pH and redox. Growing rice aerobically or anaerobically is one of the most important factors influencing the bioavailability of As in rice fields. Typically, rice is produced under flooded circumstances, which drastically lowers the soil's redox potential. When flooded, concentrations of As in soil can be as much as 16 greater than when the soil is anaerobic (Xu et al., 2008). According to recent findings by Syu et al. (2019), adding organic matter to soils contaminated with As increased the toxicity and accumulation of As. The great affinity that organic stuff has for Because of the creation of an organo-As complex, as sorption. Furthermore, Farooq et al. (2010) showed that the leaching of As deeper into the soil as a result of the formation of dissolved organic carbon (DOC) from decomposing organic matter in soils might lead to a decrease in As bioavailability in the plant rhizosphere. Therefore, it is important to apply organic matter carefully to soils with high As concentrations in order to manage As toxicity and buildup in rice. This is because organic matter has the ability to promote or decrease As mobility in soils.

In addition, anaerobic condition can make As in the soil more methylated. whereas aerobic environments are more conducive to organic arsenic's demethylation (Yoshinaga et al., 2011). Due to the plant's inability to methylate As, all organic As molecules in rice come from the soil. The initial biogeochemical model for As methylation and demethylation in rice fields under anaerobic

circumstances has been proposed by Chen et al. (2019). The model suggests that the redox condition of the soil influences the mechanisms that produce organic As in rice fields. Methylotrophic methanogenesis bacteria are in charge of demethylating methylated As, whereas anaerobes such sulphate-reducing bacteria are in charge of methylating As.

The amounts of oAs in the soil and rice grains are mostly determined by the methylation capacity of the soil. It has been demonstrated that rice fields that alternate between being wet and dry may significantly reduce the amount of As in the grain overall (Xu et al., 2008). The lowering of DMA in the grain has been demonstrated to be more influenced by intermittent flooding or aerobic rice growth than by iAs (Hu et al., 2015, Xu et al., 2008). Mlangeni et al. (2020) discovered that rice grain DMA concentrations increased in soils supplemented with Fe when subjected to intermittent floods. Anoxic environments are ideal for As methylation; adding iron may encourage bacteria that break down iron, which may increase more As methylation. The primary factors influencing the bioavailability of oAs in rice paddies remain unknown, as evidenced by the fact that soil variations in As methylation potential can range by up to two orders of magnitude.

3.2.4. Microbial activities

Arsenic speciation and mobility in agricultural soil-water systems are influenced by As biogeochemistry, which is influenced by microbial activity through several biotransformation routes (Mishra et al., 2017). A variety of microbial genes that code for transporters and enzymes involved in As transformation. Arsenic-bearing minerals provide microorganisms with growth nutrients when there are insufficient nutrients available. This leads to the release of As and the production of abiotic circumstances, which alters the speciation of As (Wang et al., 2016). It has been reported that a number of bacterial strains, including *Shewanella oneidensis*, *Desulfovibrio* strain Ben-RB, and *Desulfosporosinus auripigmentum*, cause rocks that contain As to weather and use the As that is produced as an electron acceptor (Mawia et al., 2021). Arsenite methylation via the *arsM* genes, respiration via the *arr* genes, reduction via the *ars* genes, and oxidation via the *aio* genes have all been shown to have an impact on the microbial-mediated As cycle in agricultural systems (Xiao et al., 2016). Microorganisms may methylate inorganic As species to create mono-, di-, tri-, and tetra-methyl As species by using arsenite methyltransferases. Arsenic exposure in human diets is increased by microbial transformation of organic As and subsequent root absorption because rice plants cannot methylate As (Punshon et al., 2017). Furthermore, microbes can oxidize AsIII to less harmful As species. This procedure occurs in both flooded and non-flooded

conditions. Zhang et al. (2015) showed that by employing nitrate and oxygen as electron acceptors, respectively, Paracoccus species can oxidize AsIII in paddy soil in both aerobic and anaerobic conditions. Therefore, As toxicity and buildup in rice plants can be reduced by inoculating As-polluted soils with AsIII oxidizing bacteria. Additionally, especially in anaerobic environments, microbes convert AsIII to AsV through respiratory paths and detoxifying. In paddy soils, As mobility and bioavailability may rise if AsV is reduced to more mobile and hazardous AsIII (Kumarathilaka et al., 2018). It has been shown that introducing microorganisms into growth media contaminated with As can reduce the phytotoxicity and build-up of As in plants.

3.3. Effect of arsenic toxicity in rice plant

3.3.1. Metabolism

The reduction of AsV to AsIII, which may occur both enzymatically and non-enzymatically, is the first step in the metabolism of As. AsV is reduced to AsIII during the non-enzymatic process due to the oxidation of two reduced glutathione molecules to generate oxidized GSH, which may be quickly regenerated to GSH molecules by GSH reductase. For the effective reduction of AsV, this reaction is, however, extremely sluggish. A High Arsenic Content 1 (HAC1) that can convert AsV to AsIII and helps AsIII flow back into the soil, preventing AsIII from building up in the roots and moving to the shoots in Arabidopsis (Chao et al., 2014). AsIII localization in shoots enhanced and AsIII efflux decreased when HAC1 function was lost. It has been reported that many rice arsenate reductases, including OsHAC1;1, OsHAC1;2, and OsHAC4, have been identified and their functions described (Mawia et al., 2021). The overexpression of these genes has been reported to increase AsIII efflux into the external medium and reduce As accumulation in rice, while knock-out results in reduced AsIII efflux and enhanced As accumulation in shoots and grain. These proteins facilitate AsIII efflux from the root to the soil.

After the efflux of AsIII into the soil, the remaining AsIII were metabolised into the vacuole. Arsenic stress in plants enhanced GSH content, which acts as a precursor of phytochelatin (PC) and also inhibit phytochelatin synthase at high concentration (Guo et al., 2018). Phytochelatin synthesized upon As stress formed AsIII-PC complex which was then vacuole through ABCC1/ABCC2 transporters. In rice, OSPCS1 and OsPCS2 genes are mainly orchestrate the synthesis of PCs utilizing GSH as a substrate. Glutathione S-transferases (GSTs) curated the bond between AsIII and GSH (Kumar and Trivedi, 2018).

3.3.2. Detoxification

Following the formation of AsIII-GSH/PC complexes in the cytosol, these complexes are further transported to the vacuoles for sequestration and detoxification (Ma et al., 2008). Non-protein thiol-dependent AsIII complexes are transported into the vacuoles using ABC (C-type ATP-binding cassette) transporters. Vacuolar transporter OsABCC1, an orthologous of *Arabidopsis thaliana* ABCC1, is implicated in AsIII-PC complex sequestration in the vacuole of rice. Song et al. (2014) observed that due to a malfunction in the vacuolar compartmentalization of As in the phloem of the nodal vascular bundle, the knockout of the OsABCC1 transporter gene led to a large concentration of As in their grains. The preservation of GSH homeostasis is essential for the detoxification of AsIII. Chloroquine Resistance Transporters (CRT) move the produced glutathione to the cytoplasm from the plastids. When rice's OsCLT1, a CRT-like transporter 1 gene, was mutated, the cytoplasm's GSH level dropped, which in turn caused a fall in PCs. The Osclt1 mutant shows significantly reduced PC2 levels under As treatment in comparison to the wild-type, which results in reduced As accumulation in the roots but greater or comparable As build-up occurs in the shoots (Yang et al., 2016).

3.3.3. Toxicity of arsenic and response in rice

Research has determined that AsIII is far more hazardous than AsV. The toxicity mechanisms of the two iAs species may vary. By substituting the AsV group for the phosphate group, AsV obstructs phosphate metabolism processes including phosphorylation and ATP generation. The catalytic activities of proteins, co-factors, and enzymes are inhibited when AsIII attaches to their thiol groups, changing their structures. When regular metabolic processes are disrupted, reactive oxygen species (ROS) are produced. Damage from superoxide radicals is caused by oxidative stress, which interferes with signaling pathways linked to cell division, growth, and death. Plants respond to the impacts of ROS by scavenging the free radicals with antioxidants (Kumar et al., 2015). Even if the amount of metal in the plants increases antioxidant activity, after a few days, this activity steadily decreases and the plants eventually die from damage caused by oxidative stress (Mascher et al., 2002). For example, if As builds up in rice, the concentration of H2O2 and the degree of lipid peroxidation, as measured by MDA concentration, both rise. But the concentration of ROS in the rice plants decreases when arsenic-supplemented iron is added to the growing media. When comparing rice plants treated with AsV to those supplemented with Fe, the levels of antioxidants such as CAT, SOD, AsA, and glutathione (GSH) are likewise much higher.

Fe-plaque development forms a barrier on the root surface that lowers As transfer to shoots. Therefore, lowering As stress reduces ROS concentrations and antioxidant levels in rice plants. This shows that rice plants have an antioxidant defense system in place to protect them against As stress. One compound that reduces As-induced oxidative damage is selenium (Se). It has been discovered that adding As to the medium together with Se reduces damage due to oxidation. Kumar et al. (2015) observed that supplementing the medium with Se increases the activities of glutathione peroxidase (GPX), APX, and CAT when As is present. This suggests that Se enhances antioxidant activities and guards against oxidative stress-related damage.

3.4. Alleviation of arsenic stress and reduction of rice grain arsenic

3.4.1. Alternative water regime

In typical rice cultivation, anaerobic and aerobic situations occur in paddy soils. It has been demonstrated that using alternative water management techniques results in significantly lower total As levels in rice tissues and paddy soil solutions. As alternative water management techniques have recently been investigated to reduce As accumulation in grains, aerobic and intermittent water management approaches (Liao et al., 2016). Paddy soil is inundated with irrigation water to a height of around 3-5 cm during intermittent ponding. The primary changes made to the paddy soil-water system by water management techniques are to its physico-chemical and biological components. When continuous flooding irrigation is used, for instance, the solubility of As in the soil matrix changes from AsV to AsIII, which has greater mobility compared to AsV (Sahrawat, 2015). When using intermittent and aerobic water management methods, the opposite is true and the paddy soil solution's As(V) : As(III) ratio is higher. The dynamics in the rice agroecosystem, which are controlled by various methods of water management, may also be impacted by natural rainfall. More specifically, periodically flooded rice fields show a drop in As content in the top soil during the wet season after dry season irrigation, indicating that rainfall reduces As levels in rice soils. A loss of 13–62% of the total As added to rice fields by groundwater irrigation was estimated by Roberts et al. (2010) to have occurred during the wet season, with 51–250 mg/m^2 of total As in top paddy soils being discharged into the flood water. Thus, more research is required to determine how As dynamics in the rice ecosystem are impacted by rainfall.

3.4.2. Supplementation of nutrients

There is documented evidence about the ways in which the physical characteristics, chemical composition, geographic distribution, and origin of different minerals impact their speciation and

mobility in the environment (Drahota et al., 2009). Mineral components present in the soil can have an impact on As adsorption because they can directly compete for binding sites and indirectly alter mobility (Srivastava et al., 2020). The next sections cover the effects of mineral nutrients such as Fe, Si, and P on As mobility and bioavailability, as well as their involvement in lowering As buildup in rice.

3.4.2.1. Iron

Since iron (Fe) is a necessary mineral ingredient for rice plant growth and development, it is important for understanding how As speciation and mobility in paddy soils occur. Arsenic is present as AsV in aerobic environments and is firmly adsorbed to Fe oxides, which reduces ~~As's~~ the mobility of As and plant bioavailability (Xu et al., 2008). Conversely, in anaerobic environments, AsIII mobility in paddy soil-water systems is increased by the breakdown of Fe-oxyhydroxides, making it more accessible for utilization by plants (Rinklebe et al., 2016). However, bioavailability of As is restricted by the anaerobic development of iron plaque around the rhizosphere. Iron plaque develops when dissolved Fe(II) is oxidized by oxygen moving from the leaves to the roots and then diffusing to the root surfaces (Zhao et al., 2010). Therefore, rather than directly preventing As absorption by rice plants or supply in the soil, iron plaques act as a mass scavenger of As surrounding the roots. When Fe is added to As-contaminated soils, Fe-oxide is deposited and/or Fe-plaque is formed around the roots of rice plants. As a result of AsV adsorbing to Fe-oxide, AsV availability is subsequently decreased (Bakhat et al., 2017).

3.4.2.2. Silicon

The primary way that silicon (Si) increases spikelet fertility is via raising rice production. Since Si and AsIII are chemically similar and compete for their uptake by roots through the same transporter, rice's absorption of AsIII is impeded by high soil Si concentrations (Bogdan and Schenk, 2008). The gene expression of the two Lsi genes (Lsi1 and Lsi 2) transporter genes is suppressed by the availability of silicon (Ma et al., 2008). A viable method of controlling As toxicity and buildup in rice has been demonstrated by the use of Si in As-polluted paddy soils. This is achieved by competitive reduction of arsenite absorption.

3.4.2.3. Phosphorus

Plant uptake and bioavailability of As are increased when phosphate removes AsV from binding sites in soil or Fe-plaque (Mawia et al., 2021). Since phosphate and AsV have similar chemical

structures, they share same transporter. Phosphorus influences As biogeochemistry because AsV and Phosphorus (P) compete for the same binding site. Phosphorous supplementation lowers the As concentration in Fe-plaques, increasing the solubility and bioavailability of As in the soil and rhizosphere, as several studies have shown (Bogdan and Schenk, 2009; Signes-Pastor et al., 2007). Grain As content reduced in soil with appropriate initial P and further P amendment, as reported by Dang et al. (2016). They hypothesise that competition for the root absorption of P and AsV was likely the cause of the decline in As content.

3.4.2.4. Sulfur

The addition of sulfur (S), a macronutrient that is necessary for plant development, reduces the buildup of As in rice tissues via altering the rhizosphere's mineralogy. When AsIII in the paddy soil solution reacts with S^{2-}, an As_2S_3-like compound can form. As a consequence, AsIII has less bioavailability for absorption by the rice crop. Furthermore, by altering the metabolism of the rice plant, S addition reduces the amount of As that accumulates in rice grains. Dixit et al. (2015) showed that the addition of S caused a decrease in the expression of Lsi2, which facilitates AsIII efflux toward the xylem. Additionally, sulfur can promote the production of glutathione (GSH) and phytochelatins (PCs) in rice. There is a strong affinity between these thiols and AsIII. In rice roots, OsABCC1 transports AsIII-thiol complexes for vacuole sequestration. The transportation of As(III)-PC complexes for vacuolar sequestration is likewise mediated by OsABCC1, which is found in the tonoplast of phloem in nodes.

3.4.3. Biochar application

Recently, there has been a lot of interest in applying biochar to agricultural fields because of its possible agronomic, environmental, and financial advantages. By thermally breaking down organic material in limited oxygen environments, a process known as pyrolysis, biochar is produced. The physicochemical characteristics of biochar, including pH, surface characteristics, and nutrient content, are determined by the kind of feedstock, pyrolysis temperature, heating rate, and time. When biochar is added to polluted soils, there is a noticeable decrease in toxic metal(loid) mobility and bioavailability.

Biochar has a well-developed pore structure (i.e. micropores, mesopores, and macropores) and facilitates the diffusion of As into the pores through physical adsorption (Khan et al., 2014). Because of its well-developed porous nature, biochar allows As to diffuse into the pores by physical adsorption (Khan et al., 2014). Oxygenated functional groups found in biochar have the

potential to regulate assorption through surface complexation. The Fourier Transform Infrared Spectroscopy (FTIR) bands of functional groups in the arsenic-adsorbed BCs have altered, according to several studies, demonstrating that As complexation with oxygen-containing functional groups occurs. A further significant method of As adsorption onto BC is electrostatic attraction. In paddy soils, biochar may enhance or add competitive ions like $Si(OH)_4$ and PO_4^{3-}. A high concentration of PO_4^{3-} in the soil solution may result in decreased absorption and accumulation of total As in rice grains because the same transporter acquires both PO_4^{3-} and As(V). According to research by Seyfferth et al. (2016), adding 1% of Si-rich rice husk BC reduced the amount of inorganic As by 30% in the rice grain. This is because the addition of $Si(OH)_4$ can restrict rice roots' ability to absorb AsIII. Additionally, BC may raise the concentration of dissolved organic carbon (DOC) in the soil solution, which might lead to the immobilization of As-DOC complexes (Khan et al., 2014). The rhizosphere's FeIII content may rise with prolonged BC application. As previously noted, AsIII and AsV become immobile as a result of FeIII plaque development in the rice rhizosphere. Surface modification of BC may be effectively achieved with redox-sensitive elements like Fe and Mn. Surface alteration might improve BC's physico-chemical characteristics. For instance, adding nano-zero valent Fe to biochar doubles its surface area, which in turn significantly increases the number of sites for reaction in BC (Liu et al., 2017). BC composites hence significantly reduce As accumulation in rice tissues. Using Fe-modified BC to apply Fe supplements might be a beneficial alternative to lower the bioavailability of As in paddy soil solutions. Since modified biochar contains essential plant nutrients like K, it may simultaneously improve plant development indices and prevent As accumulation in rice tissues (Awad et al., 2018).

3.4.4. Nanotechnology

One of the most well-known instruments for improving environmental sustainability and agricultural protection is nanotechnology. It works with materials of nanoscale dimensions, often known as nanoparticles, and their manipulation, processing, and application. Metal oxides were first employed to regulate As toxicity, but because of their reduced adsorption capability, their nanometal oxide equivalents are being utilized more and more to solve this worldwide issue. For the removal of heavy metals from the environment, a variety of metal oxide nanoparticles (NPs) have been employed as nanoadsorbents, including copper, iron, alumina, titanium dioxide, and zinc. Engineered nanoparticles have the ability to alter the As mobility in soil. It has been observed that low molecular weight organic acids' interactions with nano-oxides, such manganese oxide,

decrease As mobility by promoting the oxidation and precipitation of Fe, Mn, and Al oxyhydroxides (Vítkov´a et al., 2015). However, additional investigation is required to clarify specific details regarding the environmental behaviors of engineered nanoparticles in soils.

Wang et al., 2018b reported a significant decrease in total As accumulation in rice seedlings grown hydroponically and treated with a combination of ZnO NPs and AsIII, and ZnO NPs and AsV, compared with plants exposed to AsIII or AsV alone. ZnONPs were hydroponically administered by Wang et al. (2018) to reduce AsIII and AsV toxicity in rice seedlings, and they observed a reduction in both As toxicity and accumulation. There is data supporting the use of CuONPs to reduce rice's overall As toxicity. The physiological effects of CuONPs and As interaction on rice development and life cycle were assessed by Liu et al. (2018). They observed that compared with only As treatment, the application of 50 mg/L CuO NPs decreased the amount of As that accumulated in dehusked grains by 35%. The decreased mobility and bioavailability of As caused by the adsorption of AsV on the surface of CuO NPs in the soil may have contributed to the low As concentration in the dehusked grains. Effect of MnO_2 nanoparticles on As accumulation and transloation in rice plants were studied by Zhou et al. (2015). Additionally, it has been discovered that CuONPs affect the buildup of As to rice tissues. While the distribution of As and Mn in the plant organs was shown to be strongly negatively associated, the concentrations of both elements in the soil dropped. When MnO_2-NPs were administered to rice plants instead of only As-treated plants, the overall As concentration in the rice grains decreased by 65.4%. Li et al. (2019) conducted a study to examine the potential of nanostructured α-MnO_2 in reducing the build-up of As in rice grown on soil polluted with As. Silicon and arsenite have identical absorption and translocation mechanisms. Traditionally, silicon fertilization has been shown to dramatically lower the amount of As that accumulates in rice straw and grain.

Objective 1

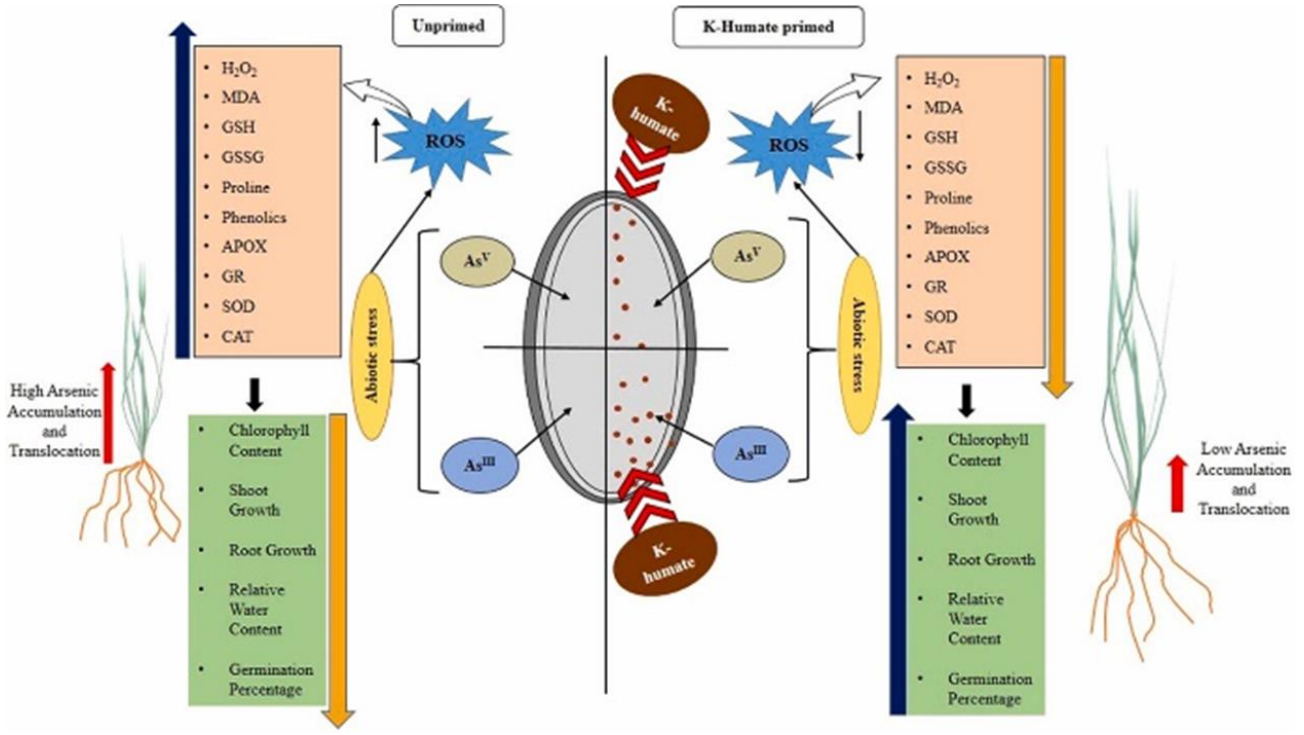
Alleviation of arsenic stress and improvement of rice seedling germination and growth with potassium humate, iron oxide nanoparticles, and selenium nanoparticles

Chapter 4

Improvement of rice seeds germination and seedling growth under arsenic stress using potassium humate (K-humate)

4.1. Rice seed priming with K-humate

Graphical Abstract



4.1.1. Introduction

Arsenic is a naturally occurring toxic pollutant and considered as class 1 carcinogen for humans (Kalita et al., 2018). Many regions in South-East Asia are heavily contaminated with groundwater As. Rice (*Oryza sativa* L.) is the most important cereal crop and staple food for half of the world's population. A major portion of rice is cultivated and irrigated with As-contaminated soil and water, and via this way As accumulates in rice grain and enters into the food chain (de Andrade et al., 2015). Plant exposed to excessive As stress through water and soil suffers from phytotoxicity which ultimately results in the inhibition of germination, reduced plant growth and yield (de Andrade et al., 2015).

Various methods have been employed to mitigate As phytotoxicity in rice plants and its subsequent accumulation in grain. Application of silicon (Si) amendments was proven to reduce the subsequent accumulation of As in rice plant (Zhang et al., 2017). Different alternate wetting and drying (AWD) water management practices were also found to reduce the As concentration in rice grain (Shrivastava et al., 2020). Moulick et al. (2016) used selenium (Se) to reduce As uptake by rice plant and elevate plant growth under As stress. Dixit et al. (2015) reported that application of sulfur (5.0 mM) reduced As uptake and its subsequent toxicity in rice plant. Apart from different soil amendments, arbuscular mycorrhiza (AM) elevates the photosynthetic efficiency and biomass yield of rice plant under As stress condition (de Andrade et al., 2015).

Seed priming is a recently blooming technology in plant stress physiology and proved to elevate plant stress tolerance against different biotic and abiotic stress. Priming with polyamines and humic acid emerged to be very effective in different stressed conditions and also promoted the germination, better growth and yield (Sheteiwy et al., 2017a, b).

Humic acid (HA) substances generally characterized by relatively higher carbon contents, and aromatic ring condensation with different amino acids (Zanin et al., 2019). HA substances have been proved to promote plant growth by increasing the membrane permeability, essential micronutrient transport through root by facilitated transport, and respiration (Hasanuzzaman et al., 2018; Noroozisharaf and Kaviani, 2018; Taha and Osman, 2018). Use of HA substances also promoted plant growth by increasing root and shoot lengths, improved foliar water content, photosynthesis, antioxidant metabolism and enzymatic activities under different stressed conditions (Kaya et al., 2018; Sheteiwy et al., 2017a). Previous studies reported the metal chelation (Pb, Zn, Cd and Fe) properties of HA substances (Musani et al., 1980; Zanin et al., 2019).

Potassium (K) on other hand is a macro element that plants need during seed germination and plant growth. Potassium is essential to maintain ionic balance and also required for various biochemical and physiological processes. Potassium also plays an important role in alleviation of various abiotic stresses like drought, heavy metal toxicity, salinity etc. (Hasanuzzaman et al., 2018; Siddiqui et al., 2012). Potassium deficiency increases the plant vulnerability under different stress condition (Hasanuzzaman et al., 2018). So, the combined beneficiary from potassium and humate ultimately increases the seed germination and plant growth under various stress conditions. Different experiment conducted with K-humate also proved to upregulate the plant growth under different stress condition (El-Nwehy et al., 2020; Taha and Osman, 2018).

Arsenic (As) toxicity in plants and especially in rice is an agro-economically relevant issue worldwide, particularly in Indian scenario and other south-east Asian countries that need to be addressed. Many rice nursery beds are using As-contaminated soil and water to germinate the seeds and grow the seedlings. Researches have been conducted to minimize the As toxicity in plant with different approaches and several studies are available which elaborate the role of humic substances in alleviation of stress from plants (Kaya et al., 2018; Sheteiwy et al., 2017a; Taha and Osman, 2018). However, no study has used humic substances like K-humate to alleviate the As toxicity in plants. The current study, possibly the first one aims to (i) investigate whether K-humate priming on rice seed can improve the seed germination, seedling growth as compared to unprimed seeds, (ii) compare the level of stress markers and antioxidant activities in primed and unprimed seedlings, (iii) compare the As accumulation and translocation in primed and unprimed seedlings under As stressed condition.

4.1.2. Materials and methods

4.1.2.1. Plant material, priming and treatments

The rice (*Oryza sativa* L.) variety IR64 (long and slender) was selected for this study. The rice seeds were surface sterilized with 70% (v/v) ethanol by continuous stirring for 1 min, and washed thoroughly with double distilled water for three times. The rinsed seeds were soaked in 5% (w/v) sodium hypochlorite solution for 5 min, followed by rinsed with double distilled water three times. The seeds were then placed in tissue paper for drying. The surface dried seeds were placed in 50 ml conical flask containing 20 ml of 100 mg/l K-humate priming solution. K-humate used for this experiment was purchased from Disha Agrotech, West Bengal, India contained 70% humic acid and 10% potassium with 95% solubility. For priming, the rice seeds were soaked in K-humate solution and the other (unprimed) seeds were soaked in water. All seeds were primed for 12 h in

absence of light at 25 ± 1 ºC. After priming, the seeds were taken out and kept in between the two layers of tissue paper for proper drying, till the seeds had moisture content of 10% ± standard error of the initial weight. The dried seeds were kept in zipper pouch for 48 h at 25 ± 1 ºC in dark, prior to germination (Moulick et al., 2016). For this experiment, six treatments were designed and detailed design of the experimental sets were: control 1 (Murashige and Skoog medium + unprimed seeds) [C1]; 50 µM AsIII + unprimed seeds; 50 µM AsV + unprimed seeds; control 2 (Murashige and Skoog medium + primed seeds) [C2]; 50 µM AsIII + primed seeds; and 50 µM AsV + primed seeds with three replica plates for each treatment and twenty seeds per plates.

4.1.2.2. Seed germination, seedling growth and vigour

For seed germination, ten seeds were taken from each experimental set, moistened with different As species (AsIII and AsV) other than the control batches and placed them on filter paper in petri plates. For germination of the rice seeds, all the petri plates were kept in darkness for three days at 27 ± 1 ºC and 70% relative humidity. After three days, the seed germination percentages were calculated. The seeds were counted as germinated when the radicle length was ≥ 2 mm. After germination, the germinated seedlings were exposed to 14 h light (270 ± 5 µmol m^{-2} s^{-1} PFD) and 10 h dark photoperiodic condition till 12 DAS (days after seeding). The seedling growth and vigour were measured at 6 DAS and 12 DAS, respectively. The seedling vigour index was measured according to the formulae prescribed by Abdul-Baki and Anderson (1973).

$$\text{Vigour Index I (VG I)} = \text{Germination \%} \times \{\text{Seedling Length (Root + Shoot)}\}$$

$$\text{Vigour Index II (VG II)} = \text{Germination \%} \times \{\text{Seedling Dry Weight (Root + Shoot)}\}$$

4.1.2.3. Relative water content (RWC)

Relative water content (RWC) of 12 DAS old rice seedling was measured according to Barrs and Weatherley (1962). Leaf laminae were weighed (fresh wt., FW) and immediately floated on distilled water in a petri dish for overnight in BOD chamber in dark. Turgid weights (TW) were obtained after drying excess surface water with tissue paper. Dry weights (DW) were measured after drying the leaves at 80 ºC for 24 h.

4.1.2.4. Chlorophyll content

Chlorophyll content was measured from 12 DAS rice seedling by acetone extraction method. About 0.1 g of seedling leaf tissue was homogenised with 5 ml of 80% (v/v) acetone solution. The extracted solution was filtered and concentration of chlorophyll a and b (mg/g FW) were measured

according to the Arnon (1949) by measuring the optical density of extracted sample at 663 and 645 nm, respectively.

4.1.2.5. Determination of oxidative stress and antioxidant parameters

Contents of H_2O_2, lipid peroxidation, proline, total phenol, GSSG (oxidized glutathione) and GSH (reduced glutathione) in root and shoot tissues of 12 DAS seedlings were estimated according to Velikova et al. (2000), Heath and Packer (1968), Bates et al. (1973), Mallick and Singh (1980) and Anderson (1985), respectively.

4.1.2.6. Antioxidant enzyme assays

For the estimation of antioxidant enzymes, about 0.2 g root and shoot of 12 DAS old seedlings were homogenized separately in pre-chilled mortar and pestle with 2 ml of 0.1 M potassium-phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% PVP (w/v) (Polyvinylpyrrolidone). The homogenized plant tissue samples were then centrifuged and the supernatant after centrifugation was collected and used for the estimation of antioxidant enzymes. The protein concentration of the enzyme extracts was measured according to Lowry et al. (1951) using BSA protein (*Bovine serum albumin*) as standard.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured according to Beauchamp and Fridovich (1971). The one unit (1 U) SOD activity was calculated in terms of 50% inhibition of nitroblue tetrazolium (NBT). The estimated SOD activity was expressed as U mg^{-1} Protein. Ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured as described by Nakano and Asada (1981). The H_2O_2 dependent oxidation of ascorbate ($\epsilon = 2.8\ mM^{-1}\ cm^{-1}$) was noted by taking the decreasing absorbance at 290 nm for 1 min. The APX activity was expressed as μmol min^{-1} mg^{-1} protein. Catalase (CAT; EC 1.11.1.6) activity was measured according to Aebi (1984) by the gradual decomposition of H_2O_2 at 240 nm. The reaction was carried out with 1.5 ml of 50 mM phosphate buffer followed by 15 mM H_2O_2 and 0.1 ml supernatant. The reaction mixture was incubated for 1 min and gradual decrease in absorbance was noted in every 1 min interval. The CAT activity was expressed as μmol min^{-1} mg^{-1} protein. Glutathione reductase (GR; EC 1.6.4.2) (U mg^{-1} Protein) was measured according to Foyer and Halliwell (1976) and one unit of GR was recorded by taking the amount of absorbance decrease per minute at 340 nm.

4.1.2.7. Determination of arsenic content and translocation in rice seedlings

To extract As from 12 DAS seedlings, Teflon-bomb digestion protocol has been followed. Detailed information of the digestion process has been described earlier (Chowdhury et al., 2018, 2020). For all the digested samples, total concentration of As was estimated using Hydride Generation-Atomic Absorption Spectrophotometry (HG-AAS) method (Varian Model AA140, USA) coupled with VGA-77 (Vapour Generation Accessory, Agilent technologies, Malaysia) with software version 5.1. Detailed information of the instrumentation and optimization conditions of HG-AAS method were mentioned earlier (Chowdhury et al., 2020). Quality control and quality assurance of the analytical work were evaluated following the protocols as described in our earlier publications (Chowdhury et al., 2018, 2020).

The translocation of heavy metals from root to shoot can be expressed as translocation factor (TF). The TF for As was calculated according to the given formula by Chowdhury et al. (2020).

$$TF = C_{Shoot} / C_{Root}$$

Where, C_{Shoot} and C_{Root} are the As concentrations in shoot and root, respectively. The value of TF >1 indicates that plants translocate metals effectively from root to shoot (Chowdhury et al., 2020).

4.1.2.8. Statistical analysis

All the experiments were carried out in triplicates and the data were expressed in average ± standard deviation (n = 3). Statistical analysis was carried out using one-way ANOVA test followed Tukey HSD ($p \leq 0.05$) test and Principal component analysis (PCA) in Origin 2019b (Origin Lab Corporation) and StatistiXL 2.0 software, respectively. The overall set of 24 variables was divided into two 12-variable subsets, which were used to conduct two PCAs of the 18 samples. The PCA results were described using variable loadings and sample scores plotted on hyperplanes defined by first and second principal components (PC1 and PC2, respectively).

4.1.3. Results

4.1.3.1. Effects of arsenic stress and K-humate priming on seed germination, and seedling growth

The germination percentage (%) in the two control sets were maximum, among which primed seeds germinated without As stress achieved highest germination percentage (91.7 ± 7.6%) ($p \leq 0.05$) (**Table 4.1.1**). However, the seeds primed with K-humate had higher germination percentage

(75 ± 5.0% and 68.3 ± 2.9%) ($p \leq 0.05$) compared to the unprimed seeds (65 ± 5.0% and 58.3 ± 7.6%) germinated under AsV and AsIII stress ($p \leq 0.05$), respectively.

The root and shoot growth in K-humate primed seedlings without As stress (Control 2) was found to be higher ($p \leq 0.05$) than the unprimed seedlings without As stress (Control 1) (**Table 4.1.1**). Similarly, K-humate primed seedlings under AsIII and AsV stress showed more promising growth rates compared to unprimed seeds (**Fig. 4.1.1**).

Table 4.1.1: Effects of seed priming and arsenic stress on seed germination and seedling growth

Treatments	Germination %	6 DAS*		12 DAS*	
		Root (cm)	Shoot (cm)	Root (cm)	Shoot (cm)
Control 1 (MS+Unprimed seed)	85.0±5.0ab	1.70±0.46ab	4.0±0.68ab	3.62±0.4a	8.43±0.32a
50 µM AsV+Unprimed seed	65.0±5.0c	0.93±0.16ab	2.66±0.28c	2.13±0.35bc	5.62±0.26c
50 µM AsIII+Unprimed seed	58.3±7.6c	0.82±0.09b	2.22±0.35c	1.75±0.13c	4.54±0.45d
Control 2 (MS+Primed seed)	91.7±7.6a	1.97±0.74a	4.20±0.3a	3.97±0.21a	8.75±0.31a
50 µM AsV+Primed seed	75.0±5.0bc	1.17±0.3ab	2.89±0.36bc	2.79±0.2b	6.72±0.43b
50 µM AsIII+Primed seed	68.3±2.9c	1.04±0.18ab	2.63±0.4c	2.55±0.22b	5.81±0.18bc

The same letters within the same column indicate mean ± standard deviation (n = 3) values are not significantly different at $P \leq 0.05$ using Tukey's HSD multiple comparison test; * DAS = days after seeding.

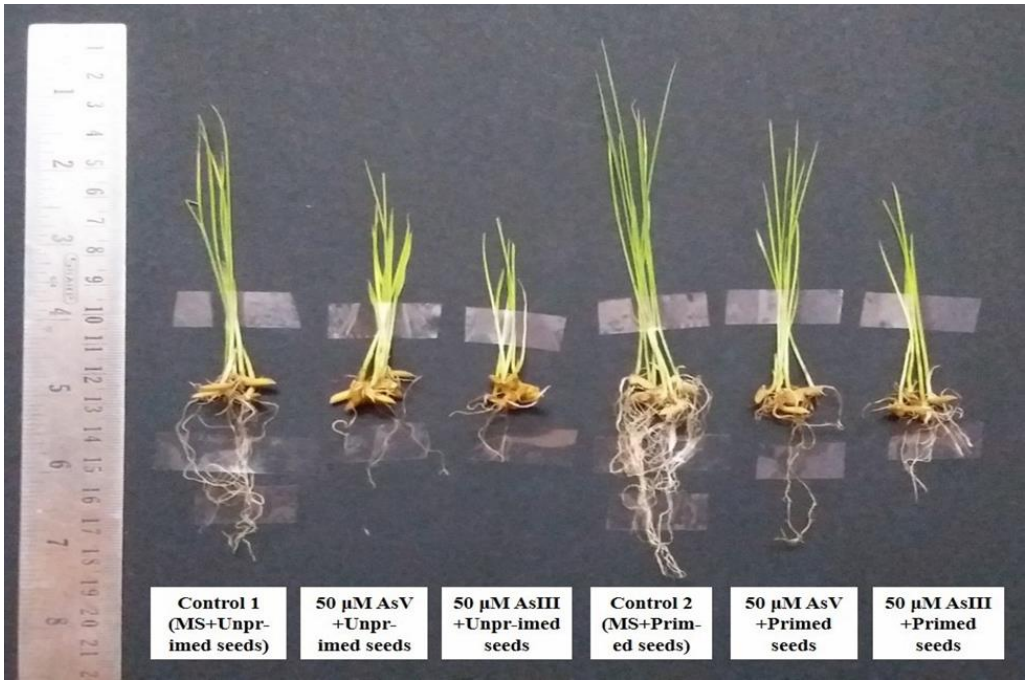


Fig. 4.1.1. Growth of primed and unprimed seedlings after 12 DAS under AsV and AsIII stress

4.1.3.2. Effects of arsenic stress and K-humate priming on relative water content and chlorophyll content

Relative water content (RWC) of unprimed rice seedlings at 12 DAS was significantly ($p \leq 0.05$) decreased by As treatments, showing 16.98% and 23.04% reduction with 50 µM As V and 50 µM AsIII, respectively as compared to control seedlings (control 1) (**Table 4.1.2**). The RWC of primed seedlings was increased by 11.16% and 13.86%, compared to unprimed seedlings under AsV and AsIII stress, respectively.

The amount of total chlorophyll (a + b) in K-humate primed seedlings were increased significantly by 24.7% and 29.7% ($p \leq 0.05$) compared to unprimed seedlings, under AsIII and As V stress, respectively (Table 4.1.2). However, there was no significant change observed in primed and unprimed seedlings for chlorophyll a/b ratio.

Table 4.1.2: Effects of seed priming and arsenic stress on relative water content and chlorophyll content of 12 DAS seedlings

Treatments	RWC (%)	Chlorophyll (mg/g FW)		Total chlorophyll (mg/g FW)	Chlorophyll a/b
		Chlorophyll a	Chlorophyll b		
Control 1 (MS + Unprimed seed)	79.01±0.69a	0.77±0.02a	0.40±0.02ab	1.17±0.01b	1.92±0.14ab
50 µM AsV + Unprimed seed	65.59±0.97d	0.46±0.03c	0.27±0.02c	0.73±0.02d	1.72±0.21b
50 µM AsIII + Unprimed seed	60.8±0.52e	0.35±0.01d	0.17±0.02d	0.52±0.03e	2.05±0.21ab
Control 2 (MS + Primed seed)	80.13±0.65a	0.83±0.02a	0.46±0.03a	1.29±0.01a	1.81±0.14ab
50 µM AsV + Primed seed	73.83±0.31b	0.61±0.03b	0.36±0.02b	0.97±0.03c	1.69±0.18b
50 µM AsIII + Primed seed	70.59±0.91c	0.51±0.02c	0.23±0.02cd	0.74±0.04d	2.25±0.08a

The same letters with in the same column indicate mean ± standard deviation (n = 3) values are not significantly different at $P \leq 0.05$ using Tukey's HSD multiple comparison test.

4.1.3.3. Effects of arsenic stress and K-humate priming on oxidative stress and antioxidant parameters

The hydrogen peroxide (H_2O_2) content in roots and shoots of 12 DAS unprimed and primed seedlings were increased significantly ($p \leq 0.05$) under AsV and AsIII stress as compared to the controls (control 1 and control 2), respectively (**Fig. 4.1.2a**). The accumulation of H_2O_2 in root of K-humate primed seedlings were reduced by 1.65 and 1.21 fold as compared to unprimed seedlings

under AsV and AsIII stress, respectively. Similarly, in shoot of K-humate primed seedlings, the amount of H_2O_2 accumulation was reduced by 1.85 and 1.21 fold as compared to unprimed seedlings under AsV and AsIII stress, respectively. The TBARS (thiobarbituric acid reactive substances) contents increased significantly ($p \leq 0.05$) in unprimed seedlings due to As treatment (both AsV and AsIII) by 1.53-2.19 fold as compared to control 1 (**Fig. 4.1.2b**). Priming with K-humate reduced the TBARS level in root and shoot by 1.38 and 1.33 fold (for AsIII treatment), and 1.25 and 1.27 fold (for AsV treatment), respectively as compared to unprimed seedlings.

The proline content in roots of primed seedlings were decreased by 1.45 and 1.28 fold as compared to unprimed seedlings under AsV and AsIII stress, respectively and proline content in shoots of primed seedlings was decreased by 1.46 and 1.19 fold as compared to unprimed seedlings under AsV and AsIII, respectively (**Fig. 4.1.2c**). The application of As in unprimed seedling growth medium significantly increased the total phenol content in root by 2.1 and 2.3 fold ($p \leq 0.05$) compared to controls (**Fig. 4.1.2d**). The supplementation of K-humate through priming significantly ($p \leq 0.05$) reduced the total phenolics content by 15.7-26.7% in roots and by 7.1-10.2% in shoots as compared to unprimed seedlings.

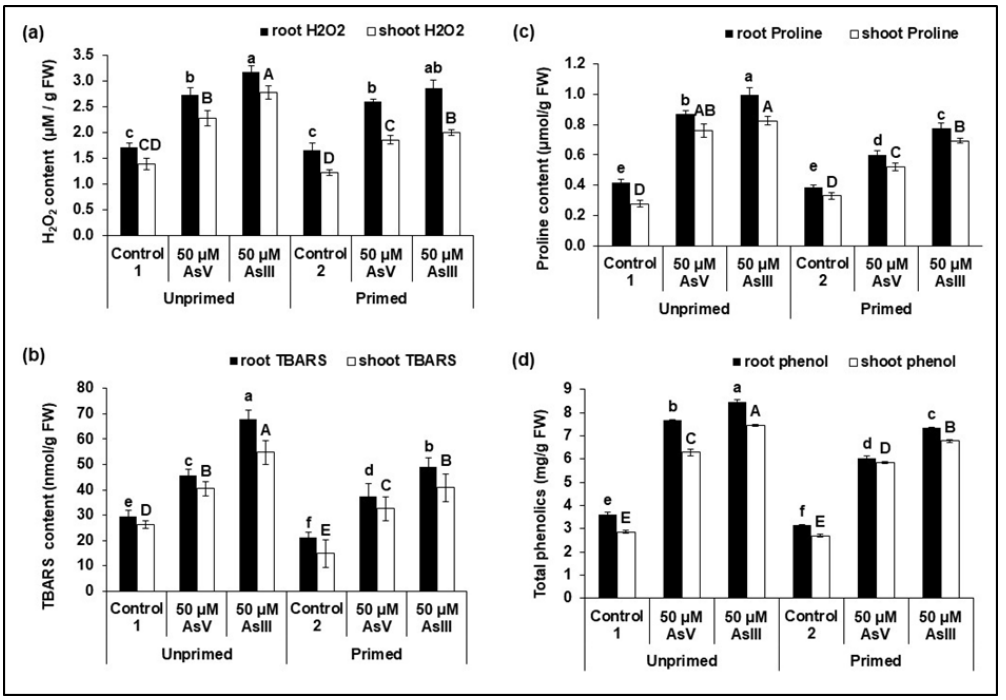


Fig. 4.1.2. Concentrations of (a) hydrogen peroxide (H_2O_2), (b) TBARS, (c) proline, and (d) total phenolics, obtained from various treatment groups. Each vertical bar with error bar represents mean ± standard deviation (n = 3). Different letters above the bars within each data series represent significantly different values (Tukey's HSD multiple comparison at $p \leq 0.05$)

GSH and GSSG content was significantly ($p \leq 0.05$) increased in root and shoot of unprimed seedlings under As stress (both AsIII and AsV) (**Fig. 4.1.3a-b**). K-humate priming reduced both GSH and GSSG content in root by 1.29 and 1.54 fold, respectively under As stress as compared to unprimed. Similarly, in shoot the GSH and GSSG content was reduced by 1.21 and 1.42 fold under As stress as compared to unprimed seedlings. However, GSH/GSSG ratio was significantly ($p \leq 0.05$) increased in primed seedlings than unprimed seedlings (**Fig. 4.1.3c**).

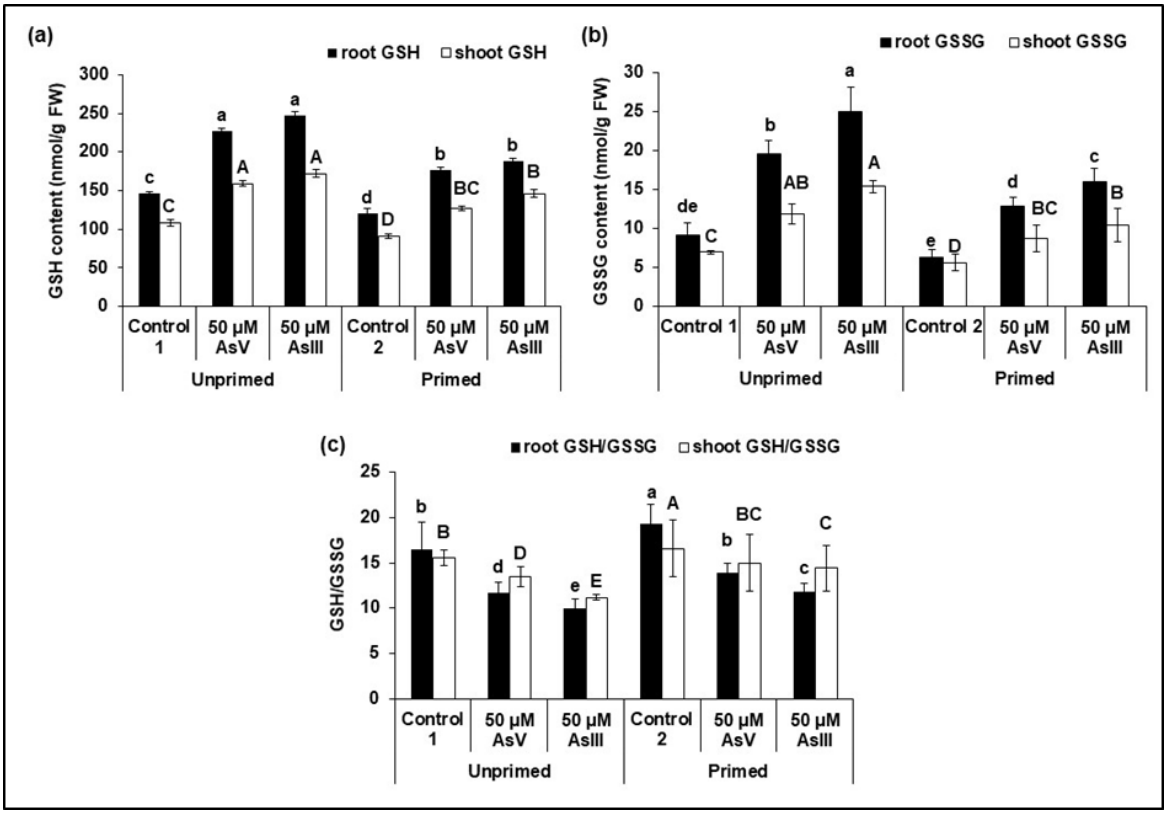


Fig. 4.1.3. Concentrations of (a) GSH, (b) GSSG and (c) GSH/GSSG ratios in 12 DAS seedlings (primed and unprimed), obtained from various treatment groups. Each vertical bar with error bar represents mean ± standard deviation (n = 3). Different letters above the bars within each data series represent significantly different values (Tukey's HSD multiple comparison at $p \leq 0.05$)

4.1.3.4. Effect of K-humate priming on antioxidant enzyme activity

The effect of As (AsV and AsIII) on CAT, SOD, APX and GR activity was measured in both primed and unprimed seedlings (12 DAS) (**Table 4.1.3**). The SOD activity in root of primed seedlings was significantly ($p \leq 0.05$) reduced by 1.13 and 1.10 fold, as compared to unprimed seedlings upon AsV and AsIII stress, respectively. The SOD activity in shoot of primed seedlings

was significantly ($p \leq 0.05$) reduced by 1.13 and 1.12 fold, as compared to unprimed seedlings, under AsV and AsIII stress, respectively.

APX activity was higher in roots than in shoots for all the treatments. The AOX activity was significantly ($p \leq 0.05$) reduced in roots and shoots of K-humate primed seedlings compared to corresponding unprimed samples. The AOX activity was considerably increased under AsIII stress as compared to AsV stress. In unprimed and primed seedlings, the mean AOX activity in seedlings increased under AsIII stress was 1.10 and 1.09 fold, respectively with respect to AsV stress. GR activity in root and shoot of unprimed seedlings was higher as compared to primed seedlings under AsV and AsIII stress. GR activity in root of primed seedlings was reduced by 1.09 and 1.21 fold, under AsV and AsIII stress, respectively. The CAT activity was not significantly changed ($p \leq 0.05$) upon As stress for any samples. However, the CAT activity in root and shoot of primed seedlings were reduced by 1.07 and 1.03 fold, respectively, as compared to unprimed seedlings, under AsV treatment. While, the CAT activity in root and shoot of primed seedlings were exhibited 1.05 and 1.01fold reduction, respectively as compared to unprimed seedlings under AsIII stress.

Table 4.1.3: Activity of Superoxide dismutase [SOD] (U mg^{-1} Protein), Ascorbate peroxidase [APX] (µmol $min^{-1}mg^{-1}$ protein), Catalase [CAT] (µmol min^{-1} mg^{-1} protein) and Glutathione reductase [GR] (U mg^{-1} Protein) in root and shoot of 12 DAS seedlings

Treatments	SOD		APX		CAT		GR	
	root	shoot	root	shoot	root	shoot	root	shoot
Control 1 (MS + Unprimed seed)	64.26±4.54cd	50.16±2.09b	1.25±0.03cd	0.63±0.04cd	82.55±4.18a	111.52±2.15a	0.57±0.02d	0.32±0.02c
50 µM AsV + Unprimed seed	80.84±5.05ab	56.03±1.43ab	1.56±0.10ab	0.85±0.04ab	84.3±4.26a	116.66±1.9a	0.70±0.02b	0.42±0.02b
50 µM AsIII + Unprimed seed	85.91±1.52a	59.94±2.14a	1.72±0.10a	0.94±0.10a	82.13±4.00a	113.32±2.3a	0.79±0.03a	0.47±0.04a
Control 2 (MS + Primed seed)	57.42±3.11d	45.00±1.93c	1.07±0.11d	0.50±0.03d	75.92±2.31a	104.47±2.23b	0.47±0.01e	0.25±0.02d
50 µM AsV + Primed seed	71.47±2.92bc	49.48±1.34bc	1.37±0.07bc	0.68±0.06bc	78.11±2.18a	112.40±1.66a	0.64±0.01dc	0.35±0.01c
50 µM AsIII + Primed seed	77.53±3.16ab	53.24±1.47b	1.43±0.07bc	0.78±0.06abc	78.12±2.21a	112.93±2.24a	0.65±0.02c	0.40±0.02bc

The same letters with in the same column indicate mean ± standard deviation (n = 3) values are not significantly different at $P \leq 0.05$ using Tukey's HSD multiple comparison test

4.1.3.5. Effect of K-humate priming on total arsenic content and translocation

Arsenic (both AsV and AsIII) exposure of rice seedlings caused greater accumulation ($p \leq 0.05$) primarily in root area for all treatments. The presence of As in both the control sets were minimal, whereas the accumulation of As was significantly higher ($p \leq 0.05$) in root and shoot for both primed and unprimed seedlings (**Fig. 4.1.4a**). The accumulation of As was higher by 1.61 and 1.72

fold in unprimed seedlings compared to primed seedlings under AsV and AsIII stress, respectively. Arsenic content in seedlings grown in AsIII stress was 20.7% and 12.7% higher than As V stress for unprimed and primed seedlings, respectively. Translocation of As from root to shoot was also high for unprimed seedlings grown in AsV and AsIII stress, respectively as compared to primed (**Fig. 4.1.4b**). The translocation of As was reduced to 25.4% and 8.33% for K-humate primed seedlings as compared to unprimed seedlings, under AsV and AsIII stress, respectively. However, the value of As translocation from root to shoot was found to be lesser than one in all treatments.

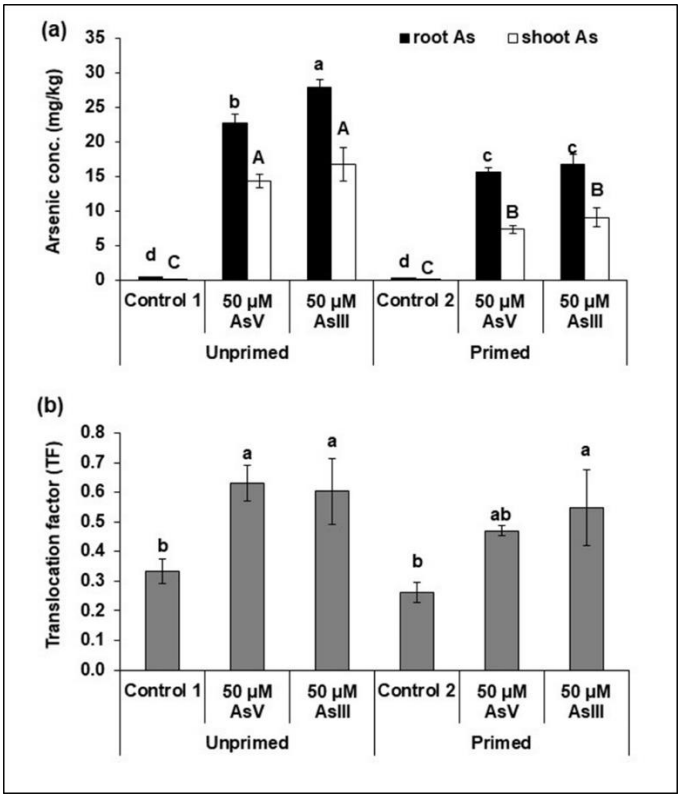


Fig. 4.1.4. Variations in (a) As contents in root and shoot and (b) As translocation (TF), in both primed and unprimed seedlings after 12 DAS, obtained from various treatment groups. Each vertical bar with error bar represents mean ± standard deviation (n = 3). Different letters above the bars within each data series represent significantly different values (Tukey's HSD multiple comparison at $p \leq 0.05$)

4.1.4. Discussion

Humic substances are most important part of soil organic matter that present in different molecular size and generally play a crucial role in the environment. However, the structure and size of humic substances are influenced by the pH, ionic strength and mineral composition of the solution (Durce

et al., 2016). However, it has been reported that different functional groups present in humic substances are accountable for direct and indirect effects on growth and nutrition of plants (Zanin et al., 2019). Humic acid is well known to increase the plant growth and yield by enabling the roots to absorb more nutrients and vitamin from soil in stressed condition, and also by increasing water holding capacity, nutrient availability and hormonal activity (Hasanuzzaman et al., 2018; Taha and Osman, 2018).

The results showed that the As application reduced the germination percentage, seedling growth as compared to control sets. This result was well matched with the observations of Shri et al. (2009). The germination and seedling vigour of rice was more reduced by AsIII stress as compared to AsV and this observation was also supported by the high phytotoxicity of AsIII (Moulick et al., 2016). The application of K-humate priming significantly enhances the germination percentage and seedling vigour of rice seeds under As stress as both humic acid and potassium are known to regulate abscisic acid and gibberellin hormones (Hasanuzzaman et al., 2018). Thus, priming with humic acid may lead to increase germination percentage and seedling vigour in rice, under abiotic stress (nano-ZnO) (Sheteiwy et al., 2017a).

Metalloids like As creates osmotic stress in plants and to measure the plant tolerance to osmotic stress, relative water content (RWC) in leaf was monitored. The present study revealed that As stress significantly decreases the RWC content (Table 4.1.2), this observation has been consistent with the findings of Hasanuzzaman and Fujita (2013). However, K-humate application increased a certain amount of RWC of leaves under As stressed condition, as K-salt helps to maintain turgidity by minimizing water loss under stress condition (Hasanuzzaman et al., 2018). Chlorophyll contents in rice seedlings were also deteriorated by As exposure. One of the reason that affected the chlorophyll content in As (AsV and AsIII) stress was the generation of reactive oxygen species (ROS) under stressed condition (de Andrade et al., 2015). The K-humate treatment increase the photosynthetic activity of chlorophyll content was increased, and the ratio between chlorophylls a and b was also improved compared to unprimed seedlings (Table 4.1.2). An earlier study observed that humic acid treatment increased the chlorophyll production rate in plants under drought condition (Kıran et al., 2019).

Uptake of toxic metals through root into plant body caused the accumulation of ROS (H_2O_2) and this oxidative stress strengthens the antioxidant defense systems. The addition of As in the growth medium consistently elevated the levels of H_2O_2 and TBARS in rice seedlings (Fig. 4.1.2a,b) and

the results were consistent with the observation of Majumder et al. (2020). The higher ROS accumulation in unprimed seedlings significantly increased GSH and GSSG contents. The lower accumulation of ROS in K-humate primed seedlings decreased the amount of GSH and GSSG contents. Since, K-humate acts as a stimulator of antioxidant enzyme during stress condition and reduces the ROS mediated damage in plant (Hasanuzzaman et al., 2018; Kaya et al., 2018). Thus, priming with K-humate significantly reduced the lipid peroxidation and helped to improve the plant physiology under As stress. The application of humic acid also reduced the level of lipid peroxidation and subsequent GSH and GSSG content in plants (Kiran et al., 2019; Sheteiwy et al., 2017a). Proline is basically an important osmolyte that helps to reduces the membrane damage caused by oxidative stress; protects protein against denaturation and water balance under As stress (Schat et al., 1997). The proline content in K-humate primed seedlings was comparatively lower than unprimed seedlings. Bakry et al. (2014) observed that the exogenous application of humic acid reduced the proline level under stress condition. Phenolic compounds act as an antioxidant under abiotic stress and As stress increased the phenolic content in rice seedlings. Elguera et al. (2013) also observed the increase phenolic content in *Lepidium sativum* when exposed to cadmium and selenium. Whereas, the supplementation of K-humate through seed priming resulting in effective reduction in the total phenolics indicates the role of K-humate in vitiate the efficiency of As stress.

In addition to oxidative stress marker and non-enzymatic antioxidant, the antioxidant enzymes (SOD, CAT, APX and GR) are the main enzymatic components to down-regulate the oxidative stress. The intracellular superoxide (O_2^{*-}) radical was primarily converted into H_2O_2 by SOD. The elevated level of SOD with AsIII and AsV exposure ultimately leads to the higher accumulation in H_2O_2. Singh et al. (2018) reported high SOD activity in rice seedlings when exposed to As. However, SOD activity in primed seedlings was reduced compared to unprimed seedlings (Table 4.1.3). These findings indicate that humic acid and potassium ameliorated the toxicity of heavy metal (Haghighi et al., 2010; Siddiqui et al., 2012). The H_2O_2 produced from superoxide detoxification was further degraded into water molecule by APX and CAT (Kalita et al., 2018). In unprimed seedlings, As induced oxidative stress generated high amounts of H_2O_2. To mitigate that amount of stress, greater activity of APX and CAT was observed in root and shoot of unprimed seedlings as compared to K-humate primed seedlings (Table 3). Sheteiwy et al. (2017a) reported that humic acid priming significantly improved APX activity which assisted in degradation of both superoxide (O_2^{*-}) and peroxide (H_2O_2), and thereby the rice plant became more tolerant to nano-

ZnO stress. GR plays a pivotal role in scavenging H_2O_2 by maintaining a favorable ratio of GSH/GSSG. GR converts GSSG to GSH and as a result, As stress causes significant accumulation of GSH in unprimed seedlings which is compatible with result reported by Hasanuzzaman and Fujita (2013).

All the above discussed oxidative stress and antioxidant activity were orchestrating by the amount of As taken up by the plant and it was evident that As content in root was higher than shoot (Fig. 4.1.4a and 4.1.4b) being an agreement with the findings of Shri et al. (2009). The priming with K-humate significantly reduced the accumulation of As in root and shoot, and this reduction in As content indicated the metal chelation property of humate, though detailed interaction mechanism between As species and K-humate is still unclear. The calculated TF < 1 suggested that least amount of As was translocated from root to shoot. This may possibly happen if translocated As from root tissue to shoot is sequestrated into the vacuole with the help of phytochelatins (Tuli et al., 2010).

The experimental results were further justified by principal component analysis (PCA) (Fig. 4.1.5). On performing PCA using ten root variables, TF and RWC, the 12 loading vectors formed two clusters on diametrically opposite sides of the hyperplane defined by PC1 and PC2, which jointly accounted for 72.4% of the total variance among these variables. One cluster consisted of loading vectors of RWC and root length, and was closely associated with scores for control 2 samples. The other cluster consisted of all other root variables and TF, and was closely associated with 50 μM AsIII + unprimed seed samples. In intermediate positions between these two extremes, scores of 50 μM AsV + primed seed and 50 μM AsIII + primed seed samples clustered together, while control 1 sample scores were positioned separately but close to control 2 scores. On performing PCA using ten shoot variables, germination % and total chlorophyll content (total Chl.), these 12 loading vectors also formed two clusters on diametrically opposite sides of the hyperplane defined by PC1 and PC2, which jointly accounted for 72.3% of the total variance. One cluster consisted of loading vectors of germination %, total Chl. and shoot length, and was closely associated with scores for control 2 samples. The other cluster consisted of all other shoot variables, and was closely associated with 50 μM AsIII + unprimed seed and 50 μM AsV + unprimed seed samples. Scores of 50 μM AsV + primed seed and 50 μM AsIII + primed seed samples almost overlapped those of corresponding unprimed seed samples, while control 1 sample scores were positioned close to but separately from control 2 scores.

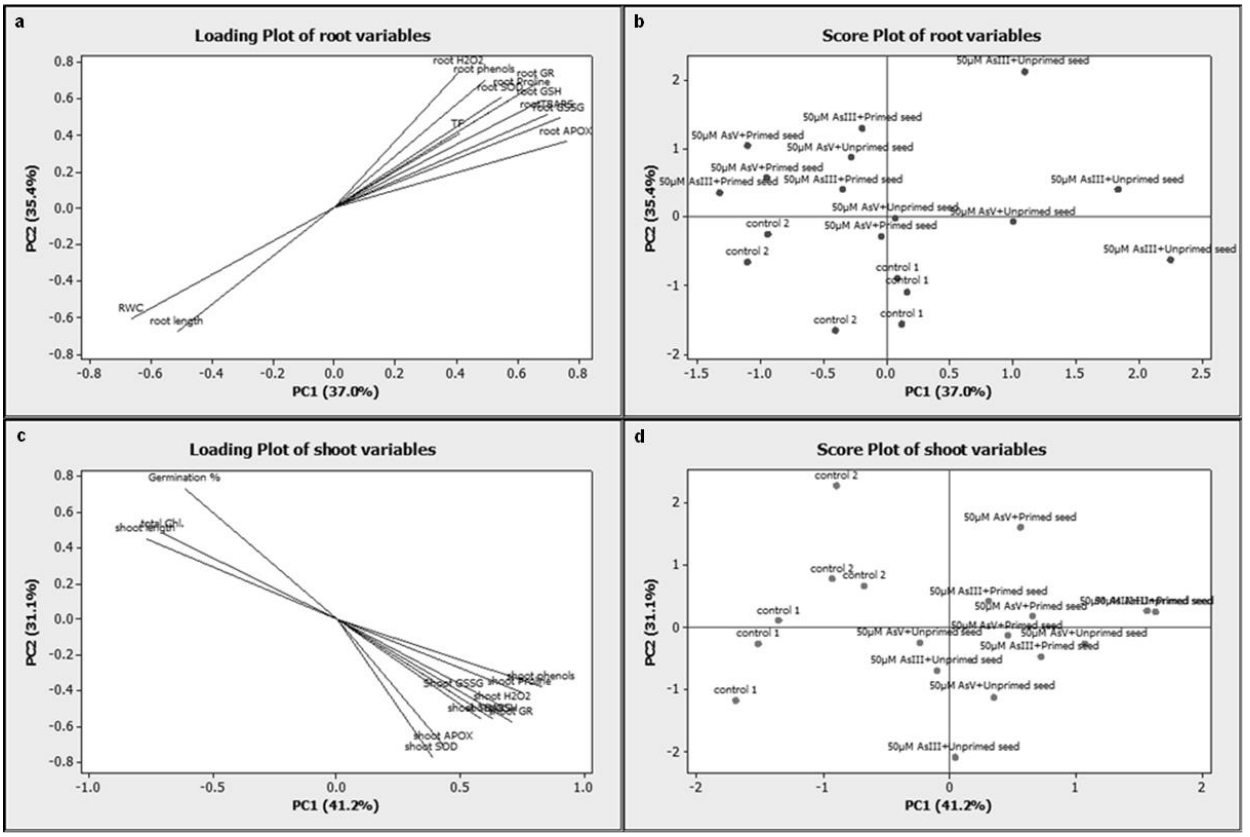


Fig. 4.1.5. PCA plots: (a) loadings plot using all root variables, RWC and TF; (b) scores plot using all root variables, RWC and TF; (c) loadings plot using all shoot variables, total chlorophyll and germination %; (d) scores plot using all shoot variables, total chlorophyll and germination %

In both PCA results, scores of the control 2 samples, characterized by As-free and primed seedlings, closely associated with variable loadings with higher values in healthy plants (e.g. germination % and RWC), were clustered farthest from scores of 50 μM AsIII + unprimed seed samples, which in turn were juxtaposed closely with stress-markers such as antioxidant variables (e.g. SOD, GSH) and TF. These two sample sets therefore established the limits on experimental data. That AsIII toxicity on unprimed seedlings exhibited extreme values of stress parameters was already apparent, even compared to AsV stressed seedlings. The lower toxicity of AsV combined with priming led to almost stress-free conditions in 50 μM AsV + primed seed samples, whose scores were located closer to control 2 samples than 50 μM AsV + unprimed seed sample scores, the latter overlapping with scores of 50 μM AsIII + primed seed samples. Since AsV ions are detoxified faster than AsIII ions, the differences in physiological status would be lesser between 50 μM AsV + unprimed seed and 50 μM AsV + primed seed samples, than between 50 μM AsIII + unprimed seed and 50 μM AsIII + primed seed samples. Similarly, the significantly different scores between control 1 and control 2 samples even in absence of As stress in both cases may be

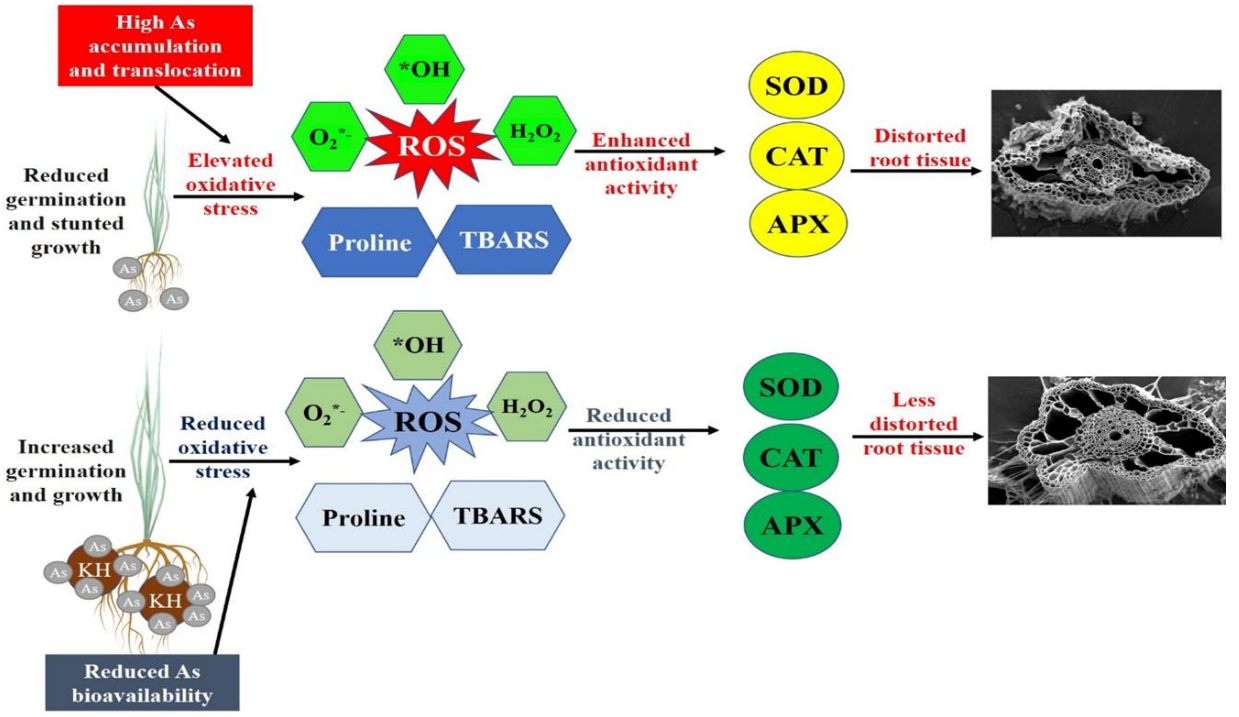
explained by improved physiological status of control 2 seedlings due to K-humate priming. Similar findings were also presented by Majumder et al. (2020). Finally, all the findings and their comparative evolution with other relevant works suggest that K-humate priming improved the germination and seedling growth of seedlings under As stress. K-humate also improved the oxidative stress and antioxidant activity of primed seedlings. K-humate also reduced bioavailability and translocation of trace elements like As.

4.1.5. Conclusion

This study demonstrated the use of K-humate as priming agent for rice seeds to mitigate As stress. K-humate priming helped to reverse the physiological effects of As induced toxicity and hence promoted seed germination and enhance plant vigour under As stress. It also improved the chlorophyll content, and antioxidant defense system in the germinated seedlings, though amelioration of toxic effects of As ions would cancel out the need for higher antioxidant activity in primed seedlings. However, these observations were carried out till the seedlings were 12 DAS old, and whether the same trends would continue with further growth and maturity of the crop, or might require additional, intermittent or enhanced doses of the priming agent is open to further investigation. Moreover, the results of the current study confirmed that AsIII stress has more inhibitory effect on rice growth and physiology as compared to AsV stress, as well, is also more mitigable with K-humate priming. Thus, seed priming with K-humate has future prospects in improvement of rice production by minimizing the detrimental impacts of As toxicity.

4.2. K-humate used in plant growth medium

Graphical Abstract



4.2.1. Introduction

Arsenic a priority toxic element, mainly geogenic in nature, polluting groundwater in various parts of the world is becoming a growing concern. Groundwater serves as the major source of drinking water and is also utilised to irrigate agricultural lands. Most agricultural lands in South-East Asia are As laden, and a significant amount of As enters through irrigation water. Rice is the staple food in South-East Asian countries, and a large amount of it is still cultivated in As laden fields, especially in countries like India, China and Bangladesh. In this way, As enters the human and animal food chain (Ahmed et al., 2016; Chowdhury et al., 2020; Das et al., 2021). Rice seeds and seedlings are very much vulnerable to As toxicity during their early stage of growth. Arsenic stress in plants reduce root and shoot growth and also decreases plant biomass (Du et al., 2017). Germination of rice seeds, seedling vigour and chlorophyll content are severely impaired due to exposure of As (Abbas et al., 2018). Furthermore, as the As accumulates inside the plant, it causes oxidative stress. (Kalita et al., 2018; Muehe et al., 2019).

Humic acid is known to mitigate abiotic stress by stimulating antioxidant defence mechanism to inhibit ROS generation (García et al., 2012). Humic acid also increases photosynthesis in plants, thus helping in plant growth and development (van Tol de Castro et al., 2021). Plant growth hormone like activities are demonstrated by humic substances that help in root growth (Yang et al., 2021). Humic acid helps to regulate the bioavailability and mobility of As by forming ternary complex with As and other metals like iron (Liu et al., 2011). Moreover, humic acid also consists of an aromatic core and functional groups like carboxyl, alcohol, acetal, alkyl radicals, carbohydrates etc. through which As can bind to form organometallic complexes (Reza et al., 2012; Tikhova et al., 2001). Humic acid in dissolved state can form complexes with As species with the help of carboxylic, phenolic and -N functional groups (Gong et al., 2020). The extent of complexation of As with humic acid increases with increasing humic acid concentration (Fakour and Lin, 2014). With the increase in concentration of humate more sites are available on which As species can bind (Costa et al., 2022). This ultimately helps in reducing the bioavailability of As.

Arsenic accumulation in plants also depends on the availability of essential nutrients. Potassium (K) is an essential nutrient which aids plants in photosynthesis, antioxidant enzyme proliferation and root growth (Wang et al., 2013). Moreover, ROS generation under abiotic stress can cause K^+ efflux from the roots, which can be compensated by providing an external source of K (Kumar et al., 2020).

Potassium salt of humic acid (KH) is effective in increasing the yield and fibre quality of cotton (Ullah et al., 2020). KH was used previously to adsorb Cd, Cu, Pd and Zn from wastewater (Havelcová et al., 2009; Shaheen et al., 2013). KH had also been reported to increase germination and chlorophyll content under water-stressed conditions (El-Nwehy et al., 2020). In an earlier study, KH application was found to increase yield and growth of bean plants under salinity stress (Taha and Osman, 2018). Numerous articles have explored the role of humic substances in alleviation of abiotic stress in plants (Liu et al., 2011; van Tol de Castro et al., 2021). However, the potential of KH in mitigation of As toxicity in plants has not yet been explored substantially. The only reported literature in this territory investigates the effect of rice seed priming using KH under As stress. This study hypothesises that hydroponic KH application reduces As toxicity in rice plants by promoting plant development through its growth hormone like activities, delivering adequate nutrition, reducing As bioavailability through complexation, and thereby reducing oxidative stress. To explore the potential of KH in As toxicity alleviation from plants, this study aims to investigate the role of KH in (1) seed germination, seedling growth, (2) oxidative and antioxidant stress management, and (3) As uptake and translocation under As stress.

4.2.2. Materials and methods

4.2.2.1. Experimental design and treatments

A 14-day long experiment was carried out with rice seeds (*Oryza sativa* L.) of variety 'Narendra'. The rice seeds for this experiment were collected from the As-affected Gaighata block of West Bengal, India (22.4629° N, 88.3968° E) (Das et al., 2021) where this rice variety (Narendra) is being cultivated. The seeds were washed a few times with double distilled water and surface sterilized using 10% H_2O_2. Finally, the seeds were repeatedly washed with double distilled water. The washed seeds were soaked in double distilled water and shaken overnight on a mechanical shaker to make them fit for fast germination. The water is then decanted and the seeds were surface soaked using two layers of absorbent paper and kept in the dark inside a zipper pouch for 24 h. Sodium arsenite ($NaAsO_2$, MW: 129.91 g mol^{-1}) salt was used for applying As (800 ppb) stress. The As concentration was chosen as per groundwater As contamination levels reported in India in previous studies (Abhinav et al., 2016; Purkait et al., 2008). The KH with 95% solubility, used in this experiment was procured from Disha Agrotech, West Bengal, India, containing 70% humic acid and 10% potassium. Hoagland's No. 2 basal salt mixture (TS1094), obtained from Himedia was used as nutrient medium in hydroponic system and the media were changed thrice a week

during the course of the experiment. Total five treatments were included in this experiment and all the treatments were set up in triplicate. In each treatment 20 rice seeds were placed for germination. The five treatments were: (a) 800 ppb As, (b) 800 ppb As + 25 ppm KH, (c) 800 ppb As + 50 ppm KH, (d) 800 ppb As + 75 ppm KH and (e) 800 ppb As + 100 ppm KH.

4.2.2.2. Evaluation of seed germination, seedling growth and vigour

All the treatments were kept in the dark for the first three days at 27 ± 1 °C and 70% relative humidity, until most of the seeds germinated and had a radicle length greater than 2 mm. In such a condition the germination percentage of the rice seeds on 3 DAS in each treatment was determined. The germinated seeds were kept in a growth chamber with a light: dark photoperiod of 14:10 h, at 27 ± 1 °C for the next 11 days. The light source had a photon flux density (PFD) of 270 ± 5 μmol $m^{-2}s^{-1}$. The seedlings' shoot and root length were measured from the shoot-root junction to the tip of the longest leaf and tip of the primary root, respectively. The fresh weights (FW) of the seedlings were taken. Individual dry weights (DW) of the seedling were taken after drying the samples overnight in a hot air oven at 60 °C. The seedling vigour indices were determined as per formulae prescribed by Abdul-Baki and Anderson (1973).

4.2.2.3. Determination of relative water content (RWC)

Fresh leaf laminae were weighed separately, and placed in separate petri dishes containing water. The petri dishes were then kept at room temperature in the dark inside a BOD chamber for 24 h and the turgid weight (TW) of the samples were taken after soaking off the excess surface water. The dry weights (DW) were taken after drying the samples at 80 °C for 24 h. The RWC was calculated according to Barrs and Weatherley (1962).

$$RWC = \frac{FW - DW}{TW - DW} \times 100$$

4.2.2.4. Determination of percentage electrolyte leakage (EL)

The percentage of EL was determined according to Cao et al. (2007). Briefly 500 mg fresh leaf tissues were segmented into pieces and vacuumed in 20 mL deionized water for 20 mins. The leaves were kept in the water at room temperature for another 2 h and the conductivities (C_1) were measured. Further the leaf samples were boiled in the deionized water for 15 mins and the conductivities were measured once again (C_2). The percentage ELs were calculated according to the following formula.

$$EL = \frac{c_1}{c_2} \times 100$$

4.2.2.5. Determination of chlorophyll content

Chlorophyll a and b were measured spectrophotometrically at 663 and 645 nm, respectively by using the protocol suggested by Arnon (1949) after extracting the chlorophyll of about 0.1 g of 14 DAS old leaf tissue with 5 mL of 80% acetone.

4.2.2.6. Measurement of ROS in root tips

For measurement of ROS, root tips of different treatments were submerged in 1 mL detection buffer (DB), DB (2.5 mM HEPES, pH 7.4) consisting of 10 mM DCF-2DA fluorescent dye (Invitrogen, Carlsbad, CA, USA). Following this the samples were incubated in the dark for 10 minutes to measure ROS (Sarkar et al., 2020) using life technologies' Floid Cell Imaging station microscope.

4.2.2.7. Determination of H_2O_2 content

Root and shoot H_2O_2 contents were determined in accordance with Velikova et al. (2000). The homogenates derived by homogenizing 1 g root and shoot tissues in 5 mL, 0.1% w/v trichloroacetic acid (TCA), were centrifuged. The supernatants were mixed with 0.05 M sodium phosphate (Na-P) buffer solution (pH 7.0) and 1 ml of 1 M KI solution. The absorbance of the reaction mixture was taken at 390 nm and the H_2O_2 contents (µmol g^{-1}) were calculated by taking the molar extinction coefficient as 0.28 $\mu M^{-1}cm^{-1}$.

4.2.2.8. Determination of lipid peroxidation

The TBARS (thiobarbituric acid reactive substances) assay was performed according to Heath and Packer (1968) to get an account of lipid peroxidation in roots and shoots. Briefly, 0.1 g of root and shoot tissues were separately homogenized in 1 mL of 0.1% TCA solution. After centrifugation of the extracts, 1 mL of the supernatants was added to 4 mL of 0.5% TBA in 20% TCA. The mixture solutions were kept at 95 °C for 25 mins. They were immediately transferred to an ice bath and kept for 5 mins. The absorbance was measured at 532 nm considering the reference point at 600 nm.

4.2.2.9. Determination of proline content

The concentration of proline in roots and shoots of 14 DAS old seedlings were determined according to Bates et al. (1973). The homogenates of 0.1 g of root and shoot tissues in 2 mL of 3% sulphosalicylic acid were centrifuged. The supernatants were mixed with 2 mL each of acid ninhydrin and glacial acetic acid. The reaction mixtures were incubated at 100 °C for 1 h. The samples were immediately transferred to an ice bath. For extraction of proline, toluene was added to the mixtures followed by vortexing. The aqueous phase consisting of chromophore containing toluene was drawn out and absorbance was measured at 520 nm. The proline concentration of the fresh tissues was measured relative to the standard curve.

4.2.2.10. Determination of antioxidant enzyme activities

The antioxidant enzyme assays were performed by homogenizing 0.2 g of root and shoot tissues of 14 DAS rice seedlings in 2 ml of chilled 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% (w/v) Polyvinylpyrrolidone (PVP). The homogenizations were done in a pre-chilled mortar and pestle. Protein content of antioxidant enzyme extracts was determined according to Lowry et al. (1951). Superoxide dismutase (SOD; EC 1.15.1.1), activities were measured by taking into account the disappearance of nitroblue tetrazolium, as prescribed by Beauchamp and Fridovich (1971). For ascorbate peroxidase (APX; EC 1.11.1.11) assay, the H_2O_2 dependent ascorbic acid (ϵ = 2.8 mM^{-1} cm^{-1}) oxidation was taken into account by measuring the declining absorbance for 1 min at 290 nm, as reported by Nakano and Asada (1981). For determination of catalase (CAT; EC 1.11.1.6) activity, the supernatants (0.1 mL each) were mixed with 1.5 ml of 50 mM phosphate buffer followed by 15 mM H_2O_2. After an incubation period of 1 min, the continuously decreasing absorbance of the mixture was recorded every 1 min at 240 nm as put forward by Aebi (1984).

4.2.2.11. Determination of K content in plant seedlings

For estimation of K content in seedling, root and shoot 14 DAS seedlings were oven dried for 72 h. The dried root and shoot were digested in a triacid mixture (HNO_3: $HClO_4$: H_2SO_4 = 5: 2: 1). After completion of acid digestion, volume make up was done with distilled water and filtered through whatman no. 42. The amount of K content was determined using Flame Photometry method (Piper, 1966).

4.2.2.12. Determination of total arsenic in plant seedlings

Roots and shoots of 14 DAS old rice seedlings were digested using Teflon Bomb digestion method, detailed description of which is provided in Chowdhury et al. (2020). Total As content of the roots and shoots were estimated using Hydride Generation-Atomic Absorption Spectrophotometry (HG-AAS; Varian Model AA140, USA) coupled with VGA-77 (Vapour Generation Accessory, Agilent technologies, Malaysia) and enabled with software version 5.1. The instrumentation and optimized conditions of the aforementioned method were mentioned earlier in Chowdhury et al. (2020) and De et al. (2021).

4.2.2.13. Quality control and quality assurance

Standard reference materials (SRMs) and spiked samples were analysed for recovery of As and K to maintain the quality control and quality assurance. The SRM samples employed for this purpose were Rice flour 1568a (NIST, Gaithersburg, MD, USA) and Tomato leaf 1573a (NIST, Gaithersburg, MD, USA). Teflon bomb digestion of the samples followed by As analysis showed 97.8 ± 0.7% and 94.9 ± 1.6% recovery, against their certified values of 0.29 ± 0.03 and 0.1126 ± 0.0014 mg/kg, respectively. The recoveries of K from digested SRM samples were 96.3 ± 0.5% and 94.5 ± 0.2%, respectively against their certified values of 1282 ± 11 and 26760 ± 480 mg/kg, respectively.

4.2.2.14. Determination of arsenic translocation factor (TF)

The translocation of As from root to the shoot is estimated by the following formula, as mentioned in Chowdhury et al. (2020):

$$TF = \frac{As\ concentration\ in\ shoot}{As\ concentration\ in\ root}$$

TF value greater than 1 suggests that the plant translocates the metal successfully from the root to the shoot.

4.2.2.15. Study of morphological changes using SEM-EDX

Transverse sections of 14 DAS old seedling roots were cut and kept in 2.5% glutaraldehyde solution for 2 h followed by dehydration through a series of different concentrations of ethanol (10%, 20%, 30%, 40%, 50%, 60%, and 70%). The dehydrated samples were sputter coated with

platinum prior to observation under SEM (Carl Zeiss Model Number – EVO18 Special Edition). Energy Dispersive X-Ray Analysis (EDX) (SmartEDX, Carl Zeiss) was performed to observe the elemental content of elements present on the root cross sections.

4.2.2.16. Data analysis and statistical inferences

All the triplicate treatment results were demonstrated as average value ± standard deviation (SD) and were subjected to one-way analysis of variance (ANOVA) in OriginPro 2019b (Origin lab corporation) software. The relationship between the data was established by Tukey-HSD test at $p \leq 0.05$, significance level.

4.2.3. Results

4.2.3.1. Variation in seed germination, seedling growth and vigour upon K-humate application on As stressed plants

The lowest germination percentage (%) was recorded in the control treatment. A significant ($p \leq 0.05$) gradual increase was observed with increasing concentrations of KH treatment. The highest increase in germination percentage was recorded to be 36.3% in the 100 ppm KH treated plants compared to the control. (**Fig. 4.2.1; Table 4.2.1**).

The control treatment also had the least development of root and shoot. . A significant gradual increase in root and shoot growth was observed with increasing concentrations of KH ($p \leq 0.05$). Upon comparing the root and shoot growths of 25 ppm humate treated plants with that of control, nearly a 3-fold increase ($p \leq 0.05$) in root growth and a 2-fold increase in shoot growth were observed (Table 1). The fresh weight of the plants also increased along with increasing of KH concentration (Table 1).

A significant enhancement ($p \leq 0.05$) both in vigour indices I and II were observed upon KH treatment. Seedlings treated with 25 ppm KH showed an increase of 145% in vigour index I compared to the control. A similar significant trend ($p \leq 0.05$) was also observed in vigour index II.

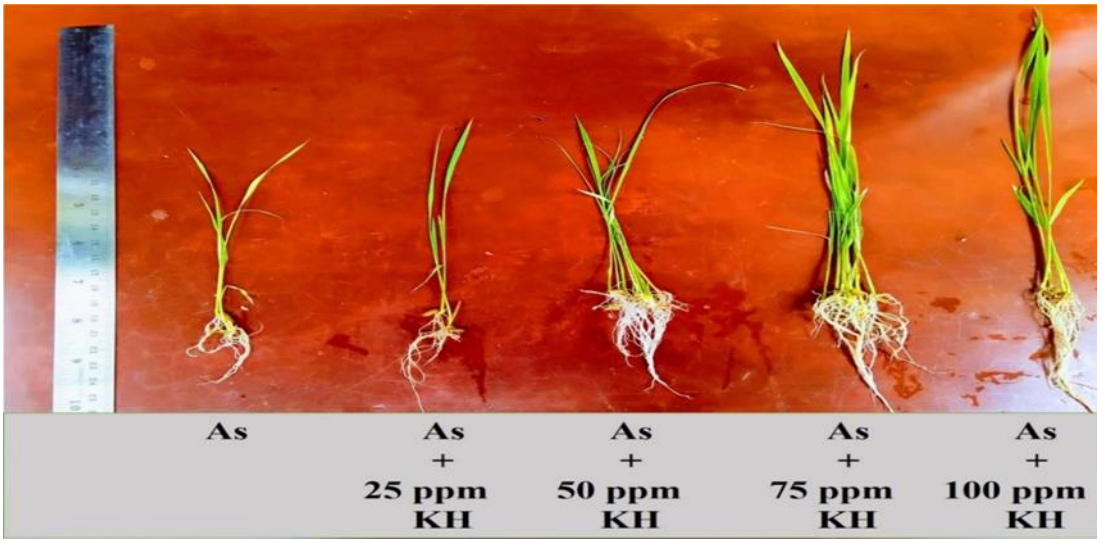


Fig. 4.2.1. Result of KH treatment in growth of seedlings under As at 14 DAS

Table 4.2.1: Variation in germination percentage, seedling length (root and shoot), fresh weight and vigour indices of 14 DAS rice seedlings

Treatments	Germination Percentage (%)	Root (cm)	Shoot (cm)	Fresh Weight (mg)	Vigour Index I	Vigour Index II
As	59.06±9.00a	1.53±0.50a	7.56±0.51a	37.3±8.21a	541.17±124.17a	0.62±0.14a
As + 25 ppm KH	68.50±6.76a	4.56±0.45b	14.83±0.56b	56.6±5.89ab	1328.40±143.05b	1.17±0.10b
As + 50 ppm KH	86.43±5.66b	6.93±0.30c	16.33±0.35c	66.86±8.39bc	2010.06±109.83c	1.60±0.12c
As + 75 ppm KH	94±2.00b	7.96±0.45cd	18.63±0.35d	86.33±5.98cd	2536.40±144.69d	1.83±0.11cd
As + 100 ppm KH	95.43±2.89b	8.26±0.30d	19.26±0.31d	104.53±18.26d	2665.50±152.72d	2.05±0.10d

The same letters in a column indicate that the values are not significantly different at $p \leq 0.05$ using Tukey's HSD multiple comparison test

4.2.3.2. Variation in RWC, EL and chlorophyll content upon KH application on arsenic stressed plants

RWC was higher in KH treated seedlings compared to the control. The RWC of rice seedlings increased significantly with increasing KH concentrations, ranging from 13% to 20.4% when compared to the control. **(Table 4.2.2)**.

The percentage EL of the leaf tissues of the plant showed a decreasing trend as the concentration of KH increased. The highest leakage percentage was observed in the control, while the lowest was observed in the 100 ppm KH treated plants.

Similar increasing trend was also observed in total chlorophyll content of the seedlings. A significant increase (27.6%; $p \leq 0.05$) in total chlorophyll was observed in the 25 ppm KH treatment compared to the control. The highest concentration of chlorophyll was observed in the 100 ppm KH treated seedlings.

Table 4.2.2: Variation in relative water content (RWC), electrolyte leakage (EL), and chlorophyll content upon hydroponic administration of KH

Treatments	RWC (%)	EL (%)	Total chlorophyll (mg/g FW)	Chl. a/Chl. b
As	80.05±0.73a	95.79±0.90a	0.87±0.01a	3.34±1.82a
As + 25 ppm KH	90.47±0.35b	94.1±0.93ab	1.11±0.07b	3.22±0.89a
As + 50 ppm KH	95.81±0.54c	92.17±0.79b	1.44±0.23c	2.26±0.17a
As + 75 ppm KH	96.05±0.27c	88.74±0.95c	2.11±0.12cd	2.15±0.14a
As + 100 ppm KH	96.39±0.54c	84.10±0.98d	2.43±0.10d	1.89±0.06a

The same letters in a column indicate that the values are not significantly different at $p \leq 0.05$ using Tukey's HSD multiple comparison test

4.2.3.3. Variation in concentrations of oxidative stress markers and antioxidant upon KH application on As stressed plants

CM-H2DCFDA staining of seedling roots provides a qualitative estimate of ROS. The As induced toxicity significantly increased the intensity of green fluorescent which indicate greater amount of ROS production in control seedlings (**Fig. 4.2.2a**). However, in vivo ROS measurement revealed gradual increase in KH concentration reduced the intensity of green fluorescence (**Fig. 4.2.2b-e**). H_2O_2 levels were also observed to attain the highest values in the control seedlings. The levels saw a gradual fall with progressive increase in KH concentration (**Fig. 4.2.3a**). The H_2O_2 levels in the roots were somewhat greater than that in the shoots.

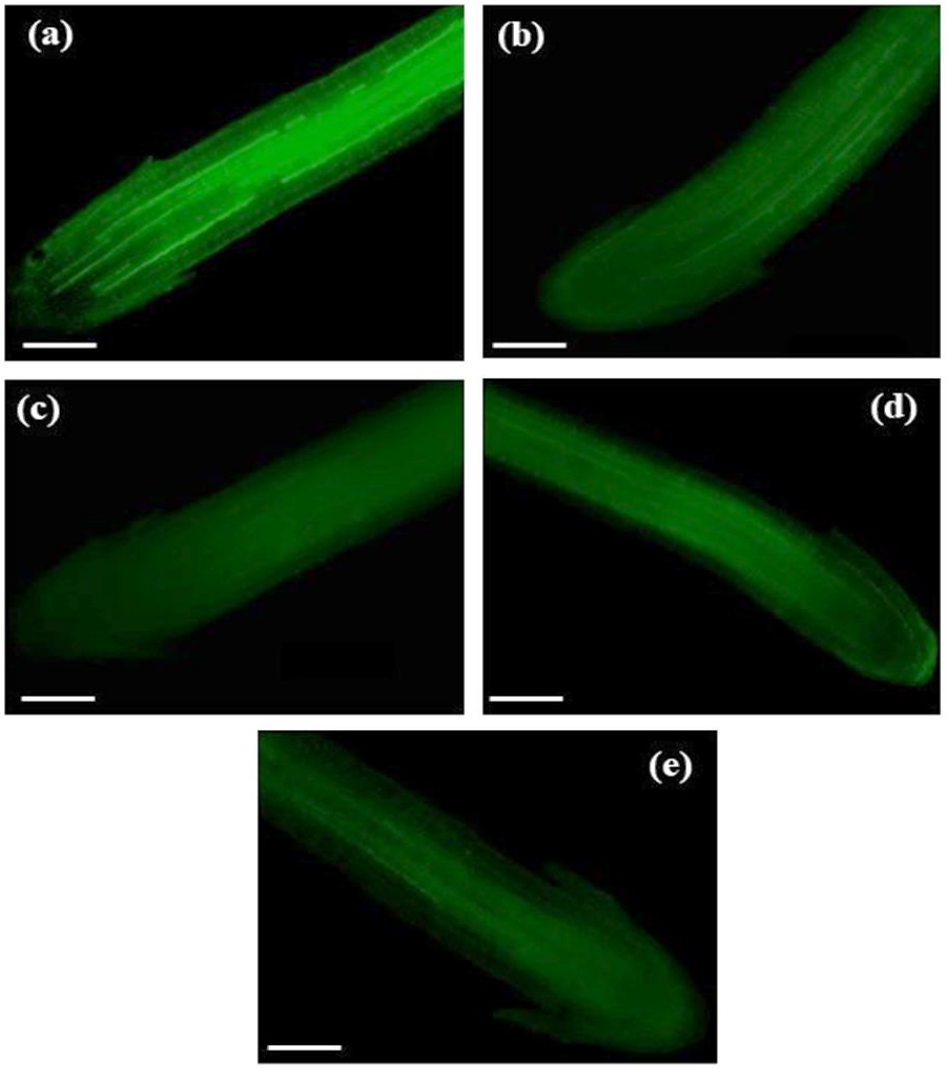


Fig. 4.2.2. Variation in ROS generation in (a) As, (b) As + 25 ppm KH, (c) As + 50 ppm KH, (d) As +75 ppm KH, (e) As +100 ppm KH observed under confocal microscope with DCF staining. The white bars indicate a scale of 100 µm

The TBARS levels were significantly ($p \leq 0.05$) lower in the treated seedlings compared to the control. The TBARS levels were quite high in the root tissues compared to that of shoot (**Fig. 4.2.3b**). Reductions of 20, 37, 44, 53% and 32, 38, 40, 56% of TBARS were observed in shoots and roots ($p \leq 0.05$) when treated with 25, 50, 75 and 100 ppm of KH, compared to control, respectively. A similar trend was observed in the case of proline content. The root tissues experienced a fraction increase in proline level than the shoot tissues (**Fig. 4.2.3c**). Reductions of 7, 37, 47, 84% and 9, 14, 48, 81% of proline were observed in shoots and roots ($p \leq 0.05$) when treated with 25, 50, 75 and 100 ppm of KH, compared to the untreated ones, respectively.

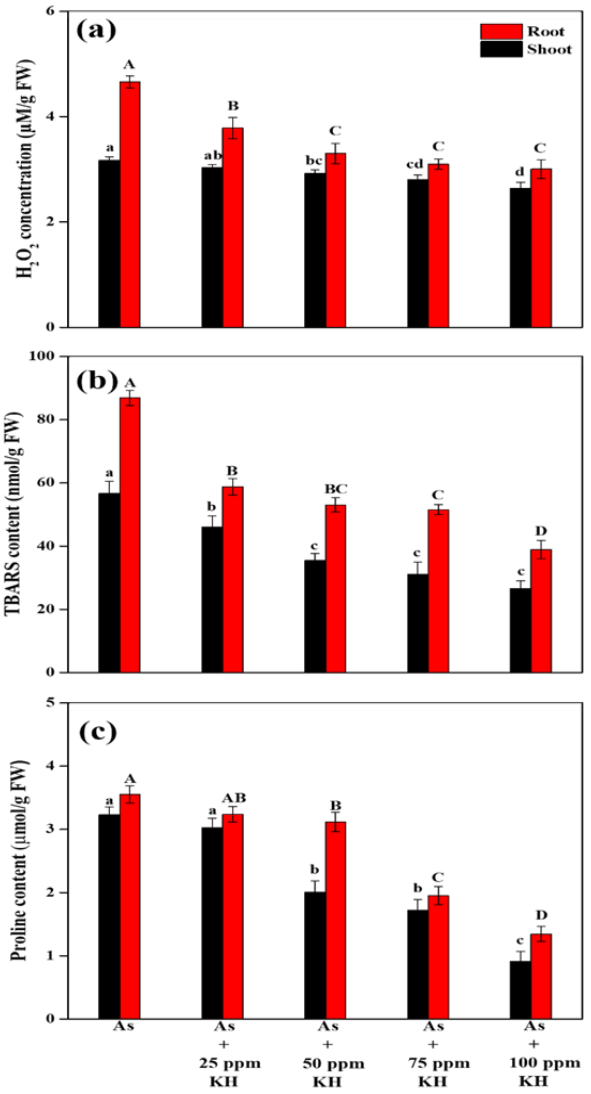


Fig. 4.2.3. Concentration of (a) hydrogen peroxide (H_2O_2), (b) TBARS and (c) proline of 14 DAS rice seedlings treated with As, As + 25 ppm KH, As + 50 ppm KH, As + 75 ppm KH and As + 100 ppm KH. Vertical bars represent mean with standard deviation and same letters in the vertical bars are not significantly different (Tukey's HSD multiple comparison at $p \leq 0.05$)

4.2.3.4. Variations in antioxidant enzyme activities upon KH application on arsenic stressed plants

The modulation in the antioxidant machinery (SOD, APX and CAT) in the seedlings (14 DAS) upon As stress with/without KH administration was evaluated (**Table 4.2.3**). A significant decrease (12%) in root SOD activity was observed in the 75 ppm KH treated plants compared to the 50 ppm ones ($p \leq 0.05$). A similar significant decreasing trend was observed in the shoot SOD activities. The differences in both root and shoot SOD activities between the control and 25, 50

ppm KH treated plants were insignificant ($p \leq 0.05$). The root APX activities decreased to a greater extent than their shoot counterpart. In the 100 ppm KH treated plants the root and shoot APX activity saw a significant decline of 42.9% and 11.1% ($p \leq 0.05$) compared to the control. KH treatment also diminished the CAT activity gradually. However, the decline in root CAT activity was less significant than that of shoot ($p \leq 0.05$).

Table 4.2.3: Variation in activity of Superoxide dismutase [SOD] (U mg^{-1} Protein), Ascorbate peroxidase [APX] (μmol $min^{-1}mg^{-1}$ protein) and Catalase [CAT] (μmol min^{-1} mg^{-1} protein) in root and shoot of 14 DAS rice seedlings

Treatments	SOD		APX		CAT	
	Root	Shoot	Root	Shoot	Root	Shoot
As	99.86±2.56a	79.95±2.62a	2.54±0.19a	1.61±0.06a	78.80±3.86a	66.92±2.09a
As + 25 ppm KH	96.71±1.45a	78.72±2.97a	2.37±0.17b	1.58±0.03b	69.68±2.78ab	65.47±1.70a
As + 50 ppm KH	94.96±1.16a	77.10±2.76a	2.21±0.12bc	1.55±0.02c	67.19±2.76ab	64.32±1.50ab
As + 75 ppm KH	83.35±2.99b	65.79±2.90b	1.74±0.24c	1.51±0.02cd	64.74±2.12b	57.36±4.03bc
As + 100 ppm KH	79.40±2.80b	63.20±2.54b	1.45±0.16d	1.43±0.03d	62.75±2.67b	53.63±3.06c

The same letters in a column indicate that the values are not significantly different at $p \leq 0.05$ using Tukey's HSD multiple comparison test

4.2.3.5. Variation in arsenic accumulation and translocation upon KH application

The concentration of As was much higher in the roots compared to the shoots. Arsenic concentration in both shoot and root saw a significant gradual decrease ($p \leq 0.05$) with consistent increase in KH concentration (**Fig. 4.2.4a**). Both the root and shoot of the lowest concentration of KH treatment (25 ppm) experienced 11% and 26% decrease in As concentration, respectively. Arsenic concentrations were also down regulated in treatments with higher concentrations of KH (50, 75, 100 ppm). The shoots experienced an average of 11% more reduction in As concentration than the roots. The evaluation of the translocation factors revealed that it remained less than 1 throughout (**Fig. 4.2.4b**).

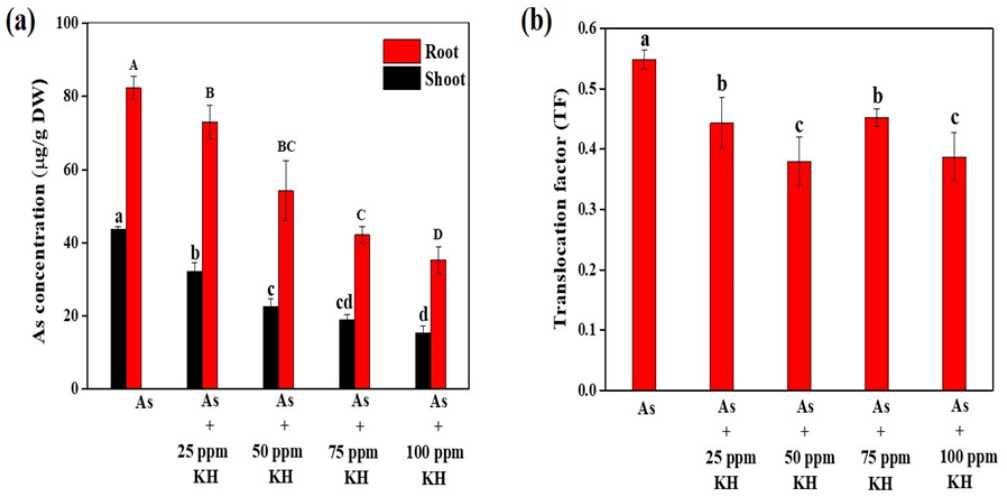


Fig. 4.2.4. Variation in (a) As concentration in root and shoot and (b) translocation As of 14 DAS rice seedlings treated with As, As + 25 ppm KH, As + 50 ppm KH, As + 75 ppm KH and As + 100 ppm KH. Vertical bars represent mean with standard deviation and same letters in the vertical bars are not significantly different (Tukey's HSD multiple comparison at $p \leq 0.05$)

4.2.3.6. Effect of KH in K uptake and its relation with arsenic assimilation in plant

In this experiment amount of K assimilated by seedlings were measured, as a portion of K enters into the seedling through KH. The shoots of the 14 DAS old seedlings contained about 13-27% more K than their roots (**Fig. 4.2.5a**). There was 16% increase in K concentration on both the roots and shoots of 25 ppm KH treated seedlings compared to the control. Finally, the 100 ppm KH treated seedlings achieved the highest concentration of K as its concentration was 1.5 times higher in both the roots and shoots compared to the control. A linear regression performed between K and As content in the roots and shoots of different treatments revealed negative correlations in both roots ($r^2 = 0.96$) (**Fig. 4.2.5b**) and shoots ($r^2 = 0.95$) (**Fig. 4.2.5c**).

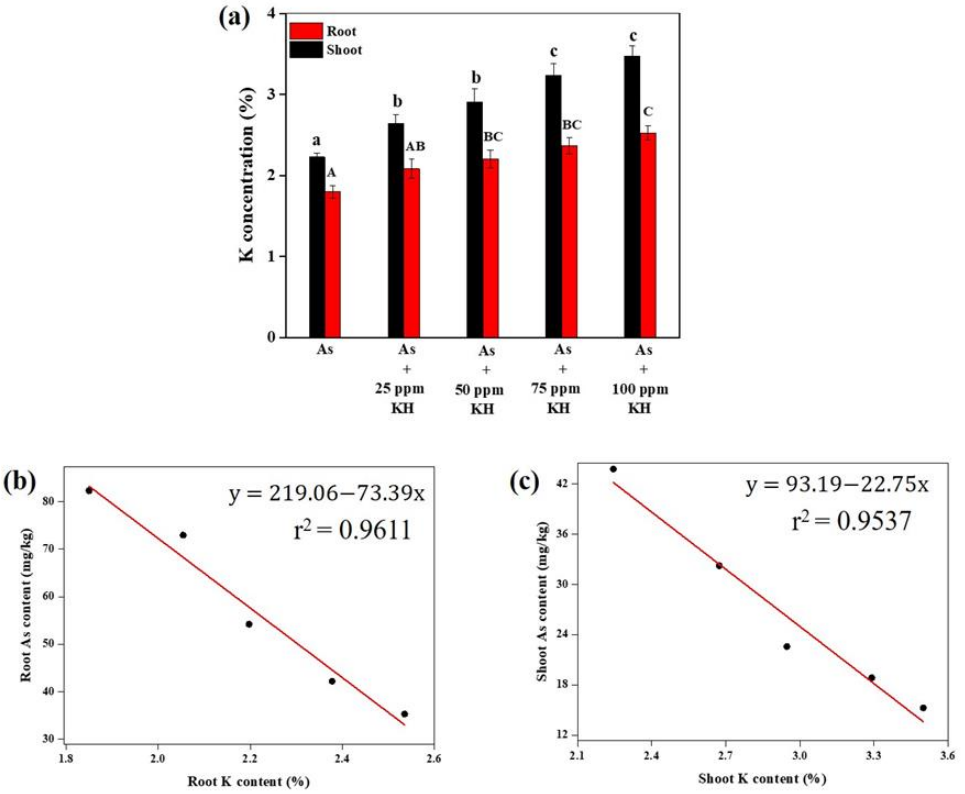


Fig. 4.2.5. (a) Variation in K concentration (%) in root and shoot of 14 DAS rice seedlings treated with As, As + 25 ppm KH, As + 50 ppm KH, As + 75 ppm KH and As + 100 ppm KH; (b) Regression between As and K content in root; (c) Regression between As and K content in shoot. Vertical bars represent mean with standard deviation and same letters in the vertical bars are not significantly different (Tukey's HSD multiple comparison at $p \leq 0.05$)

4.2.3.7. Morphological changes in root upon KH application on arsenic stressed plants

The morphological changes in root anatomical structure analysed using SEM revealed highest levels of distortions of root tissues (transverse section) in the As treated seedlings. All the tissues visible in the transverse sections, viz, epidermis, aerenchyma, endodermis, xylem and phloem showed prominent distortions due to As poisoning in the control seedlings. As the concentration of the KH treatment increased, the deformities were progressively mitigated and the cells became properly shaped. There are also signs of deposition in the internal root structures which became less prominent upon increasing the concentration of KH (**Fig. 4.2.6a-e**). Corresponding EDX studies of the SEM samples revealed the elemental content of As and K along with other elements in roots. The EDX study confirmed that the As and K weight % was in inverse proportion. The

highest As content was found in the control seedlings and the content of As gradually decreased as the K content increased (**Fig. 4.2.7a-e**).

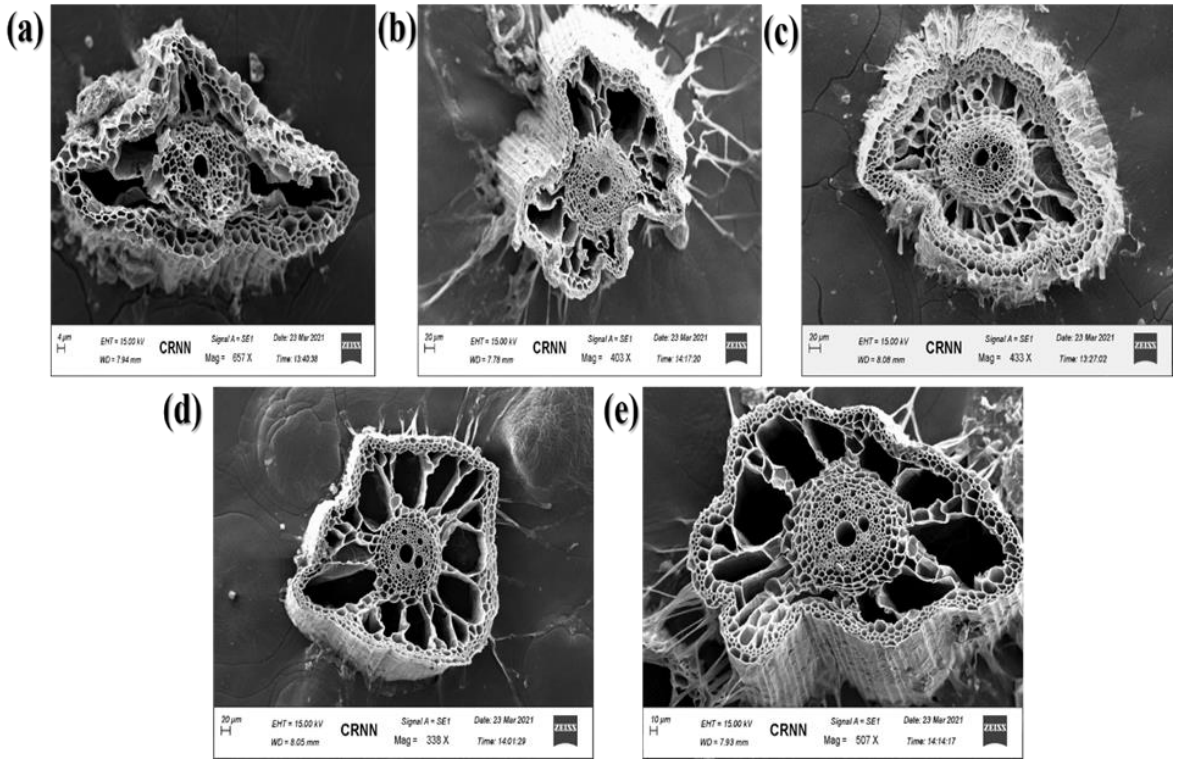


Fig. 4.2.6. Scanning electron micrographs of root of (a) As; (b) As + 25 ppm KH; (c) As + 50 ppm KH; (d) As + 75 ppm KH; (e) As + 100 ppm KH treated rice seedlings

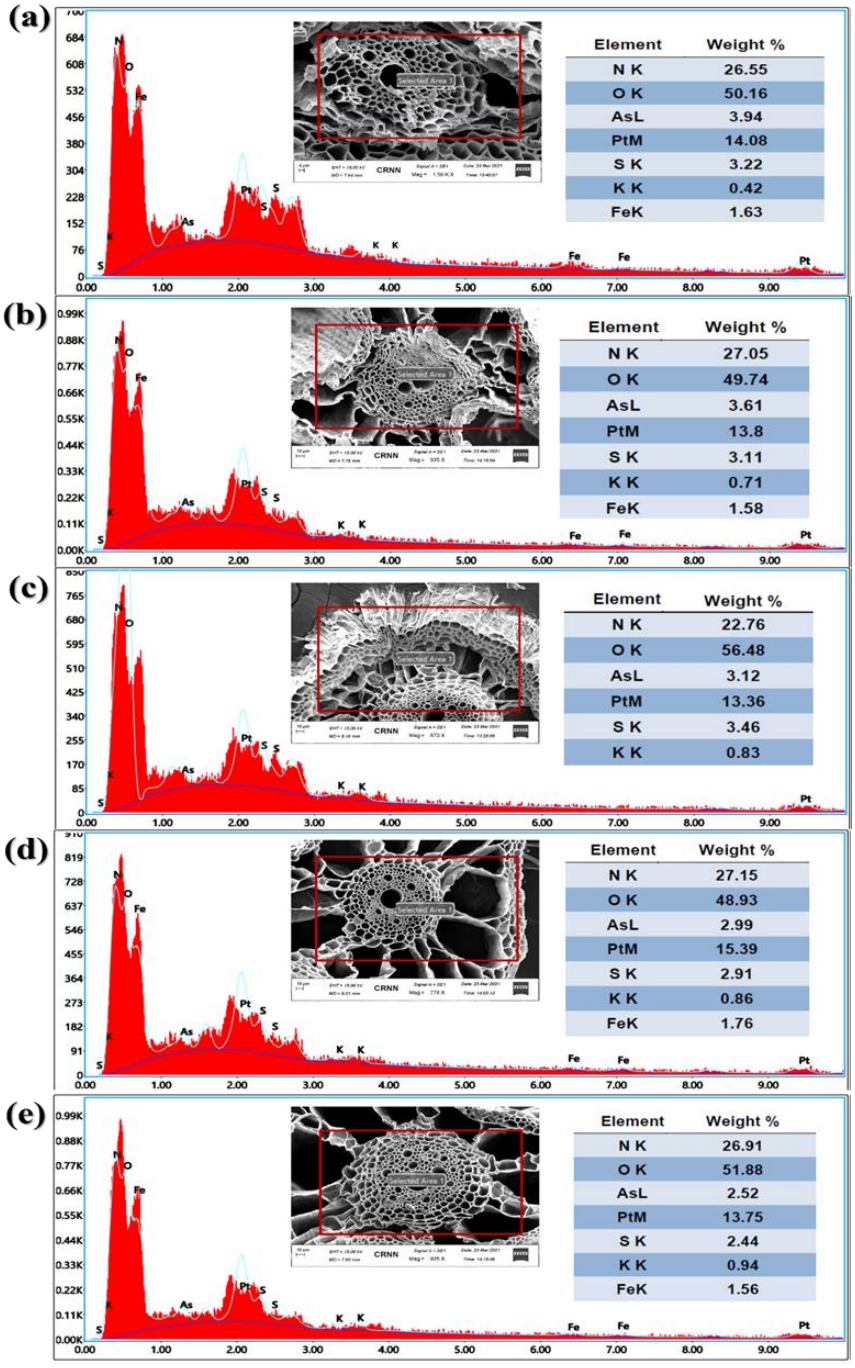


Fig. 4.2.7. Energy dispersive x-ray (EDX) spectrum of root of (a) As; (b) As + 25 ppm KH; (c) As + 50 ppm KH; (d) As + 75 ppm KH; (e) As + 100 ppm KH treated rice seedlings

4.2.4. Discussion

Exposure to As led to decrease in germination, growth and chlorophyll content of the paddy plants (Table 4.2.1 and 4.2.2). Previous studies by Choudhury et al. (2011) and Shri et al. (2009) indicate the same consequences of As toxicity. The application of KH in a hydroponic system significantly

alleviated the As toxicity as it is evident from the increased germination %, growth (root and shoot length) and chlorophyll content (Table 4.2.1 & 4.2.2). Alkali metal humate such as KH upon application to plants mimic the role of indole butyric acid, a hormone of the auxin family, which helps in root elongation (Yang et al., 2021). Apart from auxin like features, humic substances also possess cytokinin and gibberellin like characteristics (Albuzio et al., 1989; Alexander, 1990). The growth hormone indole acetic acid, responsible for cellular division and growth, is upregulated upon KH is application. Increased levels of this growth hormone also help plants to maintain their photosynthetic efficiency (Chen et al., 2022). Thus, the root as well as the shoot of the KH treated seedlings was much well developed than the untreated ones under As stress (Fig. 4.2.1). Moreover, KH can travel through the pores to enter the seeds to increase germination percentage and rate. Further, the humic acid can be absorbed by the plant, where it is broken down and releases the bound macro and micronutrients inside the plant system, thus, nourishing the plant and helping in growth and development (Alexander, 1990).

The present study revealed that As stress has adverse effects on RWC of the plants which conforms with the results of Vezza et al. (2018). However, the KH treated plants had higher values of RWC, which progressively increased with increasing concentrations of KH (Table 4.2.2). Potassium plays an important role in modulating the osmotic pressure of the plant cells by solute accumulation. It helps in lowering the osmotic potential, while increasing the turgor pressure and RWC of the plants (Wang et al., 2013). Moreover, humic acid under stressed conditions upregulates the genes responsible for triggering osmotically active compounds to maintain the osmotic balance (Chen et al., 2022). Since, KH enhanced the root development, the water and nutrient use efficiency of the plants also increased, which in turn led to the increase in RWC of the plants under As stress. Increase in EL and decrease in chlorophyll content can be attributed to ROS generation due to As stress. The ROS cause oxidative damage to lipids and proteins and eventually rupture the cell wall and makes the cell leaky (Gunes et al., 2009). Lipid peroxidation may cause damage to chloroplast. Damages incurred to the chloroplasts lead to decrease in chlorophyll content (Rahman et al., 2007). KH demonstrated neutralization of these adverse effects by maintaining the osmotic balance in the plant cells (Table 4.2.2). The results comply with that of Taha and Osman (2018).

All the major damages done to a plant due to As stress are due to the accumulation of ROS (O_2^{*-}, OH^{*}), H_2O_2 along with TBARS (a measure of highly reactive aldehyde, malondialdehyde rendering damage to tissue) which is formed as a result degradation of lipid by ROS (Janero, 1990).

The control seedlings accumulated more ROS resulting in destruction of cellular integrity (Chauhan et al., 2020). As the KH concentration increased, the bioavailable As concentration decreased due to complexation of As with different functional group present in humate. Moreover the functional groups like phenol, and quinoid/semi quinoid moiety render antioxidant property to the compound (Fakour and Lin, 2014; Zykova et al., 2018). As a result, the effective stress imposed on the plants also decreased, thus, decreasing the ROS generation (Fig. 4.2.2b-e). Eventually cell damage due to lipid peroxidation kept on decreasing, which is evident from the TBARS assay (Fig. 4.2.3b).

TBARS is a measure of lipid peroxidation and also is an indirect measurement of ROS generated in the plants. Potassium is known to trigger both the enzymatic and non-enzymatic antioxidant defence mechanism and also maintain water balance in the plant tissues, both of which is instrumental in decreasing the ROS generated due to abiotic stress (Ahanger and Agarwal, 2017). Humic acid, itself also possesses antioxidant properties due to presence of functional groups like phenol and quinone/semiquinone (Zykova et al., 2018). Thus, eventually the level of lipid peroxidation decreased, due to which membrane stability and integrity increased, which is also evident from the scanning electron micrographs (Fig. 4.2.6b-e).

Plants, when exposed to As stress, tend to accumulate free proline, which being an osmolyte is responsible for maintaining optimum osmotic potential. Proline is also responsible for inhibiting enzyme denaturation, assisting in protein synthesis and scavenging hydroxyl radicals under heavy metal stress. However, excess levels of proline can hamper substrate level phosphorylation and inhibit certain metabolic reactions (Alia and Saradhi, 1991). Application of KH reduced the levels of proline consistently (Fig. 4.2.3c), which is consistent with the results of our earlier result. This can be inferred as an evidence of decreasing As stress due to increasing KH concentration.

To scavenge away the ROS, plants depend on certain antioxidant enzymes such as SOD, CAT and APX (Abbas et al., 2018). The present study found highest levels of ROS and antioxidant enzymes (SOD, CAT, APX) in the control plants and their levels kept on decreasing as the concentration of KH increased (Table 4.2.3). These results comply with the findings of other researchers (Duan et al., 2020; Saidimoradi et al., 2019). The superoxide anion (O_2^{*-}) can directly give rise to the most harmful ROS, i.e., hydroxyl radical. SOD converts the superoxide radical to H_2O_2, which in turn is converted to water and molecular oxygen by CAT and APX (Ighodaro and Akinloye, 2018). KH, with its own antioxidant properties is capable of scavenging the ROS, thus assisting the

antioxidant enzymes in the process. Due to this assistance the requirement for the antioxidant enzymes decreases, leading to their decreased concentrations. Another plausible explanation for decrease in the antioxidant enzyme concentrations can be the reduced bioavailability of As due to organometallic complexation with certain functional groups present in humic acid (Tikhova et al., 2001; Zykova et al., 2018). A decrease in As uptake leads to decrease in oxidative stress. As a result, the need for antioxidant enzymes to scavenge ROS reduces. The fact that KH has successfully alleviated oxidative stress and membrane degradation is evident from the decrease in antioxidant enzyme concentrations.

The determination of As concentration in roots and shoots by AAS reveals a reduction in As bioavailability. Arsenic can bind to humic acid by forming metal bridge with the help of Fe, Ca or Al (Gong et al., 2020). Adsorption studies conducted for removal of As from aqueous media found evidences of such ternary complex formation with the help of metal bridge. However, the more probable mechanism of As-humate complexation in the present experiment may be binding of As with the functional groups like carboxyl, carbonyl, hydroxyl, methoxyl, phenolhydroxy present in humic acid (Gong et al., 2020). The aromatic structure of humic acid also provides certain other functional groups available for complexation. Humic acids are formed as a result of heteropolycondensation of phenolic substances like phloroglucinol, resorcinol, pyrogallol etc. The aliphatic side chains of such phenolic compounds may provide binding sites for As (Burges et al., 1964; Reza et al., 2012). Thus, the provision of multiple binding sites for As-Humic acid organometallic complexation leads to decrease in As accumulation in the roots and shoots. These results conform to previous studies. The TF value of less than 1 provides evidences that the plant adopts some mechanism to arrest the translocation of the up taken As from root to the shoot. Arsenic, once inside the plant system triggers the synthesis of phytochelatins, which are metal binding peptides. Thus, As binds with these peptides and gets sequestered in the vacuoles with the help of C-type ATP-binding cassette (ABCC) transporters, barring its translocation from roots to the shoots (Song et al., 2014). KH treatment not only reduces the bioavailability of As to the plants; however, also increase the K content in seedlings as K salt of humic acid was used in this experiment. The negative correlation between root and shoot K and As concentration in all the five treatments confirms the role of KH in decreasing the bioavailability and eventually accumulation of As in the rice seedlings (Fig. 4.2.5b-c).

Highly distorted tissue structures revealed from anatomical studies (Fig. 4.2.6a) are due to lipid peroxidation and osmotic imbalance. Xylem tissues facilitating the translocation of As leads to its

distortions (Khan et al., 2021). Elemental composition of the transverse sections of the roots revealed that as the content of K increased due to KH application, As content decreased (Fig. 4.2.7b-e). The reason for decrease in As content may be due to complexation of As at different binding sites present in humate which in turns reduce the bioavailability of As, barring its entry into the plant root and thus eliminating distortions (Fakour and Lin, 2014).

4.2.5. Conclusion

This investigation attempted to illustrate the effects of hydroponic application of KH at varying doses to As stressed plants. The findings of the study can be summarised as follows:

i. KH administration to As exposed rice seedlings led to increased seed germination, seedling vigour, chlorophyll content.

ii. The morphology and anatomy of the plants improved due to implementation of KH.

iii. The oxidative stress experienced by the plants due to As exposure decreased and eventually the antioxidant defence machineries were also regulated.

iv. The present analysis confirmed that increasing concentrations of KH leads to decreased uptake of As by the rice seedlings.

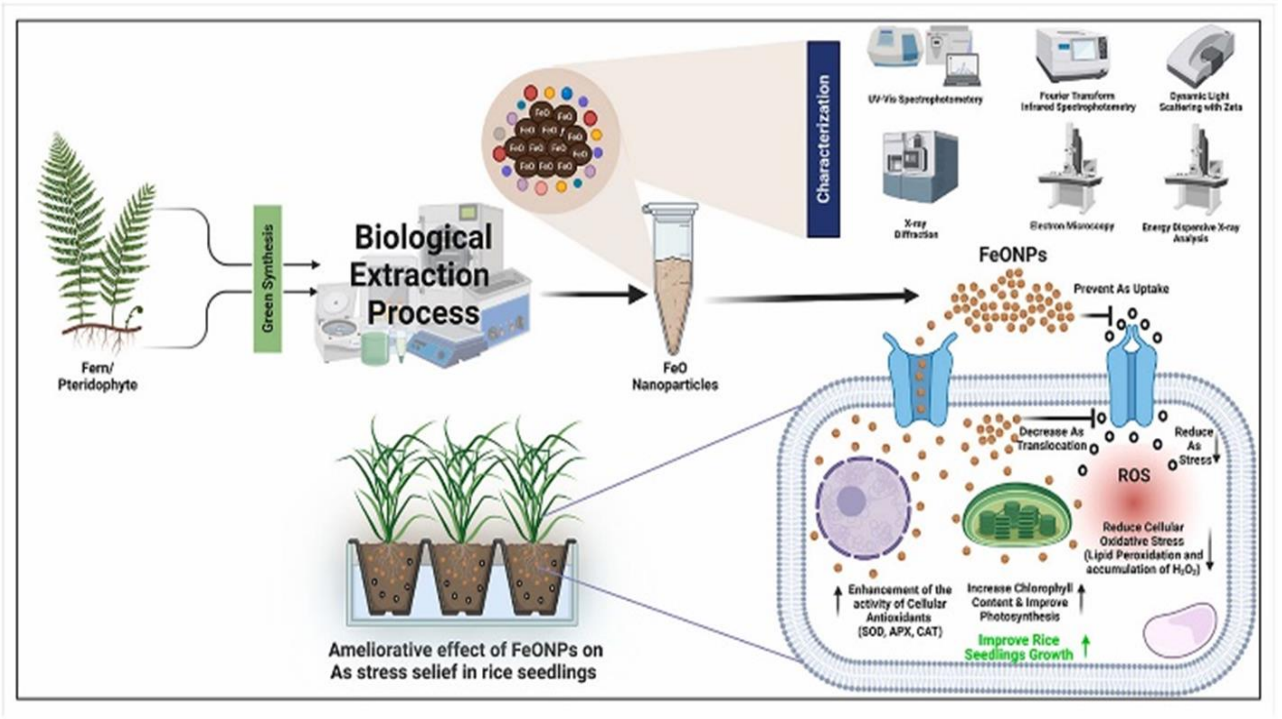
Arsenic dose was kept unchanged throughout this study. Further studies can be conducted to assess the ameliorative capacity of KH under varying doses of As. There can be an estimation of the threshold dose of As as well as KH beyond which amelioration will be difficult. Further field trials with KH need to be done to check the effect of KH in improvement of rice production by reducing the phytotoxic effects of As.

Chapter 5

Biosynthesis of iron oxide and selenium nanoparticles and their potential to mitigate arsenic stress in Oryza sativa L. *seedlings*

5.1. Iron oxide nanoparticles

Graphical Abstract



5.1.1. Introduction

Nanoparticles are the particulate scatterings or strong particles with the Nano range between 10-1000 nm. Nanoparticles are of broadly two types' non-metallic nanoparticle (NMeNP) and metallic nanoparticle (MeNP). NMeNP includes various bio-polymers, carbon-related compounds etc (Dasgupta et al., 2017). Metallic nanoparticles (MeNPs) are in increasing demand due to their innate metallic properties. They are widely used in the field of sensing (El-Ansary and Faddah, 2010), catalytic (Astruc, 2020), agriculture (Y. Ghidan and M. Al Antary, 2020) Medicine, etc. (Murthy, 2007). Iron nanoparticles (FeONPs) are being widely used due to their size and magnetic properties. It was first widely employed for the clean-up of polluted soil and groundwater. FeONPs are also applied as (i) absorbent of toxic chemicals and/or (ii) photocatalysts for conversion/degradation of toxic pollutants to non-toxic forms (Xu et al., 2012). FeONPs have been successfully employed for wastewater treatment and removal of toxic elements like As (Jang et al., 2008; Xu et al., 2012; Zou et al., 2016) and also as exceptional nano fertilizers (Rui et al., 2016). Among the different phases of iron nanoparticles, α-Fe_2O_3 nanoparticles are a widely studied material, which has a wide array of applications.

Synthesis of FeONPs is mainly carried out by chemical route using different reducing chemicals (Gupta et al., 2010). The chemicals, used during chemical synthesis and by-products generated after these chemical reactions, are hazardous in nature (Das et al., 2017). Therefore, biologically synthesized nanoparticles are preferred over chemically synthesized nanoparticles due to their less toxicity and environment-friendly nature. Plant contains a variety of terpenoids, polysaccharides, phenols, and flavonoids which act as reducing agents and facilitate the formation of stable MeNPs (Sarkar et al., 2020). FeONPs were synthesised using tea, gardenia, and *Aloe vera* leaf extracts in several investigations (Ahmmad et al., 2013; Karade et al., 2019; Mukherjee et al., 2016).

Lower groups of plants like algae (Saif et al., 2016), bryophytes (Acharya and Sarkar, 2014; Saif et al., 2016), fungi preferred cryptograms for producing metal nanoparticles. However, the potential of several pteridophytes (fern and fern allies) for the synthesis of nanoparticles has received less attention. Until recently, only a few species have been utilized, including *Pteris tripartite* (Baskaran et al., 2016), *Adiantum capillus-veneris* (Chatterjee et al., 2019), *Adiantum philippense* (Chatterjee et al., 2019), *Asplenium scolopendrium* (Chatterjee et al., 2019), *Actinopteris radiata* (Chatterjee et al., 2019), *Azolla microphylla* (Chatterjee et al., 2019), and others (Sarkar et al., 2020). However, *Adiantum lunulatum* Burm. f. is well-known for its

antibacterial, antioxidant, and other therapeutic characteristics (Mengane, 2016). The plant extract may have anti-hyperglycemic properties, as well as some therapeutic properties against influenza and TB. A recent study also confirmed that the synthesis of copper oxide NPs using *Adiantum lunulatum* plant extract has an ameliorative effect on defense and antioxidant enzymes activities of *Lens culinaris* (Sarkar et al., 2020).

Arsenic (As) contamination of soil and water is a major problem in various parts of the world (Chowdhury et al., 2020a,b). Arsenic exposure to humans can also cause potential carcinogenic and non-carcinogenic effects (Joardar et al., 2021a,b). In Southeast Asia, Ganga-Meghna-Brahmaputra flood plain is considered as worst As affected area (Das et al., 2021a,b; De et al., 2021). Rice is a major crop and main source of food for people living in this part of the world. However, a substantial amount of rice is still cultivated in As contaminated soil and water (Chowdhury et al., 2020a). The presence of inorganic As species (AsIII and AsV) in soil and water causes a phytotoxic effect when enters the plant (Sarkar et al., 2021). Several kinds of research had been attempted to remove or immobilise As from water and soil using different chemically synthesized nanoparticles. However, the non-toxicity of green synthesized FeONPs make them a lucrative option to remediate As from soil and water. Previously, green synthesized α-Fe_2O_3 NPs were used for the removal of As from wastewater (Mukherjee et al., 2016). Likewise, green synthesized iron oxide nanoparticles using *Excoecaria cochinchinensis* leaves were utilized to stabilized soil As fraction (Su et al., 2020). Different doses of chemically synthesized FeONPs were also utilized to remediate As stress in plants (Bidi et al., 2021; Shabnam et al., 2019). However, limited research has been carried out with green synthesized FeONPs to alleviate As toxicity from the plant (Khan et al., 2021). Therefore, the main goal of this study was designed to synthesize FeONPs through a green route by using *Adiantum lunulatum* plant leaves extract as a reducing agent and investigate the efficiency of synthesized FeONPs in the alleviation of As stress from rice plants during the seedling stage.

5.1.2. Materials and methods

5.1.2.1. Synthesis and characterization of FeONPs

Freshly plucked *Adiantum lunulatum* Burm. f. was collected from a local area in Garia, Kolkata, West Bengal (22.4629°N 88.3968°E). Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) and sodium hydroxide (NaOH) pellets were purchased from Merck, Germany. The chemicals were further used without any purification. During plant extraction and nanoparticle synthesis, deionized water

was used. The collected plant was washed out with tap water to remove any particulate contamination and thereafter with deionized water, respectively. The superficial water was soaked from the plant surface with a paper towel. Further, the pinnules of the plant were taken and a paste was made out of it in the mortar and pestle and 100 ml of deionized water was added to it (Sarkar et al., 2020). Finally, the extract was attained with the help of a Whatman's filter paper no.1 in a conical flask thrice to get the plant extract.

The green synthesis of iron oxide (Fe_2O_3) nanoparticles using plant extract was performed by a bottom-up process approach and reduction technique by a single pot system revamping the procedure by Sarkar et al. (Sarkar et al., 2020). At first, the measured amount (100 ml of 3 mM) of $FeCl_3.6H_2O$ was added to a beaker. Then the plant extract was added slowly into the solution. Finally, the $FeCl_3$ solution reacted with 100 ml of filtered plant extract. The entire reaction was done at pH 11 by adding 1 M NaOH. The mixture was stirred continuously for a minimum of 1 h and the formation of a dark brown colour solution confirmed the synthesis of FeONPs. The synthesized FeONPs were separated by centrifugation at 12,000 × g for 15 min and the pellet containing FeONPs were washed (3 times) with deionized water followed by drying at 80 ºC for 3 h and stored for further use.

The UV-visible spectra of the synthesized FeONPs had been recorded using a Hitachi 330 spectrophotometer. The morphological, topographical, size and purity of the nanoparticles were analysed by a transmission electron microscope (TEM) [Tecnai G2 spirit Biotwin (FP 5018/40), operating at around 80 kV accelerating voltage] and a scanning electron microscope (SEM) (Hitachi S 3400N) outfitted with an Energy Dispersive X-ray Spectroscopy (EDX), respectively. The zeta potential (Charge distribution) of the nanoparticles was studied using a Beckman Coulter DelsaTM Nano Particle Analyzer (USA) by illuminating the solution with a He–Ne laser (658 nm) in a sample cell. The crystallinity of the FeONPs was analysed by XRD. The diffracto gram was documented by PANalytical, XPERTPRO diffractometer using Cuk (Cu Kα radiation, λ 1.54443) as X-ray source running at 45 kV and 30 mA. To identify the functional groups, present in synthesized FeONPs, Fourier transform infrared spectroscopy (FTIR, Shimadzu 8400S) was employed to record and analyse the spectral data range between 4000 and 400 cm^{-1}.

5.1.2.2. Plant material, seed preparation and experimental design

Rice (*Oryza sativa* L. c.v. Ranjit) seeds were collected from the Madhusudankati village (22°54'14.51" N, 88°46'25.36" E), Gaighata block, West Bengal, India which is one of the worst

As affected area of India. The rice seeds were surfaced sterilized with H_2O_2 (30%) solution for 10 min and then washed with distilled water three times. The seeds were then kept for germination in their respective treatment. Total five treatments were designed for this experiment and all the treatments were set up in triplicate. In each treatment 20 rice seeds were placed for germination. The experiment has 50 µM of both AsIII ($NaAsO_2$, MW: 129.91 g mol^{-1}) and AsV (Na_3AsO_4, MW: 207.88 g mol^{-1}) along with 100 mg/L of FeONPs. The details of the five experimental sets were: control (Hogland medium); 50 µM AsIII; 50 µM AsV; 50 µM AsIII + FeONPs and 50 µM AsV + FeONPs. The treatments were produced by adding As and FeONPs, respectively in the Hogland solution. Before application, the FeONPs were sonicated using a probe sonicator for 5 min to achieve homogenised distribution in the nutrient solution. The pH of the nutrient medium was maintained between 5.7 and 6.0 using 0.1 M KOH and HCl, and the nutrient medium was changed after every 3 days. The entire experiment was conducted for 14 days after seeding (DAS).

5.1.2.3. Seed germination and seedling vigour

The seeds in their respective treatment were grown hydroponically under a controlled environment. For rice seed germination, all treatments were kept in dark at 27 ± 1 ºC with a humidity of 60 - 70 %. The germination of seeds was counted at 3 DAS and a seed was considered as germinated only when the radicle length was found ≥ 2 mm. After 3 DAS, the germinated seeds were kept under controlled photoperiod (14/10 hr light/dark cycle) till 14 DAS (Zhang et al., 2021). The seedling vigour at 14 DAS was measured.

Vigour Index I (VG I) = Standard Germination % × {Average Seedling Length (Root + Shoot)}

5.1.2.4. Determination of relative water content (RWC)

For the determination of RWC in seedlings, the fresh leaf was weighed and placed in a petri dish containing distilled water. The petri dish was then kept in dark at room temperature inside a BOD incubator for 24 h. Afterwards, the turgid weight (TW) of the leaf was taken. The leaf sample was again kept in the oven for drying at 80 °C and after 24 h, the dry weight (DW) was measured. The RWC of rice seedlings were calculated according to Barrs & Weatherleyt (1962) (Barrs and Weatherley, 1962)

$$RWC = \frac{FW - DW}{TW - DW} \times 100$$

5.1.2.5. Estimation of chlorophyll content

The chlorophyll (a and b) content of the rice seedlings was measured based on the Arnon method (Arnon, 1949). The seedling leaf was homogenised with 80% acetone and centrifuged at 10,000 × g for 10 min. The absorbance of the leaf extract was measured spectrophotometrically at 663 nm (chlorophyll a) and 645 nm (chlorophyll b).

5.1.2.6. Estimation of electrolyte leakage

The fresh leaf tissues (500 mg) were segmented into pieces and vacuumed for 20 min in 20 ml deionized water. Conductivities (C1) were measured after the leaves were maintained in the dark for additional 2 hours at room temperature. The conductivities (C2) were tested again after the leaf samples were put in boiling water for 15 minutes. The percentage of electrolyte leakages were estimated according to the following formula (Cao et al., 2007).

$$EL = \frac{c_1}{c_2} \times 100$$

5.1.2.7. Determination of H_2O_2, lipid peroxidation and proline content

The amount of H_2O_2, TBARS (thiobarbituric acid reactive substances) and proline content in root and leaf of rice seedlings were measured according to the proposed methods of Velikova et al. (2000) (Velikova et al., 2000), Heath and Packer (1968) (Heath and Packer, 1968) and Bates et al. (1973), respectively.

5.1.2.8. Antioxidant enzymes activities

The antioxidant enzyme assays were performed by extracting 0.2 g of root and shoot tissues of rice seedlings in 2 ml of chilled 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% (w/v) Polyvinylpyrrolidone (PVP). The protein content of antioxidant enzyme extracts was measured according to Lowry et al. (1951). Superoxide dismutase (SOD; EC 1.15.1.1), activities were estimated by measuring its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT), as prescribed by the scientific manuscript of Beauchamp and Fridovich (1971) Beauchamp and Fridovich (1971). For the ascorbate peroxidase (APX; EC 1.11.1.11) assay, the H_2O_2 dependent ascorbic acid (ϵ = 2.8 mM^{-1} cm^{-1}) oxidation was taken into account by measuring the decrease in absorbance for 1 min at 290 nm Nakano and Asada (1981). The catalase (CAT; EC 1.11.1.6) activity was determined after 0.1 ml of enzyme extract was mixed with 1.5 ml of 50 mM phosphate buffer followed by 15 mM H_2O_2. After an incubation period of

1 min, the continuously decreasing absorbances of the mixtures were measured every 1 min interval at 240 nm as per the protocol of Aebi (1984).

5.1.2.9. Arsenic and iron content in rice seedling

For estimation of As and Fe content in rice seedling, 0.02 - 0.1 g of dried root and shoot tissues were digested overnight with 2 ml of HNO_3 and 1 ml H_2O_2. The digested samples were then placed on a hotplate for evaporation. Afterwards, the evaporated samples were subjected to volume makeup and filtered through Whatman filter paper (0.45 µm). The analysis of As and Fe content of root and shoot tissues were performed through Atomic Absorption spectrometry (AAS) (Chowdhury et al., 2020).

The standard reference materials (SRMs) of rice flour 1568a and tomato leaf 1573a and spiked samples were analysed during the estimation of As and Fe to maintain the quality control and assurance of the analysis. The digestion of SRM 1568a (rice flour) and 1573a (tomato leaf) samples, followed by As analysis, yield 96.3 ± 0.4% and 94.2 ± 0.8 % recovery, against their certified value of 0.29 ± 0.03 and 0.1126 ± 0.0014 mg/kg, respectively. The recovery of Fe from the SRM 1568a (rice flour) and 1573a (tomato leaf) were 93.7 ± 1.2 % and 95.3 ± 0.9 %, against their respective certified values of 7.4 ± 0.9 mg/kg and 368 ± 7 mg/kg.

5.1.2.10. Translocation of arsenic and iron

The translocation of As and Fe from root to shoot of rice seedling was calculated according to the following equation (Chowdhury et al., 2020)

$$Translocation\ factor\ (TF) = \frac{Concentration\ of As\ or\ Fe\ in\ shoot\ (mg/kg)}{Concentration\ As\ or\ Fe\ in\ root\ (mg/kg)}$$

The value of TF > 1 indicates that the plant can translocate metal or metalloid effectively from root to shoot (Chowdhury et al., 2020).

5.1.2.11. Statistical analysis

All experimental results were collected in triplicate and the data was demonstrated as average value ± standard deviation (SD). The one-way ANOVA test followed Tukey-HSD ($p \leq 0.05$) test was carried out to present the difference between treatments in Origin 2019b (Origin Lab Corporation).

5.1.3. Results and discussion

5.1.3.1. Characterization of green synthesized FeONPs

The stimulation of the surface plasmon resonance of FeONPs gave the reaction solution its distinctive brown-red colour, which served as a useful spectroscopic hallmark of their production (**Fig. 5.1.1**). In the identical experimental circumstances, neither the positive control group ($FeCl_3$ Solution) nor the negative control group (ALE) showed any significant colour change. Using a UV–Visible spectrophotometer, the reduction of ferric oxide was analysed spectroscopically. This revealed a 270 nm absorbance peak (**Fig. 5.1.2a**), which was unique to FeONPs (Saif et al., 2016). The FTIR spectra of the green synthesized FeONPs highlighted the functional groups present in FeO-NP (**Fig. 5.1.2b**). The peak that appeared near 660 cm^{-1} could be assigned to the stretching vibration of the Fe–O–Fe bond in FeONPs. The peaks found in between 3400 and 3300 cm^{-1}, 1630 cm^{-1}, 1400 cm^{-1} and 1057 cm^{-1} were assigned to stretching vibration of O–H bond, stretching vibration C=O bond, bending vibration of C–H and stretching vibration of C–N bond, respectively (Karade et al., 2019; Mukherjee et al., 2016). The occurrence of these bonds can be attributed to the presence of the phenolic hydroxyl group, phenolic acids, terpenoids-phenols, and aliphatic amines. These functional groups are also responsible for the reduction of Fe^{3+} ions and behave as the capping agent of the nanoparticles (Karade et al., 2019). The XRD measurement is frequently found to be a valuable analytical tool for determining the crystalline nature of freshly generated compounds and their phases. The XRD pattern of the FeONPs, shown in **Fig. 5.1.2c**, clearly identified the structure of α-Fe_2O_3 nanoparticles (JCPDS file, No. 89-2810). XRD peaks noticed at 2θ values of 28°, 33°, 35°, 41°, 49°, 54°, 57°, 62°, and 64° for the generated nanoparticles corresponded to the (012), (110), (113), (202), (024), (116), (018), (214), and (300) planes of the α-Fe_2O_3 nanoparticles, respectively. During this measurement, a sequence of diffraction peaks was detected that were consistent with the theory. The prominent peaks of the XRD pattern suggest that the green synthesized FeONPs were well-crystallized α-Fe_2O_3.

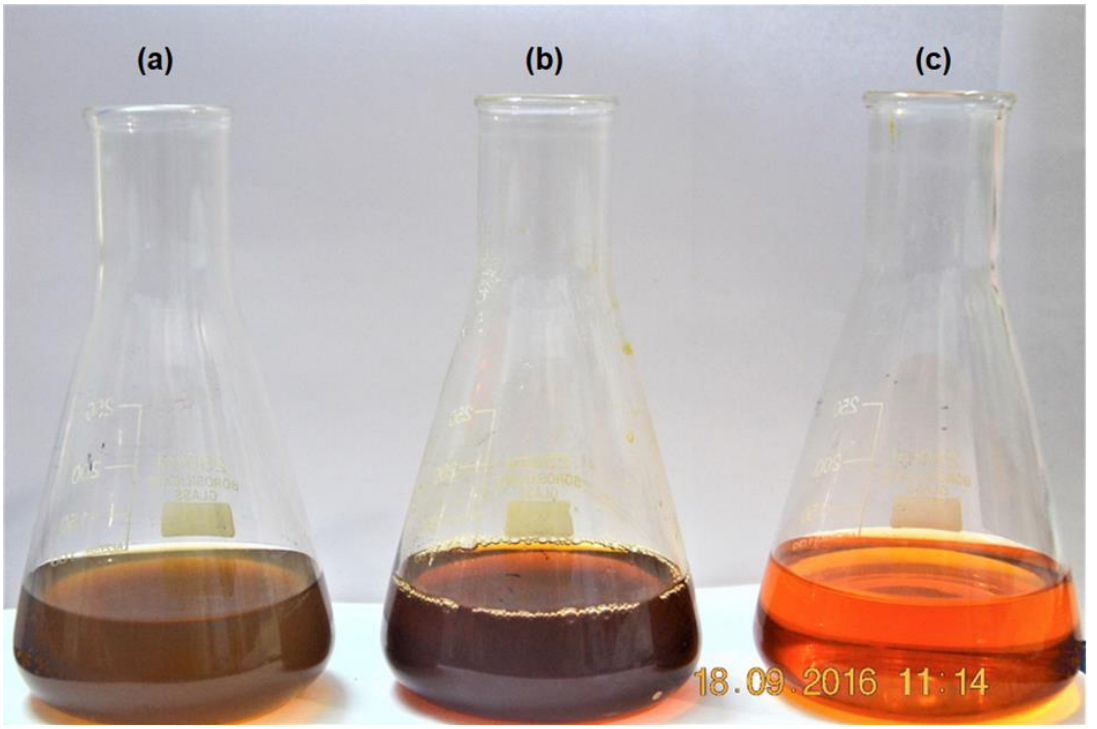


Fig. 5.1.1. Three coniocal flask containing (a) only *A.lunulatum Extract* (ALE), (b) the reaction mixture of ALE and $Fecl_3$ solution, (c) only $Fecl_3$ solution, respectively

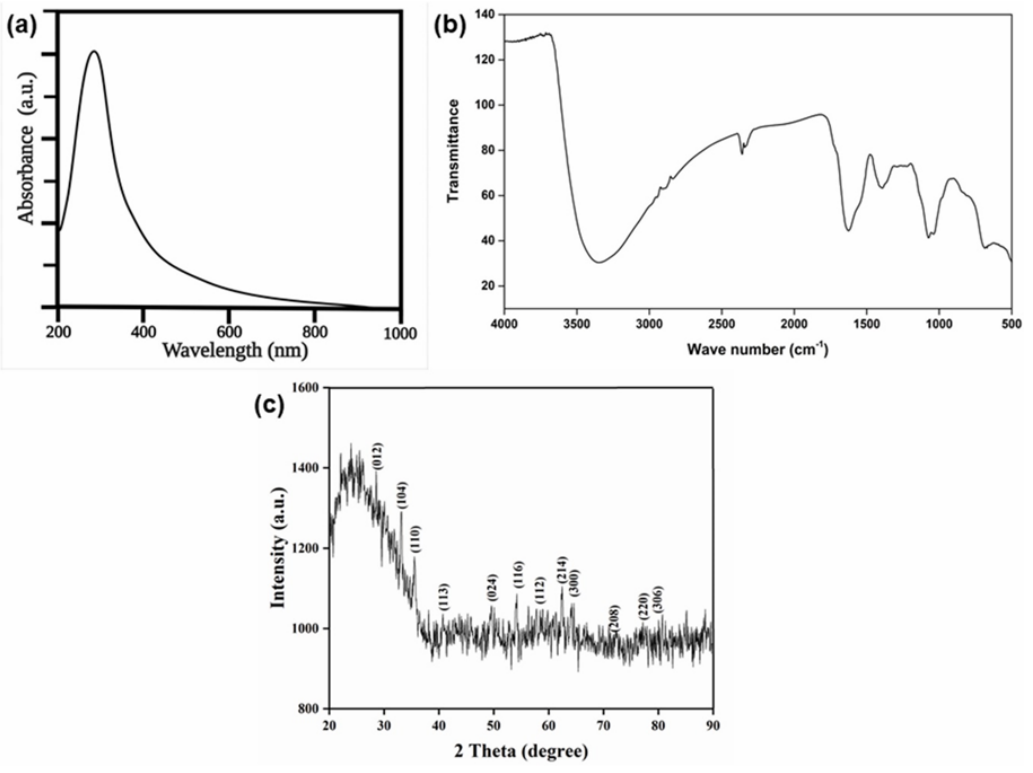


Fig. 5.1.2. The UV–visible absorption spectrum (a), FTIR spectra (b), and XRD pattern (c) of green synthesized FeONPs

The EDX spectrum from one of the densely populated ferric oxide nanoparticles regions, obtained in the spot-profile mode, is shown in **Fig. 5.1.3a**. EDX spectra showed two distinct signals of iron (Fe) and oxygen (O) in synthesized FeONPs (Mukherjee et al., 2016). Peak related to any other element did not appear in the EDX spectrum, which confirmed that the synthesized FeONPs were made of Fe and O. A transmission electron microscope (TEM) study was carried out to analyse the size and morphology of the synthesized FeONPs. The average size of these FeONPs was 5 ± 1 nm (Range: 3 – 10 nm) (**Fig. 5.1.3b)** (Mukherjee et al., 2016). In nature, particles were found to be quasi-spherical and mono-disperse (**Fig. 5.1.3c**). Further analysis of FeONPs using SAED patterns indicated that the shells of large-size nanoparticles, as well as a fraction of small nanoparticles, are made up of highly crystalline iron oxide (**Fig. 5.1.3d**). A similar type of findings has been observed earlier. The majority of the FeONPs in the TEM picture was not in physical touch but were separated by a suitably undeviating inter-particle distance.

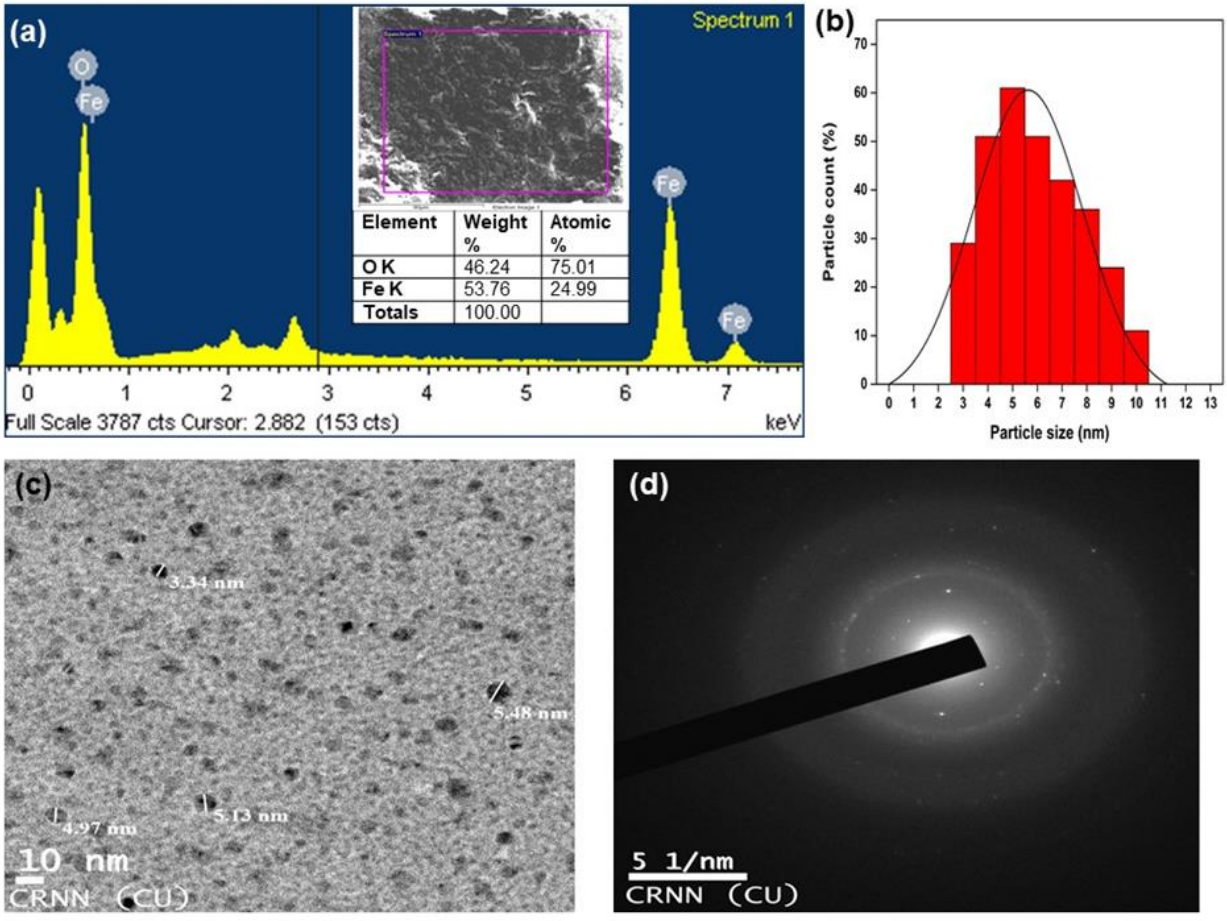


Fig. 5.1.3. EDX spectrum (a), Particle size (b), TEM (c) and SAED pattern (d) of green synthesized FeONPs

5.1.3.2. Effect of FeONPs and arsenic stress on seed germination and seedling growth

One of the most important parameters to evaluate in this study is seed germination. The control group had the highest germination percentage (93.33%) of all of the treatments (**Table 5.1.1**). The germination of rice seedlings was affected by both AsIII and AsV stress (Table 5.1.1). Previous studies by Choudhury et al. (2011) and Shri et al. (2009) also indicate the same consequences of As toxicity. Rahman et al. (2012) reported that the moisture content and nutrient absorption in seeds were considerably decreased with an increase in As concentration. However, the application of FeONPs significantly ($p \leq 0.05$) improved the germination by 9.8 % and 15.4 % as compared to seeds germinated in AsIII and AsV, respectively. Khan et al. (2021) reported that the application of 5 ppm FeONPs increased the rice seed germination by 65 % under As stressed condition. Earlier study observed that FeONPs diffuses through the seat-coat of mung bean (*Vigna radiata*) seeds and increases the water uptake, activity of amalyse and starch metabolism of seeds, which in turn led to increase the germination of mung bean plants.

The root and shoot growth of rice seedlings were significantly ($p \leq 0.05$) affected under AsIII and AsV stress (**Fig. 5.1.4**). The root growth was hindered under As stress due to the initial exposure of the root to the As stressed environment. The As toxicity also restrict the shoot growth by inhibiting the cell division, hydrolytic enzyme activity and translocation of nutrients from root to shoot. The biomass yield was also minimal due to the lowered root and shoot growth. AsIII had a greater phytotoxic effect on seedling growth than AsV, which is consistent with previous research. Arsenic (III) inhibits the metabolic process by interacting with thiol groups. However, FeONPs significantly ($p \leq 0.05$) improved the biomass and the seedling vigour (Bidi et al., 2021). FeONPs improved seed germination, root and shoot length, fresh weight, and vigour index, reducing As absorption and protecting seedlings from abiotic stress (Khan et al., 2021). Moreover, FeONPs promotes plant growth by improving hormonal (gibberellin and cytokinin) regulation, photosynthetic activity and redox process of the plants under heavy metal stress.

Table 5.1.1: Effects of FeONPs and As stress on seed germination, root growth, shoot growth, seedling fresh weight and seedling vigour

Treatments	Germination (%)	Root length (cm)	Shoot length (cm)	Fresh wt. (mg)	Vigour index
Control	93.33±5.77a	9.22±0.43a	18.76±0.3a	148.27±7.76a	2611.37±148.63a
AsIII	61.66±2.88d	2.13±0.35e	7.46±0.31e	90.33±4.8c	591.31±104.2e
AsV	65±5dc	4.38±0.2d	10.33±0.35d	96.34±5.69c	956.15±137.4d
AsIII + FeONPs	68.33±2.88c	5.58±0.65c	14.2±0.45c	109.45±6.64bc	1351.56±157.69c
AsV + FeONPs	76.88±5.77	6.74±0.3b	15.6±0.3b	117.62±5.3b	1717.49±149.8b

The same letters within the same column indicate values are not significantly different at $P \leq 0.05$ using Tukey's HSD multiple comparison test.

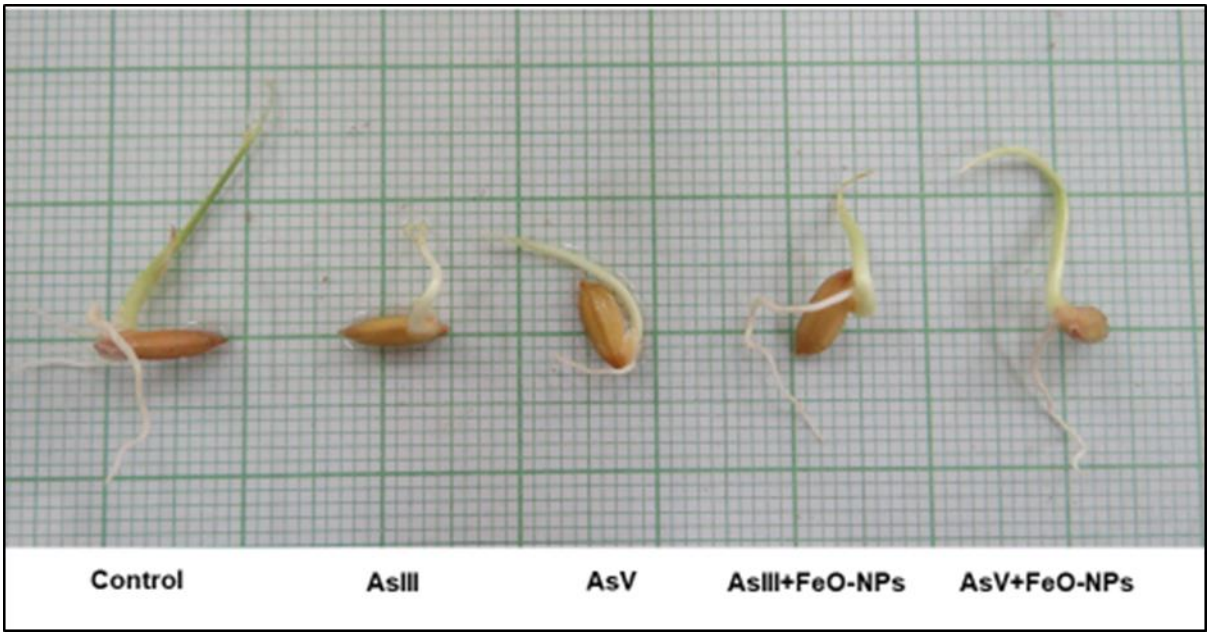


Fig 5.1.4. Seedling growth under different treatment at 5 DAS

5.1.3.3. Effect of FeONPs on arsenic and iron uptake and translocation

Higher As content was recorded in root and shoot tissue of rice seedling grown in only AsIII and AsV, respectively, as compared to those grown in different As species along with FeONPs (**Fig. 5.1.5a**). The As species enters into the plant system via different transporters and aquaporin channels present in plant root. Plant roots inadvertently take up AsV through phosphate transporters, whereas AsIII enters through silicic acid trans-porters and some aquaporin channels (Wang et al., 2017). FeONPs application in growth medium significantly ($p \leq 0.05$) reduced the As accumulation in the root by 46.36 % and 48.17 % under AsIII and AsV stress, respectively. A similar reduction in heavy metals uptake by rice plants under FeONPs fertigation was also confirmed in other studies. The FeONPs adsorbed the As ions on its surface, hence the bioavailability of As to plants was reduced (Chowdhury et al., 2020). The competitive uptake of As and Fe at the root surface could be a possible reason for the reduced uptake of As in rice seedlings (Bidi et al., 2021). Supplementation of FeONPs also reduced the translocation of As from root to shoot by 13.6 % and 14.2 % as compared to seedlings grown in only AsIII and AsV (**Fig. 5.1.5b**). Application of FeONPs increased the Fe accumulation and its subsequent translocation from root to shoot in As stressed rice seedlings (**Fig. 5.1.5c and 5.1.5d**). The accumulation of Fe in root and shoot of rice seedlings treated with FeONPs were significantly ($p \leq 0.05$) increased by 61.5 % and 65.4 %, respectively. Fe is classified as a micronutrient and

required for plant metabolism. Fe also plays a crucial role in both redox reactions and photosynthetic pigment synthesis. The deficiency of Fe in plants caused leaf necrosis, stunted growth; Fe deficiency also disrupts the electron transport chain. An earlier study found As toxicity hampered the rice plant growth by minimizing the Fe accumulation and translocation and these observations were also matched with our findings where uptake and translocation of Fe were comparatively less in seedlings treated only with As (AsIII and AsV) as compared to control. As a result, fertigation with FeONPs counteracts the phytotoxic effect of As by restoring Fe homeostasis.

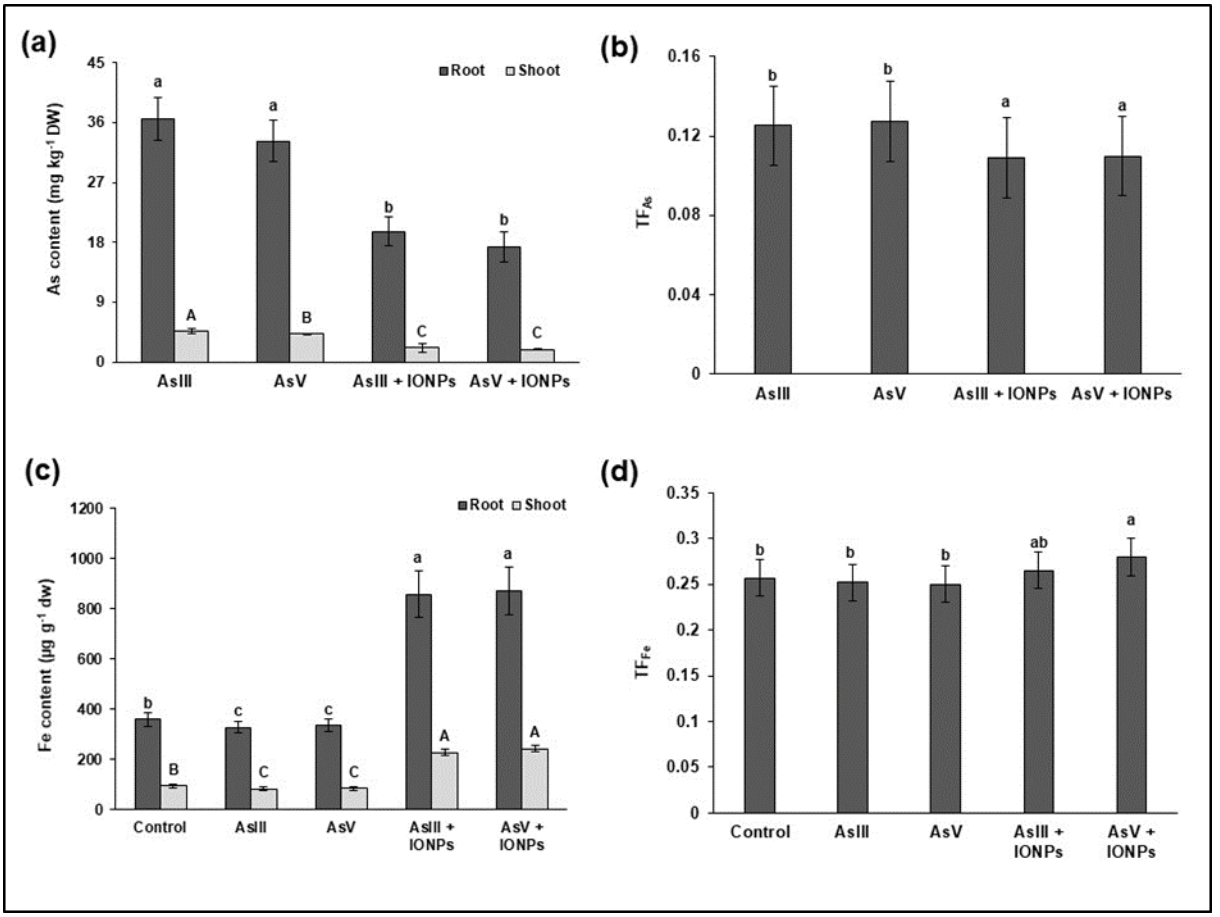


Fig. 5.1.5. Arsenic content (a), arsenic translocation factor (b), iron content (c) and iron translocation factor (d) of 14 DAS rice seedlings. Vertical bars represent mean with standard deviation and same letters in the vertical bars are not significantly different (Tukey's HSD multiple comparison at $p \leq 0.05$)

5.1.3.4. Effect of FeONPs and arsenic on oxidative stress, lipid peroxidation, electrolyte leakage and proline

The As phytotoxicity in plants triggers the accumulation of reactive oxygen species (ROS) in plants which further causes oxidative stress in plants. A significant ($p \leq 0.05$) increase in H_2O_2 content was observed in root and shoot of rice seedling under only As (AsIII and AsV) stressed conditions as compared to the seedlings treated with FeONPs (**Fig. 5.1.6a**). The FeONPs treatment reduced the shoot H_2O_2 content by 35.84% and 47.66% as compared to AsIII and AsV treatment, respectively. The increment in H_2O_2 content can cause oxidative damage to lipids and proteins and eventually rupture the cell wall and makes the cell leaky.

TBARS is a measure of lipid peroxidation and also is an indirect measurement of ROS generated in the plants. Arsenic accumulation in root and shoot increased accumulation of H_2O_2 which in turn damages biomacromolecules such as membrane lipids and induces membrane lipid peroxidation (Bidi et al., 2021). The amount of TBARS in seedling root was increased by 25.9 % and 32.9 % under AsIII and AsV treatment, respectively, as compared to AsIII + FeONPs and AsV + FeONPs treated seedlings (**Fig. 5.1.6b**). Mousavi et al. (2020) also observed a similar level of lipid peroxidation due to oxidative stress caused by As toxicity. The hike in lipid peroxidation caused an increase in electrolyte leakage in seedlings treated only with As (AsIII and AsV). However, the application of FeONPs reduced the electrolyte leakage (EL) in rice seedlings by 16.6% and 19.7% as compared to the seedlings treated with only AsIII and AsV, respectively (**Fig. 5.1.6c**). The IONP mediated reduction in oxidative stress markers and EL was also reported in an earlier study.

Plants, when exposed to As stress, tend to accumulate free proline, which is an osmolyte that is responsible for maintaining optimum osmotic potential. However, excess levels of proline can hamper substrate-level phosphorylation and inhibit certain metabolic reactions. To mitigate oxidative damage caused by As exposure in plants, higher levels of proline was observed in root and shoot of seedlings treated only with AsIII and AsV, respectively (**Fig. 5.1.6d**). The application of FeONPs in growth medium significantly ($p \leq 0.05$) reduced the shoot proline content by 47.12% and 33.51% as compared to only AsIII and AsV treatment, respectively. Bidi et al. (2021) also observed a similar reduction in proline level when rice seedlings were fertigated with FeONPs under As stressed conditions (Bidi et al., 2021). Moreover, FeONPs succeeded in the reduction of H_2O_2, TBARS, EL and proline level in rice seedlings by lowering the As uptake and translocation.

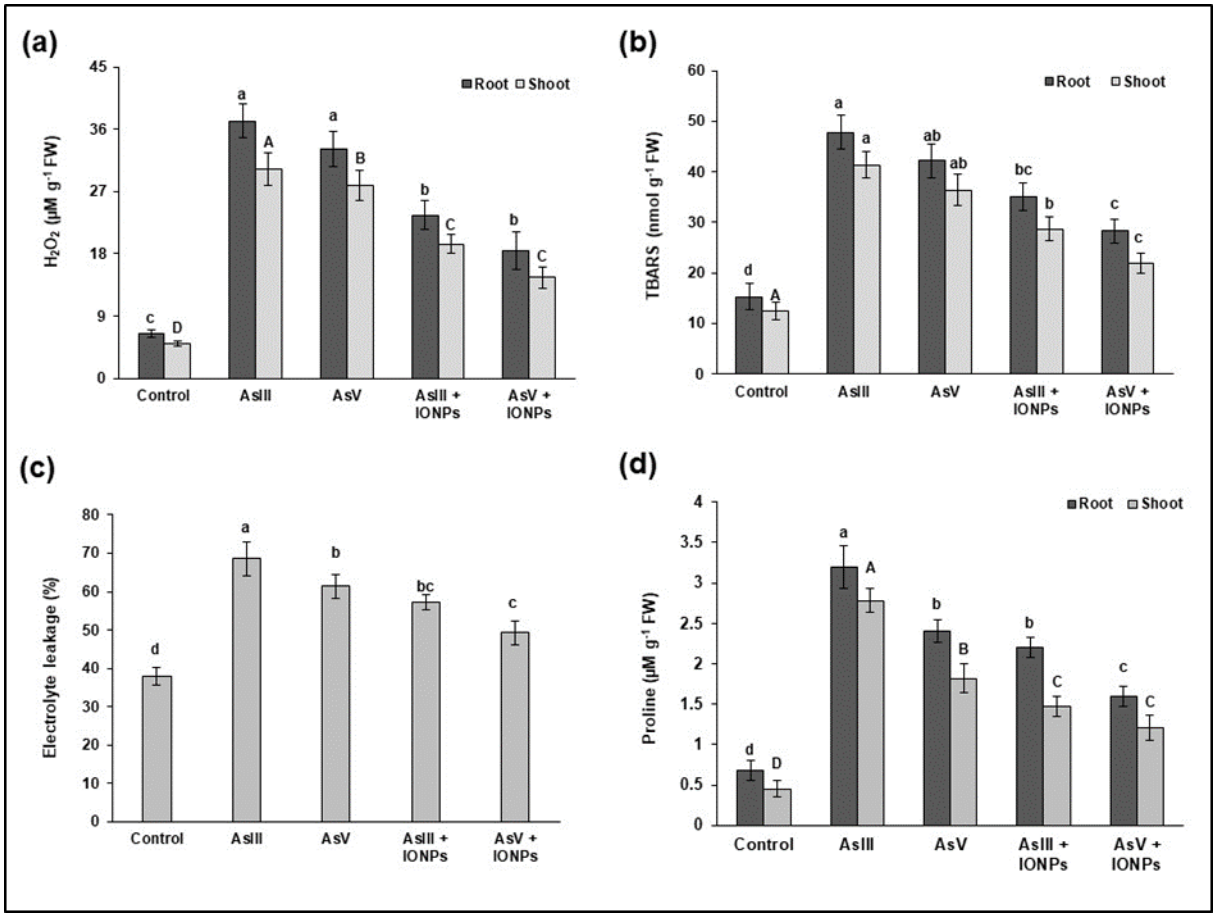


Fig. 5.1.6. Hydrogen peroxide (H_2O_2) (a), TBARS (b), electrolyte leakage (c) and proline (d) content in 14 DAS rice seedlings. Vertical bars represent mean with standard deviation and same letters in the vertical bars are not significantly different (Tukey's HSD multiple comparison at $p \leq 0.05$)

5.1.3.5. Effect of FeONPs and arsenic stress on chlorophyll and RWC

Photosynthetic pigments are an integral part of the plant's machinery and are used as a stress indicator. This study confirmed that the chlorophyll content of seedlings was found to decrease due to As toxicity (**Fig. 5.1.7a**). Chlorophyll (a and b) content of rice seedlings was found minimal under AsIII and AsV treatment by 61.41%, 45.14%, 54.06% and 50.13%, 29.1%, 40.6% as compared to control, AsIII+FeONPs and AsV+FeONPs, respectively. The generation of ROS under As stressed conditions triggered lipid peroxidation that may cause damage to the chloroplast. As stress in plants can also induce chlorophyllase activity or reduce the synthesis of precursor molecule (δ-aminolevulinic acid) and enzyme activities that involved in the biosynthesis of chlorophyll. However, the application of FeONPs was found to elevate the chlorophyll content in rice seedlings which were grown under AsIII and AsV stress. Iron is a micronutrient that is

necessary for chloroplast production, photosynthesis, respiration, and DNA synthesis (Mushtaq et al., 2020). FeONPs were earlier proven to increase the chlorophyll content in As stressed rice plants and Cd stressed wheat plants. The study also suggests that FeONPs up-regulate photosynthetic genes which in turn led to the increase in chlorophyll content of the plants. The current study found that As stress harms RWC content (**Fig. 5.1.7b**), which conforms with the results of other studies. However, the RWC content in seedlings treated with FeONPs has been increased by 16% and 10.6% as compared to the seedlings treated only with AsIII and AsV, respectively. Since FeONPs enhanced the root development, the water use efficiency of the plants also increased, which in turn led to the increase in RWC of the plants under As stress. A similar study using FeONPs found that the drought tolerance ability of soyabean plants was improved.

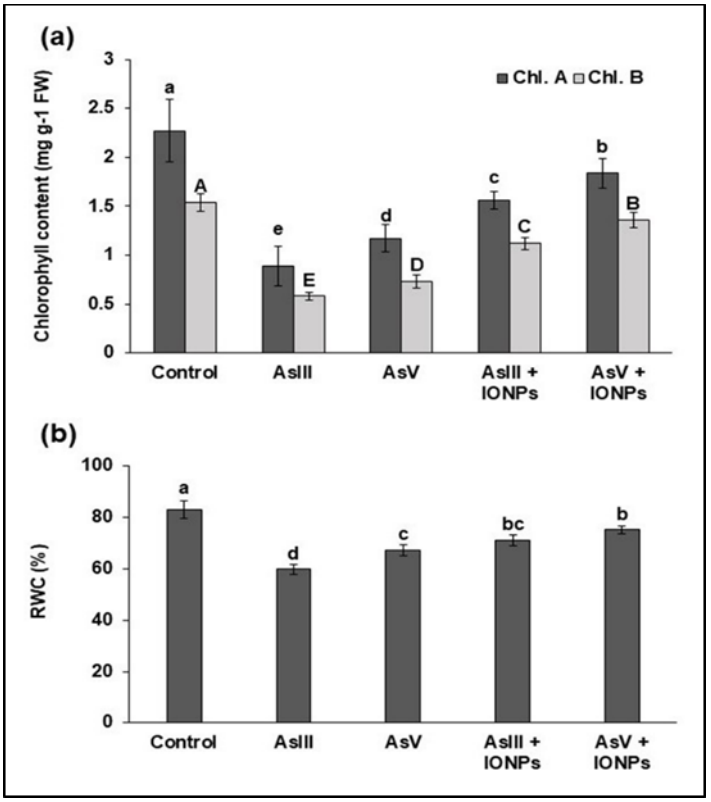


Fig. 5.1.7. Chlorophyll a, b content (a) and RWC (b) in 14 DAS rice seedlings. Vertical bars represent mean with standard deviation and same letters in the vertical bars are not significantly different (Tukey's HSD multiple comparison at $p \leq 0.05$)

5.1.3.6. Effect of FeONPs and arsenic on antioxidant enzyme activities

To scavenge away the ROS mediated phytotoxicity, plants depend on certain enzymes such as SOD, APX and CAT. In the present study, As stress has significantly ($p \leq 0.05$) enhanced the

activity of SOD, APX and CAT. SOD initially converts the intracellular superoxide ($O2^{*-}$) radical into H_2O_2. The AsIII and AsV stress increased the activity of SOD in the shoot of rice seedling by 55.1%, 21.12%, 37.6% and 48.8%, 10.1%, 28.8% as compared to control, AsIII+ FeONPs and AsV+ FeONPs, respectively (**Fig. 5.1.8a**). In our earlier work, it was observed that increased SOD activity in rice seedlings when exposed to AsIII and AsV stress. The produced H_2O_2 was further converted to water and molecular oxygen by CAT and APX. The higher accumulation of H_2O_2 in root and shoot tissues of rice seedlings caused a significant ($p \leq 0.05$) increase in APX and CAT activity (**Fig. 5.1.8b and 5.1.8c**). CAT diminished different types of reactive oxygen during stress conditions and inhibit cell wall destruction. However, the lesser accumulation of ROS caused a subsequent reduction in the activity of SOD, APX and CAT in root and shoot of rice seedlings treated with FeONPs. Different investigations have confirmed that using FeONPs lowered antioxidant enzyme activity under abiotic stress settings, which is consistent with our findings.

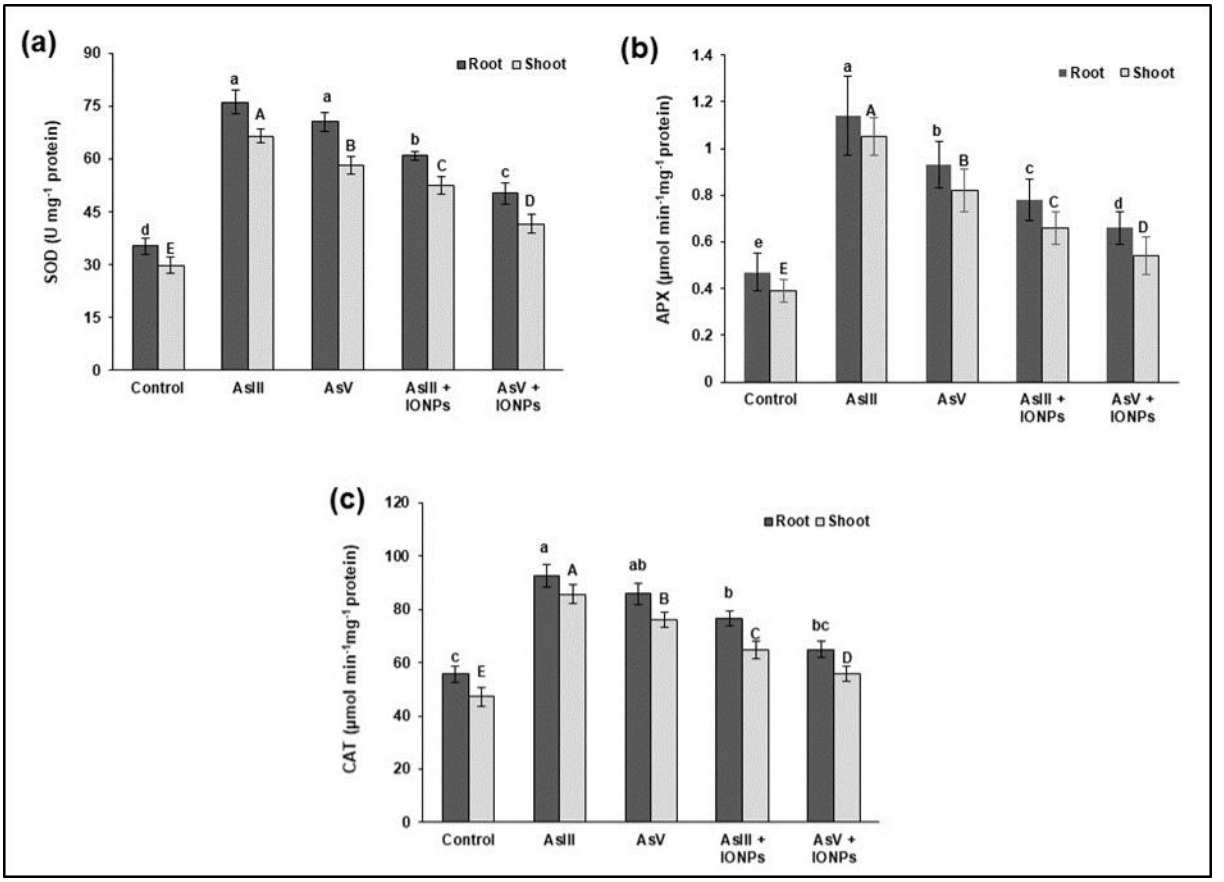


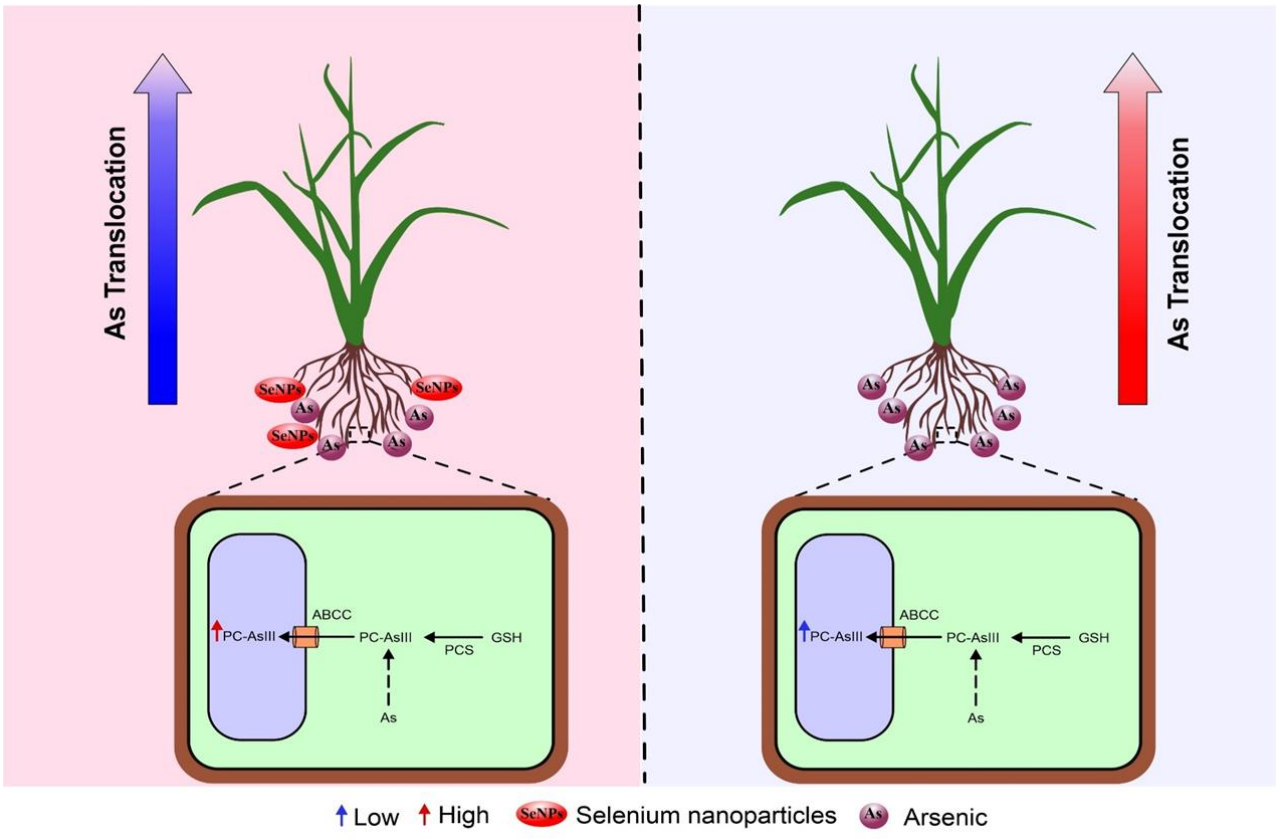
Fig. 5.1.7. Superoxide dismutase [SOD] (a), Ascorbate peroxidase [APX] (b) and Catalase [CAT] (c) activities in root and shoot of 14 DAS rice seedlings. Vertical bars represent mean with standard deviation and same letters in the vertical bars are not significantly different (Tukey's HSD multiple comparison at $p \leq 0.05$)

5.1.4. Conclusion

The present study confirmed that *Adiantum lunulatum* leaf extract can be used for the green synthesis of environmentally safe FeONPs in a non-toxic way. The green synthesized FeONPs were quasi-spherical with a mean size of 5 ± 1 nm. This study also demonstrated that the application of FeONPs alleviates the As induced toxicity from the rice plant during the seedling stage. The ameliorative effect of FeONPs was prominent against As induced oxidative stress. FeONPs reduced the bioavailability of As to rice seedlings which in turn minimize oxidative stress and improve the seedling growth. Thus, findings from this study furnish the prospect for the utilization of green synthesized FeONPs to alleviate the As toxicity in plants. However, further field-scale study needs to be done to check the efficiency of FeONPs in this aspect. Study on optimization of FeONPs dosage need to be done to obtain the best possible efficacy against As toxicity. Despite its prominent ameliorative effects against As toxicity in plants, iron nanoparticles can be detrimental to microbes and soil animals, necessitating further investigation.

5.2. Selenium nanoparticles

Graphical Abstract



5.2.1. Introduction

Arsenic (As) is a very toxic metalloid that has been categorized by WHO as a Class 1 carcinogenic substance (WHO, 2018). Arsenic exposure also induces acute oxidative stress in plant tissue which impairs the growth of plant. Naturally occurring arsenite (AsIII) and arsenate (AsV) are the two inorganic As species and highly toxic in nature. As rice is mainly cultivated in anaerobic condition, this leads to the formation of AsIII under such condition. Due to the molecular similarity between AsIII and silicic acid, AsIII is absorbed into the rice plant via the silicon (Lsi-1, Lsi-2, and Lsi-6) transporters. Lsi-1 and Lsi-2 are predominantly expressed in the roots; however, Lsi-6 is expressed in both the roots and the shoots (Ma et al., 2008). AsV is mainly present in an aerobic environment and it is taken up into the plant via transporters (Phts) (Zeeshan et al., 2022). Plants naturally respond to oxidative stress by activating a variety of enzymatic or non-enzymatic antioxidant systems. In addition to their antioxidant defense system, plants have developed a variety of other defense mechanisms to cope with pollutants. These defensive mechanisms include limiting the distribution of metal(loids) in tissues through vacuolar sequestration mediated by phytochelatins (PCs) (Huang et al., 2021).

Rice is one of the most extensively cultivated cereal crops in the world (around 160 million hectares), owing to the fact that rice is the staple diet of more than half of the world's population (Samal et al., 2021). Rice plants grown in As-containing soils have been shown to exhibit toxicity signs such as reduced growth, scorched leaves and brown patches (Bakhat et al., 2017). Hence, reducing As toxicity in agricultural crops is of great significance. Selenium (Se) is a vital trace element that plays crucial functions in controlling the activity of antioxidant enzymes, preventing the excessive production of reactive oxygen species (ROS), and fostering growth (Azimi et al., 2021). Earlier studies have also shown that Se improved the antioxidant defense, growth, and yield in a variety of plant species, including Chinese cabbage (*Brassica campestris* L. ssp. *Pekinensis*) (Zhao et al., 2019), tobacco (Jiang et al., 2015), potatoes (Shahid et al., 2019), and basil (*Ocimum basilicum*) (Oprica et al., 2018). Selenium also played an important function in amelioration of As toxicity while also decreasing As accumulation and translocation in rice (Singh et al., 2018). It has been noted that both Se^{IV} and Se^{VI} compete for same aquaporin transporters Lsi1 and Phts with AsIII and AsV, respectively (Trippe and Pilon-Smits, 2021). Metal(loids) distribution at the subcellular level and localization in plant tissues are directly related to their toxicity. A crucial defence mechanism in plants for metal detoxification involves the chelation of metal(loid)s and their subsequent sequestration into cellular components including cell walls and vacuoles (Huang

et al., 2021). Studies observed that addition of Se increased vacuolar sequestration of toxic metal(loid) in plants by improving phytochelatin (PC) synthesis (Farooq et al., 2022; Huang et al., 2021; Zeeshan et al., 2022).

In recent years, SeNPs have gained interest in agricultural applications due to their favorable physicochemical properties, unique bioactivities and high degree of biosafety. SeNPs have mostly aided plants in surviving circumstances of heavy metal or other abiotic stress (Sarkar et al., 2023). Specially, SeNPs produced by biological means are found to be more stable due to the presence of surface biomolecules (Menon et al., 2019). Low levels of SeNPs shield plants against metal toxicity by reducing metal-induced oxidative stress, lowering metal uptake and translocation, and reducing cell membrane damage (Gupta and Gupta, 2017). In comparison to other Se species, SeNPs have excellent biological activity, greater biocompatibility, antioxidant properties, and lower toxicity (Azimi et al., 2021).

Previous studies claimed that SeNP application reduced As uptake, translocation, and related toxicity; however, they did not highlight how SeNP affects As uptake and reduces As translocation into shoot. These aspects are crucial to have in depth knowledge on role of SeNPs in As uptake and accumulation in plants. Thus, the present study explored the role of SeNPs in 1) As uptake and translocation, 2) the modulation of genes involved in As uptake and translocation, 3) regulation of stress responses, and 4) subcellular distribution of As with special emphasis on vacuolar sequestration.

5.2.2. Materials and methods

5.2.2.1. Synthesis and characterization of SeNPs

The organisms used in this study, *Trichoderma harzianum* were procured from the Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany, University of Calcutta. The cultures were incubated in 500 mL Erlenmeyer flasks containing 250 mL each of potato dextrose broth (PDB) medium at pH 7.5 and the culture was maintained for 15 days at temperature of 35±2 °C and light intensity of 75 μ mol photons m^{-2} s^{-1} PAR. The culture flasks were shaken manually 2-3 times (150 rpm) every day. After the incubation period, the media was filtered and the fungal cultured filtrate (FCF) was kept for further use. In order to synthesize SeNPs, culture filtrate (100 ml) was mixed with sodium selenite (Na_2SeO_3) solution (1 mM) (Sigma, St. Louis, MO, USA). This was agitated for 24 h at room temperature (25 °C). For control, flasks containing pure PDB medium with 1 mM Na_2SeO_3. The synthesis was turned off when the color of the

reaction solution changed to red. The newly synthesized SeNPs were separated out by centrifugation (at 12000 rpm for 10 min) and ultrasonication. The newly synthesized SeNPs were further utilized for their characterization.

5.2.2.2. Rice seed germination and seedling growth

Rice seeds (Satabdi; IET 4786) were surface sterilized with 30% H_2O_2 for 15 min and then washed properly with distilled water. For germination, Rice seeds were kept in dark at 27 °C. After germination, seedlings were placed in a growth chamber with a photoperiod of 14 h, day/night temperature cycle of 27/22 °C and relative 70% relative humidity. The uniformly grown seedlings were transferred to 50% Hogland solution. Treatment groups of this experiment were as follows: control (only Hogland); 50 mg/L SeNPs; 25 µM AsIII; 50 µM AsIII; 25 µM AsIII + 50 mg/L SeNPs; and 50 µM AsIII + 50 mg/L SeNPs. The hogland solution (pH: 5.8-6.0) was replaced twice a week. Before application, fresh hogland solution with various treatments were ultrasonicated for 30 min. The seedlings were maintained for 3 weeks and one day before harvest, total RNA and subcellular fractions from plant root and leaf samples were isolated. After harvesting, some plants from each treatment were oven-dried at 65 °C for 24 h for extraction of plant As and remaining plants were instantly frozen in liquid nitrogen and stored (–80 °C) for subsequent assays.

5.2.2.3. Assessment of chlorophyll and carotenoid

The total chlorophyll and carotenoids content in plant leaves were measured using the prescribed protocol of Lichtenthaler (1987). Leaves (0.1 g) were crushed with 5 mL ice cooled acetone (80% v/v) and the absorbance of the extract solution was recorded at 470 nm, 645 nm and 663 nm.

5.2.2.4. Assessment of lipid peroxidation, H_2O_2, and thiol compounds

Briefly, plant tissue (0.1 g) was homogenized with 5% trichloroacetic acid (TCA). The plant extract was centrifuged (4000 rpm for 10 min) and the supernatant was collected to analyse TBARS (thiobarbituric acid reactive substance). The amount of lipid peroxidation in leaf and root and leaf tissue were measured as the quantity of TBA (thiobarbituric acid) according to Heath and Packer (1968). Assessment of H_2O_2 was measured according to the methodology proposed by Velikova et al. (2000).

The amount of total non-protein thiols (NPTs) in rice seedlings was estimated using the procedure of Ellman et al. (1959). The concentration of reduced glutathione (GSH) and oxidised glutathione

(GSSG) was measured using the procedure provided by Hissin and Hilf (1976). Total phytochelatins (PCs) concentration was calculated as PCs = NPTs – (GSH+GSSG).

5.2.2.5. Histochemical analysis

Intracellular H_2O_2 in root tissue was measured *in situ* after stained with 10 mM DCF-2DA (2',7'-dichlorodihydrofluorescein diacetate) fluorescent dye for 10 min in dark. For measurement of intracellular O_2^{*-} in root tissue, the rice roots were stained with 15 mM DHE (dihydroethidium) for 15 min at 25 ºC (Yamamoto et al., 2002). The intracellular H_2O_2 and O_2^{*-} were measured under Floid Cell imaging station microscope (Life Technologies).

5.2.2.6. Assessment of antioxidant enzymes

The plant protein was extracted and determined using Bradford (1976) method. The activities of catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD) enzymes were measured according to Aebi (1984), Nakano and Asada (1981), and Beauchamp and Fridovich (1971), respectively.

5.2.2.7. Sub-cellular distribution of arsenic

The sub-cellular fractions of As in plant root and shoot were performed as per the method of Zhao et al. (2017). Root and leaf samples weighing about 0.5 g were homogenized in an extraction solution that had been pre-cooled (1.0 mM dithioerythritol, 250 mM sucrose, and 50 mM Tris buffer, pH 7.5). The extract was centrifuged at 3000 rpm for 15 min at 4 ºC, the isolated pellets was regarded as cell wall (F1). The supernatant was again centrifuged at 15000 rpm for 45 min at 4 ºC, the isolated bottom pellet was regarded as organelles (F2) and the remaining supernatant was soluble fraction (F3) which includes cytosols and vacuoles.

5.2.2.8. Measurement of arsenic and selenium and determination of arsenic translocation

The plant material, subcellular fractions and cell wall components were digested with a mixture of HNO_3 and H_2O_2 (2:1) for 12 h and finally at 120 ºC for 2 h using a tube heating block. The final volume of the digested samples was adjusted with mili-Q water and then filtered using a 0.45 µm syringe filter before to estimate As and Se by Atomic Absorption Spectrophotometry (AAS) and ICP-MS.

Translocation factors (TFs) of As was calculated as follows:

$$TF = {As\ in\ shoot}/{As\ in\ root}$$

Where, As in shoot and root (mg/kg) refer to mean As concentrations in the rice shoots and roots, respectively

5.2.2.9. RNA extraction, cDNA synthesis, and RT-PCR analysis

Fresh shoot and root of rice (100 mg) from both treated and control plants were used to extract total RNA using the Trizol reagent (Ambion, life technologies) according to the standard instructions. The iScript cDNA synthesis kit (Bio-Rad) was used to reverse-transcribe total RNA from each sample. The transcript levels of target genes using specific primers were subsequently measured using quantitative real-time PCR in both treated and control samples using Quant Studio 3 (Thermo Fisher scientific). Gene specific primer sequences are depicted in **Table 5.2.1** and relative expression levels of target genes were calculated using the $2^{-\triangle\triangle Ct}$ method, with 18S rRNA as the control gene (Sarkar et al., 2023).

Table 5.2.1: Details of genes and gene-specific primers

Gene	Gene ID	Forward primer	Reverse primer
OsPCS1	Os05g0415200	AGTTAGGAGACAAGAGGAAGGA	AGTATCACGGATTTGCTGTAGG
OsPCS2b	Os06g0102300	TCTGTCCCGCTTAGTGAAATC	GCTTAATCCTGATCCTCCTTCC
OsLsi1	Os02g0745100	AGAGCTTGAGCTGCTTGTT	GAGTTGTTGCTGGCCATTTC
OsLsi2	Os03g0107300	GCTCCTCAACCTCAAGTCAAT	CAGCACATATACGGTGGGTAAA
OsABCC1	Os04t0620000-01	AACAGTGGCTTATGTTCCTCAAG	AACTCCTCTTTCTCCAATCTCTG

5.2.2.10. Statistical analysis

Statistical analysis was carried out using one-way ANOVA test followed by Tukey HSD ($p < 0.05$) test in SPSS version 18 and Origin 2019b (Origin Lab Corporation). NPs size and fluorescent intensity were analysed using Image J software, and heml 2.0 software was used for heat map analysis of gene expression.

5.2.3. Results

5.2.3.1. Characterization of SeNPs

In this work, *Trichoderma harzianum* was used for the synthesis of Selenium nanoparticles (SeNPs). The culture filtrate after addition of aqueous Na_2SeO_3 (1 mM) showed a time dependent gradual change in color at room temperature, from light yellow to radish yellow color and finally to dark red (**Fig. 5.2.1a**). The appearance of the red colour indicated the occurrence of the reaction and the formation of the nontoxic, insoluble, and red colored elemental nano-Se (Rehan et al., 2019). In case of positive control (only culture filtrate) and negative control (sodium selenite solution alone), no change in color was observed. TEM images clearly reveals the mono scattering and nearly spherical morphology of the particles (**Fig. 5.2.1b**). Additionally, average particle size was re-confirmed by Image J analysis (based on TEM image) and the average particle size was observed 7 nm (**Fig. 5.2.1c**). Further the elements were confirmed by EDS mapping analysis. The mapping (**Fig. 5.2.1d**) analysis detected the presence of higher weight selenium elements percentage in nanoparticle. The SeNPs X-ray plans (100), (101), (110), (102), (111), (201), (112), (202), (210), (113) and (301) were matched at diffraction peaks at 2Ɵ of 23.64°, 29.85°, 41.34°, 43.73°, 45.50°, 51.67°, 55.90°, 61.40°, 65.33° and 71.70° (**Fig. 5.2.1e**). All the diffraction peaks in the 2Ɵ range corresponds to the hexagonal structure of selenium with lattice constants a = 4.357 Å, c = 4.945 Å and good agreement with the standard JCPDS data (JCPDS No. 06-0362). FTIR measurements were carried out to identify the possible interactions between selenium salt with culture filtrate. The FTIR absorption spectra of synthesized SeNPs was shown the presence of bands at around 3672, 2930, 1696, 1535, 1404, 1233 cm^{-1} (**Fig. 5.2.1f**). The peak at 3672 and 2930 cm^{-1} were characteristic of the –OH and C–H stretching vibration. The bands at around 1650, 1535 and 1233 indicated the presence of amide II and III of proteins, respectively. The bands at 1404 cm^{-1} confirmed the symmetrical stretching vibrations of –COO. The two band at 1233 cm^{-1} showed a resemblance to the C–N stretching vibrations of amines. The IR spectra was similar to Sarkar et al. (2012). With the overall observations, it can be concluded that the proteins might have formed a coating over the nano selenium, which in turn supports their stabilization. Due to this, the nano-Se persisted for several months in liquid suspension.

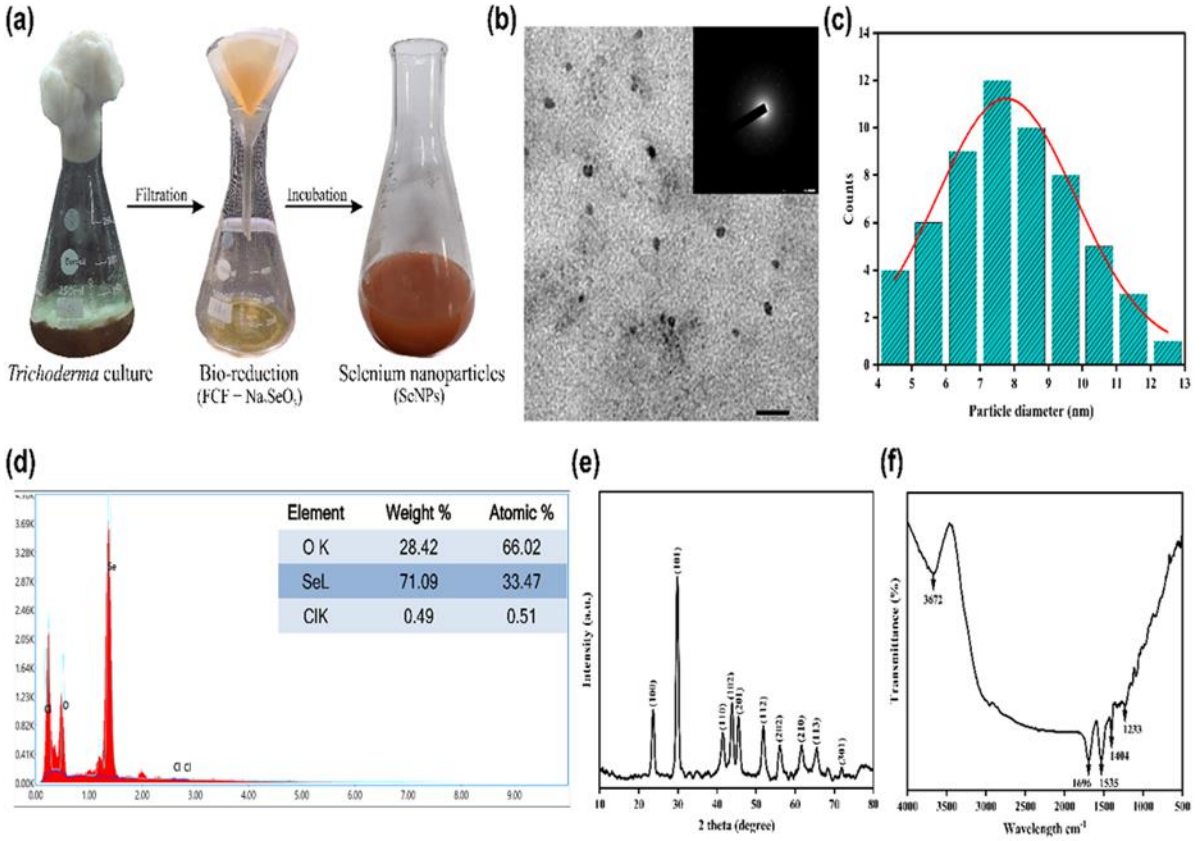


Fig. 5.2.1. Characterization of selenium nanoparticles (SeNPs) (a) SeNPs synthesis, (b) TEM and SAED pattern, (c) particle size distribution, (d) EDX, (e) XRD, and (f) FTIR of SeNPs

5.2.3.2. Seedling growth and photosynthetic pigments

In order to evaluate the effect of As on rice seedlings, growth and photosynthetic pigment concentrations were measured (**Fig. 5.2.2a-e**). In comparison to the control, the height of rice seedlings in 25 µM and 50 µM As were decreased by 17% and 35%, respectively (**Fig. 5.2.2b**). However, the difference in height of rice seedlings in SeNPs and control was not significant. Although, plant height was improved when SeNPs was supplemented together with 25 µM and 50 µM As as compared to their respective As treatments. Further, the plant dry weight was reduced by 30.4% and 31.9% in 25 µM and 50 µM As, respectively and slightly increased by 3% in SeNPs treatment as compared to control (**Fig. 5.2.2c**). Moreover, the plant dry weight in 25 µM As + SeNPs and 50 µM As + SeNPs were improved by 22% and 12.4% as compared to 25 µM and 50 µM As, respectively.

The photosynthetic pigments content in rice seedlings were heavily affected by As exposure. The total chlorophyll content in rice plant was reduced by 37% and 42% in 25 µM and 50 µM As

treatments, respectively as compared to control. (**Fig. 5.2.2d**). Similarly, the carotenoid content was also decreased by 34% and 35% in 25 µM and 50 µM As treatments, respectively as compared to control (**Fig. 5.2.2e**). However, SeNPs treatment in As stressed rice plants improved both chlorophyll and carotenoid concentration in comparison to respective As treatments.

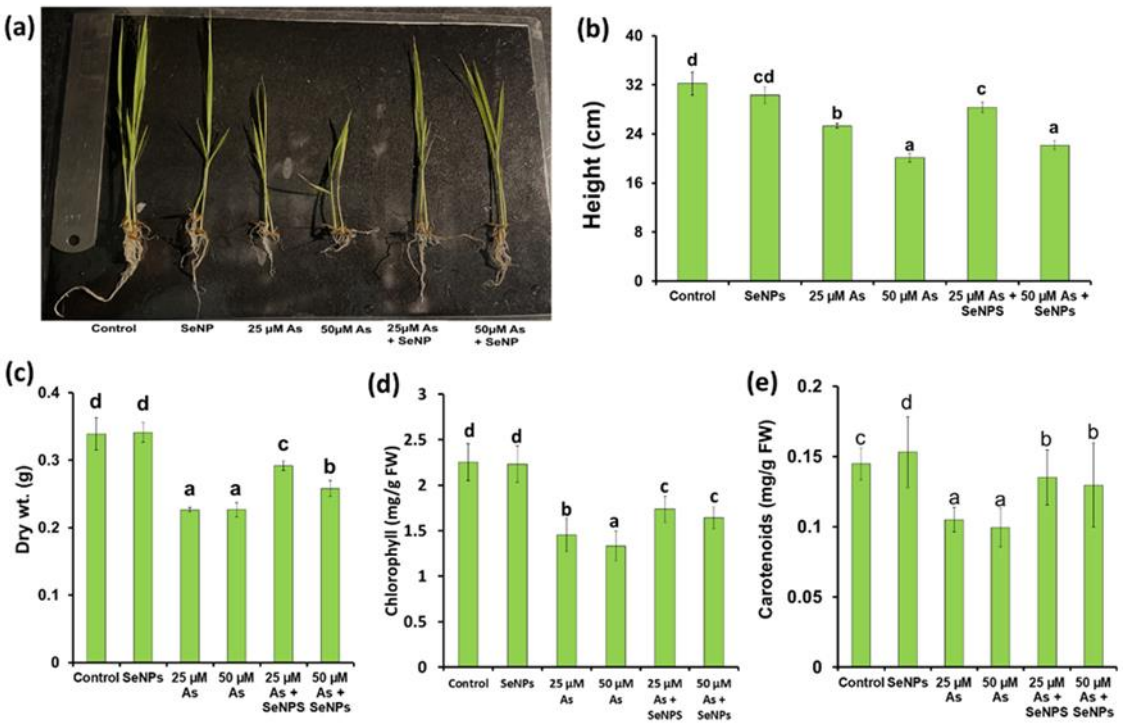


Fig. 5.2.2. Seedlings growth after 15 days (a), height (b), dry weight (c), chlorophyll (d), and carotenoids content (e) in rice seedlings. Different letters indicate significant differences ($P < 0.05$) among different treatments ($n = 3$)

5.2.3.3. Oxidative stress indicators and histochemical detection of H_2O_2 and O_2^{-*} in rice seedlings

Arsenic stress in rice plant significantly ($p<0.05$) increased the TBARS level in root (47-57%) and shoot (48-60%), compared to control (**Fig. 5.2.3a**). The SeNP treatments again significantly reduced the TBARS level in root (28-31%) and shoot (20-29%) of As stressed rice seedlings, compared to rice seedlings exposed only to As stress. Arsenic (25 µM) stress caused higher generation of H_2O_2 compared to control (**Fig. 5.2.3b**). In 50 µM As treatment, H_2O_2 content in root and shoot were increased by 60% and 56%, respectively as compared to control. In contrast, SeNP+As treatments effectively reduced the H_2O_2 level in the root (28-30%) and shoot (21-26%) compared to only As treatments.

The fluorescent intensity of H_2O_2 and O_2^{*-} in root tissue of rice seedlings were visualized after stained with fluorescent dye (**Fig. 5.2.3c**). The high intensity of green and red fluorescence indicates high oxidative damage in tissue. It was observed that the green (H_2O_2) and red (O_2^{*-}) fluorescence intensity in the SeNPs+As-treated root tips were lower than that of the As-treated root tips. The positive impact of SeNPs helps to reduce the ROS generation in plant which yielded lesser production of H_2O_2 and O_2^{*-} in root tissue.

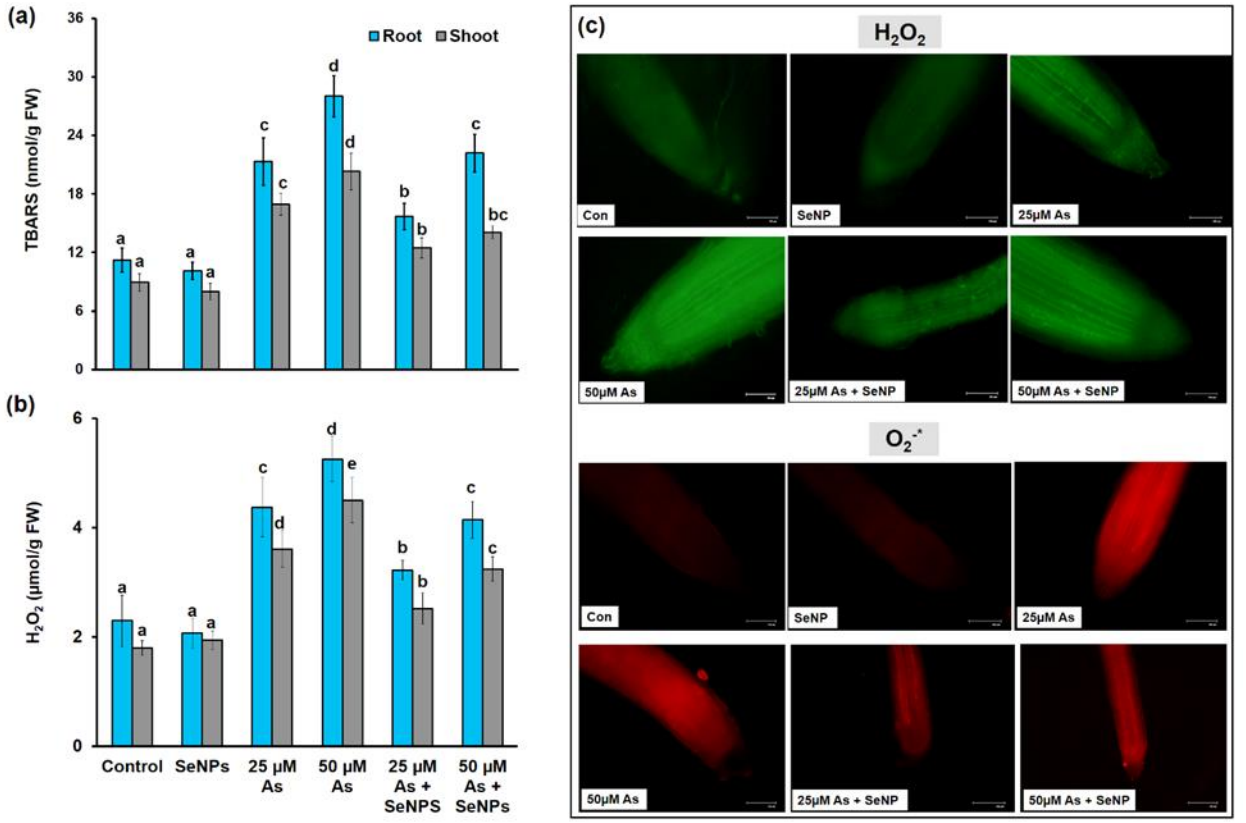


Fig. 5.2.3. TBARS (a), H_2O_2 (b), and histochemical detection of H_2O_2 and O_2^{-*} (c) in root of rice seedlings. Different letters indicate significant differences ($P < 0.05$) among different treatments ($n = 3$)

5.2.3.4. GSH and phytochelatins contents in rice seedlings

The concentration of thiol compounds was higher in root as compared to shoot in all treatments. Compared to individual As treatments, As+SeNP treatments have greater levels of GSH, GSSG, and NPTs content in rice shoot and roots (**Fig. 5.2.4a-c**). In shoot, the level of GSH, GSSG and NPTs were more or less similar. Similarly, in shoot, the difference in GSH, GSSG and NPTs levels were insignificant among two As+SeNP treatments. SeNP supplementation also increased the NPT content in rice root and shoot. Similarly, SeNP treatment improved the PC contents in rice

seedlings (**Fig. 5.2.4d**). SeNP treatment increased the PC content in both root and shoot under 25 µM As and 50 µM As. Interestingly, PC concentration in root of SeNP and 25 µM As and 50 µM As were remain indifferent, whereas in shoot, two individual As have greater accumulation of PCs.

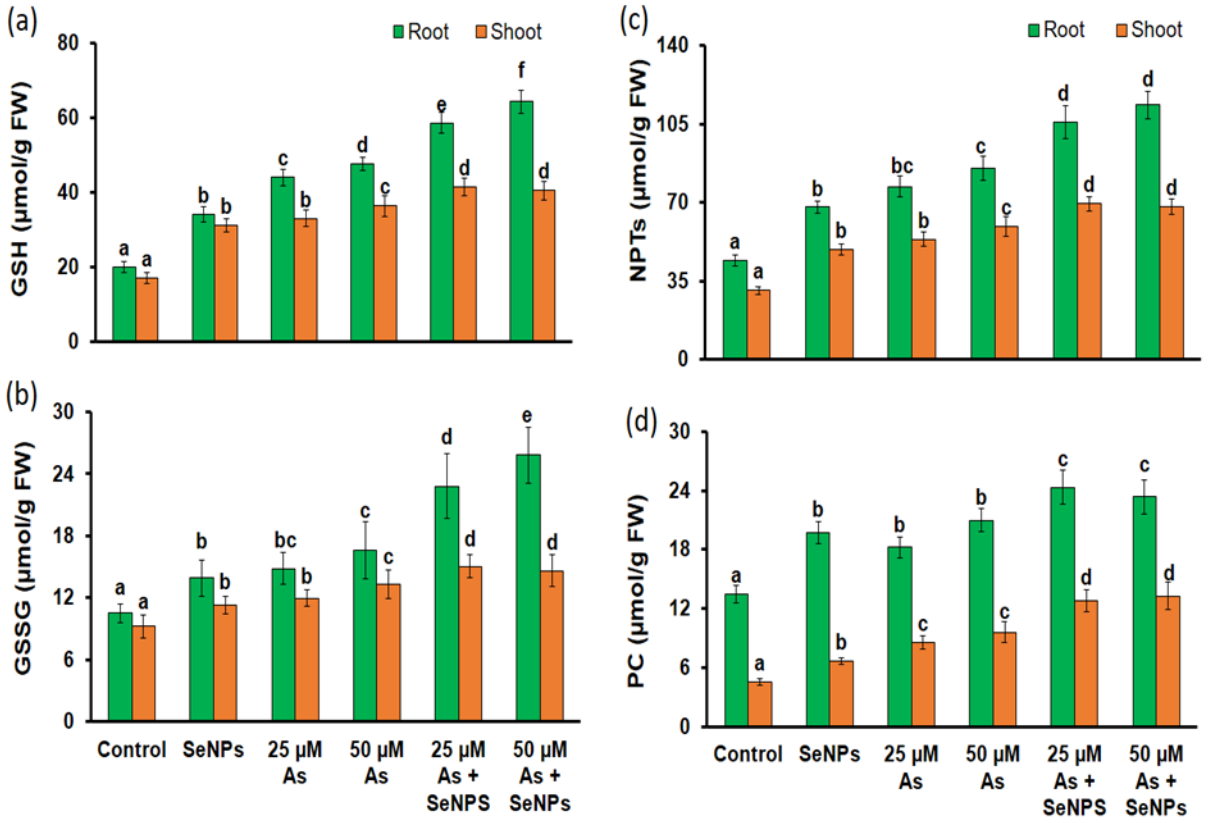


Fig. 5.2.4. GSH (a), GSSG (b), NPTs (c) and PC content (d) in rice seedlings. Different letters indicate significant differences ($P < 0.05$) among different treatments ($n = 3$)

5.2.3.5. Activity of antioxidant enzymes

The activity of SOD, CAT and APX was increased after 25 µM As and 50 µM As treatment (**Fig. 5.2.5a-c**). Similarly, SeNP treatment increased the CAT, APX and SOD activity in both root and shoot of rice seedlings. The SeNP treatment significantly ($p<0.05$) increased the CAT, SOD and APX.

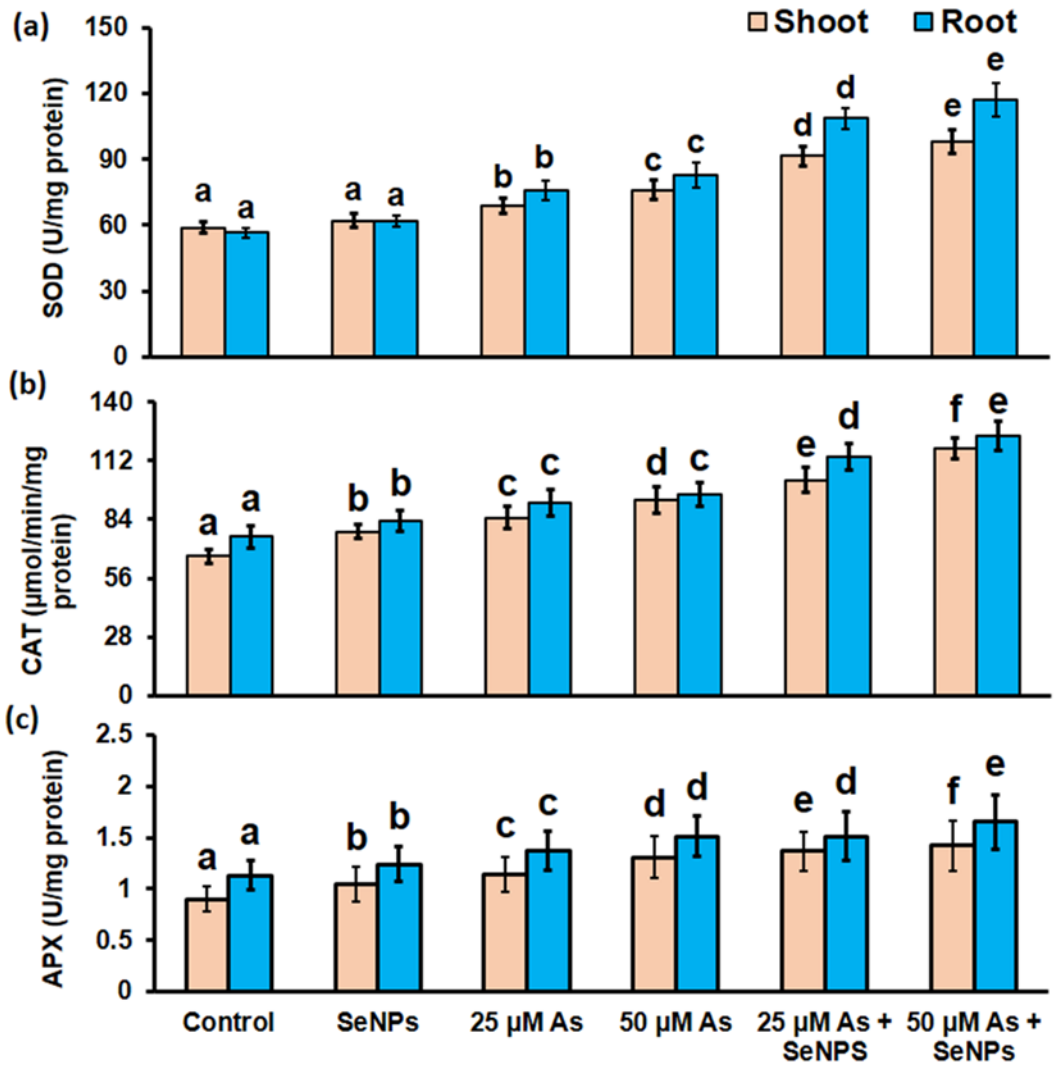


Fig. 5. SOD (a), CAT (b), and APX activity (c) in rice seedlings. Different letters indicate significant differences ($P < 0.05$) among different treatments ($n = 3$)

5.2.3.6. Accumulation of arsenic and selenium

The concentration of As in different As-treated rice shoots was substantially reduced by the addition of SeNP (**Fig. 5.2.6a**). Arsenic content in shoot under 25 µM As + SeNPs and 50 µM As + SeNPs were reduced by 38% and 35.2%, respectively as compared to their respective As treatments. While the As content in root showed no significant change among different treatments. The shoot Se content was significantly increased due to application of SeNP (**Fig. 5.2.6b**). However, the Se content in shoot under 25 µM As+SeNP was decreased significantly ($p<0.05$) by 25% with respect to SeNP treatment. There was no significant difference in shoot Se content among 50 µM As +SeNP and SeNP treatment. The root Se content among different SeNP treated groups including As have showed similar Se accumulation pattern in root. However, SeNP

application significantly ($p<0.05$) reduced the As translocation in rice seedlings (**Fig. 5.2.6c**). SeNP treatment reduced the translocation of As by 34.3-30.2%, as compared to only As treated groups.

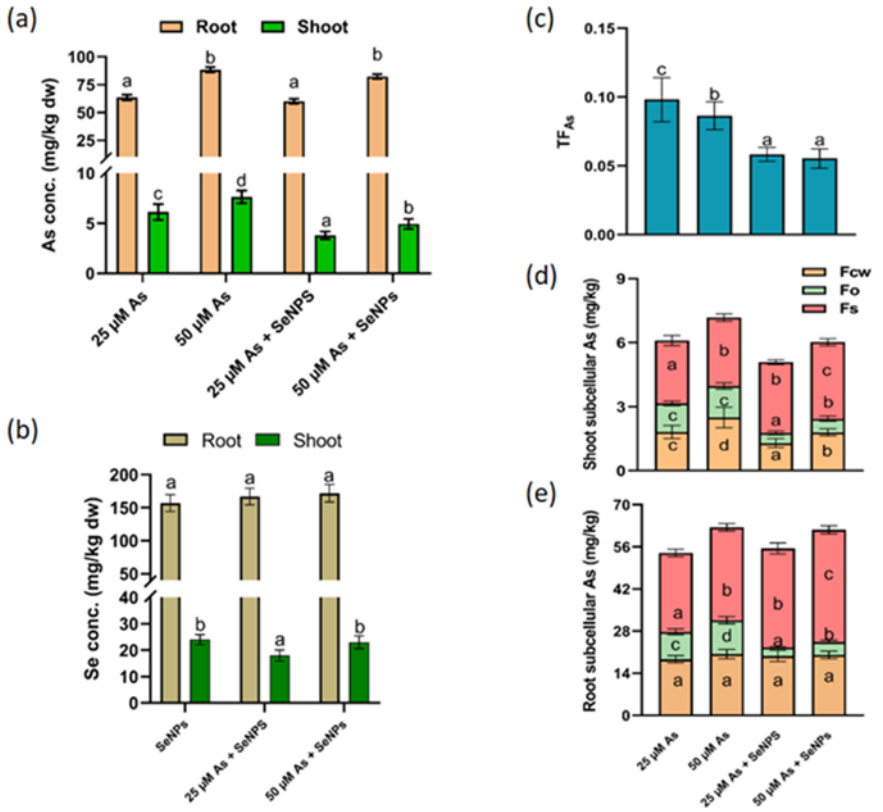


Fig. 5.2.6. Arsenic accumulation (a), Selenium accumulation (b), Arsenic translocation (c), shoot subcellular distribution of arsenic (d), and root subcellular distribution of arsenic (e). Where, Fcw = Cell wall fraction, Fo = Organellar fraction, Fs = Soluble fraction. Different letters indicate significant differences ($P < 0.05$) among different treatments ($n = 3$)

5.2.3.7. Accumulation of arsenic in different cell fraction

Arsenic accumulation in different subcellular fractions were similar in all treatment groups *i.e.* soluble > cell wall > organelle fraction (**Fig. 5.2.6d-e**). However, the trend of As accumulation in different subcellular fractions were changed in SeNP treatments, except cell wall. Arsenic accumulation in soluble (vacuole) fraction was increased after SeNP treatments. Following SeNP treatment in 25 µM and 50 µM As, the accumulation of As in the soluble fraction increased by 20.1% and 17.1% in the root when compared to the corresponding only As treatment groups at 25 µM and 50 µM As. Similar trend was also observed in shoot. Moreover, the application of SeNP decreased the accumulation of As in the organelle fraction in both the root and shoot.

5.2.3.8. Transcriptional response of genes

By comparing the relative expression of rice genes under As exposure with that of corresponding control treatments, the effect of As treatment was assessed (**Fig. 5.2.7**). As compared to 25 µM As and 50 µM As treatments, SeNP treatment does not alter the expression of OsLsi1 and OsLsi2 genes in the root of plants in presence of 25 µM and 50 µM As. In both the 25 µM As and 50 µM As, the expression of OsPCS1 and OsPCS2b were considerably up regulated in root and shoot. Again, SeNP treatment under 25 µM As and 50 µM As stress enhanced OsPCS1 and OsPCS2b expression by 1.39-1.62 fold and 1.45-1.58 fold, respectively in the root and 1.22-1.24 fold and 1.70-2.09 fold, respectively in shoot; as compared to both 25 µM As and 50 µM As treatments. Similarly, the expression of OsABCC1 expression in root significantly upregulated in As+SeNP treatment as compared to only As treatments.

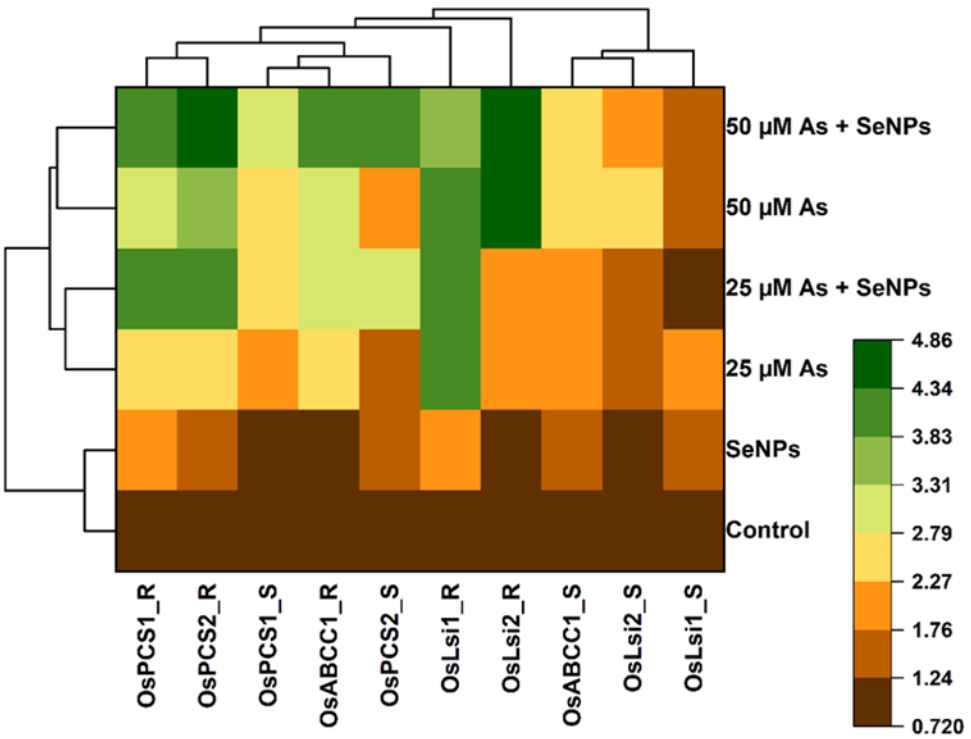


Fig. 5.2.7. Heat map and clustering analysis of targeted genes in rice seedlings. In the heat map, the rows represent genes while the treatments are shown as columns. The row dendrogram represents the gene clusters with similar pattern and column dendrogram represents the treatment clusters with similar pattern. The color code corresponds to the different level of gene expression i.e. low (brown) to high (green). S and R in the gene name represent root and shoot

5.2.4. Discussion

5.2.4.1. Improvement of the growth of arsenic stress and photosynthetic inhibitory effects by SeNPs

Arsenic is a hazardous environmental contaminant that is harmful to both humans and plants. Arsenic toxicity reduced the growth of plants, photosynthesis, and yield. Arsenic exposure significantly reduces the growth of rice seedlings (Fig. 5.2.2a-c). In our previous study, it was shown that different As species had an adverse effect on the growth of rice seedlings. In this study, SeNP application reduced the As accumulation and its translocation (TF_{As}; Fig. 5.2.6c) to shoot. The reduced accumulation of As in rice seedlings upon SeNP application improved overall growth of rice seedlings. Selenium is vital for human health and is considered to be good to plants (Trippe and Pilon-Smits, 2021). In the present study, biosynthesized SeNPs readily reduce As toxicity and increase rice seedling height and biomass in comparison to As stressed plants. Previous studies also demonstrated that different forms of Se can reduce the metal(loid)s toxicity in plants (Farooq et al., 2022; Zeeshan et al., 2021). Azimi et al. (2021) reported that, under As stress conditions, Se-NPs had a growth-promoting impact on *Dracocephalum moldavica*. The beneficial role of SeNPs on promotion of growth of rice seedlings under As stress is well supported by Domokos-Szabolcsy et al. (2012). The study report showed that SeNPs had a positive effect on biomass growth and organogenesis of *Nicotiana tabacum* roots.

One of the key indicators of heavy metal oxidative stress in plants is the efficiency of photosynthetic activity. Photosynthetic pigments like chlorophyll and carotenoids are useful bio-indicators of plant growth status. Photosynthetic pigment levels were decreased in As-treated plants (Fig. 5.2.2d,e). Nitrogen (N) is a critical component of chlorophyll and is directly impacted by As stress, which limits its bioavailability to the plant. Thus, the reduced N bioavailability and uptake under As stress led to a decrease in the concentration of chlorophyll. Reduced chlorophyll production caused by enzyme inhibition and low levels of chlorophyll and carotenoids may be related to the disruption of pigment-protein complexes (Rai et al., 2022). Siddiqui et al. (2020) found that As stress substantially raised the expression of the chlorophyllase in *Vicia faba*. In this study, the application of SeNPs to As-stressed rice seedlings helped to restore the photosynthetic pigments by reducing the As accumulation in rice seedling shoot. According to Qi et al. (2021), supplementing Se improves carbohydrate metabolism, restores chloroplast ultrastructure, increases chlorophyll production, and inhibits chlorophyll breakdown, all of which promote the

growth of *Brassica napus*. SeNP application was reported to upregulate the expression of PS-II related genes in rice under combined lead and cadmium stress (Wang et al., 2021). The administration of SeNP in this study increased the amount of carotenoids in rice seedlings under As stress. Additionally, as carotenoids are involved in quenching singlet oxygen species and other free radicals, a rise in their concentration in response to Se application is a sign that Se has stress-protective properties (Collins 2001). Carotenoids are known to stabilize proteins in light harvesting complexes and thylakoid membranes (Gill et al., 2011).

5.4.2.2. Effect of SeNPs on oxidative stress management and antioxidant enzyme activity

Excessive free radical production results in oxidative stress, which further breaks down different biomolecules. Arsenic stress alters ion transport and balance inside the plant cell, causing oxidative and osmotic stress. Under As treatment, high levels of O_2^-, H_2O_2, and lipid peroxidation (shown as TBARS) were found as a marker of ROS formation (Fig. 5.2.3a-c). Histochemical assessment of O_2^- and H_2O_2 in rice root revealed that As stress first elevates O_2^- and H_2O_2 that damages cell membranes, resulting in increased lipid peroxidation in plant cells. Only As-treated groups would have greater levels of oxidative stress in their leaves and roots, which might disrupt their metabolism and disrupt their membrane structure (Singh et al., 2018). SeNPs supplementation reduced H_2O_2 and TBARS accumulation in plant tissues, indicating that As stressed plants were more likely to escape oxidative damage when SeNPs were applied. In earlier studies, the potential of different forms of Se to mitigate oxidative stress in several plant species under metal(loid) stress was noted (Qi et al., 2021; Shahid et al., 2019; Singh et al., 2018; Zeeshan et al., 2022).

Arsenic-induced ROS produces oxidative damage, which activates the plant's antioxidant defense mechanism. Plants have a variety of antioxidant enzymes (e.g., CAT, SOD, APX) that assist in eliminating ROS and protect cells from oxidative damage caused by metal(loid) stress. Consequently, by regulating ROS generation and elimination, CAT, APX, and SOD lessen the effects of stress. For instance, SOD is a crucial enzyme that converts O_2^- into H_2O_2 and is used to neutralize ROS. The produced H_2O_2 is then subsequently scavenged by CAT into H_2O and O_2 utilizing ascorbate (Zeeshan et al., 2021). In addition, APX, which is a component of the ascorbate-glutathione cycle, reduces the accumulation of H_2O_2 in tissue by converting it into H_2O. Antioxidant enzyme activities in the present investigation revealed activation of CAT, APX, and SOD over As stress as compared to control (Fig. 5.2.5a-c). In contrast, the current investigation observed that SeNPs application enhances antioxidant enzyme activity when exposed to As, which

is consistent with earlier study on SeNP (Farooq et al., 2022). Under adverse conditions, antioxidant enzyme activities are increased, which assists in reducing ROS levels and maintains plants' physiological function. According to Mozafariyan et al. (2016), the beneficial effect of Se on improving CAT, APX, and SOD activities in tomato can be explained by stimulating antioxidant defense gene expression. Additionally, Se treatment increased the uptake of micronutrients (like Zn, Mn, and Fe), which are crucial components of antioxidant enzymes thus increasing the activity of these enzymes (Azimi et al., 2021). According to our outcomes, increasing antioxidant enzyme activities by SeNPs may mitigate the harmful effects of As-stress by eliminating As-induced oxidative stress.

5.2.4.3. Effect of SeNPs on arsenic uptake and accumulation in subcellular fractions

Arsenite (AsIII) is taken up by rice plants, and mainly accumulated in their roots and further translocated to their shoots. Aquaporins OsNIP2;1 (OsLsi1) and OsNIP2;2 (OsLsi2) are mainly responsible for AsIII uptake in rice root (Mawia et al., 2021). OsLsi1 (As/Si influx transporter) and OsLsi2 (As/Si efflux transporter) were located at distal side of exodermis and proximal side of endodermis, respectively. In this study, different levels of As treatments upregulate the expression of OsLsi1 and OsLsi2 expression in root, which increased the As accumulation in root and its translocation in shoot (Fig. 5.2.7). Interestingly, SeNP treatment did not downregulate the OsLsi1 and OsLsi2 expression in root as compared to only As treatments, which was consistent with our As accumulation data in root. It was observed that Selenite (Se^{IV}) accumulates in plants by competitive uptake through Lsi1 transporter (Chauhan et al., 2020). However, SeNPs in this experiment could not inhibit uptake of AsIII through Lsi transporters, thus similar uptake and expression pattern of Lsi1 and Lsi2 were observed in both As and As+SeNP treatments. However, conflicting results were observed in different experimental works where addition of Se^{IV} only inhibit the As translocation from root to shoot (Camara et al., 2018; Kramarova et al., 2012). The position of Lsi1 and Lsi2 in exo- and endo-dermis make it difficult to accurate expression of these genes utilizing expressional studies. Thus, more specific studies involving Se is required in this area for better understanding. Wang et al. (2021) also observed that SeNP treatment did not affect the AsIII uptake in root. In growth medium at pH 5.5–5.8 and pH < 8, arsenite is present as an uncharged molecule of $As(OH)_3$ (Ma et al., 2008), and Si influx transporters are more permeable to uncharged substrates such silicic acid, glycerol and arsenite in the lower pH range (such as pH 5.5) (Bakhat et al., 2017).

Plant roots accumulate As, which is subsequently transported through the xylem to the shoots. Se has an antagonistic function in As translocation from roots to shoots, based on this study's findings that SeNPs substantially decreased the As content in rice shoots (Fig. 5.2.6a, c). Wang et al. (2021) also confirmed that SeNP significantly reduces the As translocation and accumulation in shoot. A key defence mechanism in plants for metal detoxification, involves the chelation of metal(loid)s and their subsequent sequestration into cellular components including cell walls and vacuoles (Huang et al., 2021). A large amount of As accumulated in the root subcellular fraction when exposed to different As stress, as opposed to the shoot subcellular fraction suggests that rice may have used an extrusion technique to reduce the As transport to above ground plant parts. Among different sub-cellular fractions, As was mainly accumulated in the soluble fraction, which contains the cytoplasm and vacuoles, than in the cell wall and organelle fractions. Cell wall is reservoir of different functional groups which facilitate the bindings of metal(loid) in it before metal(loid) actually enters the cell. Proteins and polysaccharides (including cellulose, hemicellulose, and pectin) found in plant cell walls serve as the first line of defense against metal(loid) toxicity and prevent them from entering cell protoplasts (Zeeshan et al., 2022). However, in this investigation, SeNP treatment facilitated vascular sequestration rather than improving As cell wall sequestration. The plant's internal detoxification system stores excess of hazardous substances in the non-sensitive compartment known as the vacuole in order to shield the cell from the harmful effects of these toxic materials. SeNP treatment enhanced As accumulation in the vacuole (soluble fraction) to shield cell organelles from the deteriorating consequences of As toxicity. This demonstrates how the application of SeNPs altered the subcellular distribution of As to reduce As toxicity in rice plant. Arsenic exposure increases the amount of GSH in the cytosol, which also serves as a precursor to phytochelatin (PC) (Mawia et al., 2021). Both GSH and PC are important for chelation of toxic metals and their transportation into vacuole. In Capsicum, free AsIII in the cytosol formed complexes with GSH or PC (AsIII-PC/ AsIII-GSH) for detoxification, which were then transported to the vacuole (Kaya et al., 2022). In this work, the addition of SeNP under As stress significantly improved both GSH and PC content in root and shoot compared to only As stressed rice seedlings. Addition of Se or SeNP increased the GSH and PC content plant to combat metal(loid) stress in plants (Farooq et al., 2022; Huang et al., 2022; Zeeshan et al., 2022). As previously stated, the addition of SeNP enhanced the As concentration in the vacuole, suggesting that excessive PC generation aids in As sequestration. This study evaluated the gene expression of PC synthase (PCS), which is required for the synthesis of PC from GSH. Two PCS, OsPCS1 and OsPCS2, have been identified in rice. In comparison to OsPCS1, OsPCS2 is the major PCS isozyme that regulates

PC production, contributing significantly to rice's ability to tolerate As toxicity and its translocation (Sun et al., 2023). In comparison to only As treatment, As+SeNP treatment significantly enhanced the expression of OsPCS1 and OsPCS2b in both root and shoot. In *Brassica rapa* under Cd stress, Yu et al. (2023) found that the addition of selenite increased PCS1 and PCS2 expression by around 120% when compared to the control group. C-type ABC transporters (ABCC) facilitate As sequestration into vacuoles after being chelated by PC. Overexpression of ABCC1 enhances As tolerance only when co-expressed with PCS, showing the cooperation of PC synthesis and AsIII-PC complex transporters in plant As detoxification (Mawia et al., 2021). Song et al. (2014) revealed that OsABCC1 is required for vacuolar AsIII-PC sequestration, since OsABCC1 knockout results in enhanced As sensitivity. Based on these results, SeNP can improve As vacuolar sequestration in roots by elevating OsPCS1, OsPCS2b and OsABCC1 expression.

5.2.5. Conclusion

This study was conducted to explore the role of SeNPs on As uptake, translocation and toxicity alleviation. The increased Se transport and reduced As concentration in leaf improved the photosynthetic pigment content, and SeNP application also improved the antioxidant activity in rice seedlings which helps to mitigate the toxic effect of ROS. In summary, SeNP did not inhibit the accumulation of As in root which confirmed through gene expression of AsIII transporter in root, as no down regulation of OsLsi1 and OsLsi2 was observed in root. However, cellular fractionation analysis revealed As accumulation in different fractions were in order of soluble (vacuole) > cell wall > organelles. SeNP increased As accumulation in soluble fractions. Increased concentrations of GSH, PC and their related genes OsPCS (OsPCS1 and OsPCS2b) and OsABCC1 under SeNP treatments might aid the vacuolar sequestration of As in root. The enhanced vacuolar sequestration restricts the As translocation from root to shoot. These findings provide new insights into the role of SeNP in As accumulation and its translocation from root to shoot.

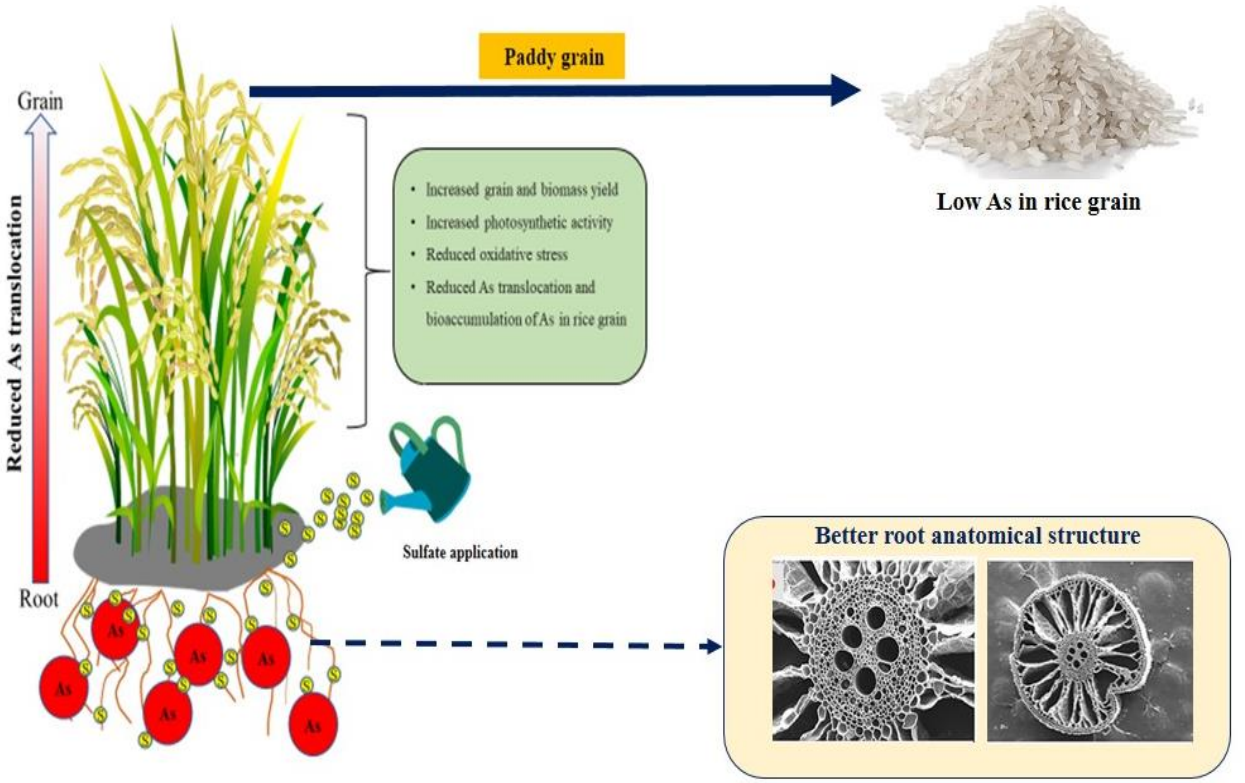
Objective 2

Pot study with different amendments [sulfate (Na_2SO_4), and iron modified mango leaf biochar (FeMBC)] to improve rice plant growth and reduce rice grain arsenic

Chapter 6

Effect of sulfate application on inhibition of arsenic bioaccumulation in rice (Oryza sativa L.*)*

Graphical Abstract



6.1. Introduction

Arsenic is a naturally occurring, toxic metalloid that can cause potential carcinogenic and non-carcinogenic effect on human upon exposure (Joardar et al., 2021a). A large part of Ganga-Meghna-Brahmaputra (GMB) flood plain with an area 569,749 km2 and population over 500 million is one of the worst As affected regions in south Asia due to the presence of elevated levels of As in groundwater (Chakraborti et al., 2018). The situation is at its worst in Bengal delta, Bangladesh and West Bengal, India as over 100 million populations in the zones is increasingly facing problems of using As contaminated groundwater for drinking, cooking and irrigational purposes (Chakraborti et al., 2013). Rice is a staple crop and people living in these regions are consuming substantial amount of As through rice grown with As contaminated water (Chowdhury et al., 2020a, b). Toxicity of As in rice is due to the presence of AsIII and AsV, as methylated forms of As (DMA and MMA) are less toxic to humans (Halder et al., 2014). AsV is predominantly present in aerobic condition, and reduced to AsIII under anaerobic or flooded condition (Mitra et al., 2017). The primary entry route of different As species from soil into the plant system is through root. AsV enters into the plant root via phosphate transporter (PHT) proteins, as AsV is analogous to inorganic phosphate (iP) (Finnegan and Chen, 2012). AsV and iP compete for uptake through same transporter and in presence of low iP in soil, AsV outperforms iP for entry into the plant root. However, primary route of AsIII entry into the plant is via Nodulin 26-like intrinsic proteins (NIPs) (Bienert et al., 2008; Ma et al., 2008; Meharg and Jardine, 2003). Silicon transporter protein Lsi1 serves as a major uptake route for AsIII into the rice root (Ma et al., 2008). Rice plant cultivated in As contaminated soils can bio-accumulate As inside plant and lead to disruption of various physiological processes. Arsenic exposure can reduce the germination percentage and seedling growth during the early growth stage of rice. The yield of rice grain and biomass can be reduced substantially due to high exposure. The concentration of rice grain As is basically dependent on the amount of As present in soil as As translocates from root to grain via different plant parts. Rice grain As is a major burden and a good amount of As (219–664 μg/kg) is consumed through cooked rice in As endemic area of West Bengal, India (Mandal et al., 2019). A major portion of As enters into the human body through As contaminated water and foodstuffs, especially from rice, since rice plants accumulate a good amount of As due to the reducing environment in soil (Chowdhury et al., 2018a). Elevated level of As in rice grain and consumption of cooked rice high in As concentration for long period may cause potential risk to human health (Chowdhury et al., 2020a). Exposure of As in human through dietary route can promote the development of different arsenical

manifestations in exposed population. Apart from non-carcinogenic risk, As exposure can also lead to the development of cancer (Joardar et al., 2021a, b).

Sulfur (S) is an essential nutrient that plays a pivotal role in plant growth, metabolism and defense. By decreasing soil pH through acidification, S application increased the availability of macro and essential micronutrients to plants (Yang et al., 2007). Sulfur treatment improved soil microbial biomass, particularly the amount of *Thiobacilli* and aerobic heterotrophic S-oxidizing bacteria (Yang et al., 2010). Cysteine in plants formed through the reduction of sulfate to sulfide (Smith et al., 1995). Cysteine a major S-containing amino acid, serves as a precursor for various stress responsive compounds, like S-adenosylmethionine (SAM), methionine, glucosinolates and glutathione (GSH) (Rausch and Wachter, 2005). GSH, a reactive oxygen species (ROS) scavenging molecule and also used as substrate for phytochelatin (PC) synthesis, is a S containing molecule (Cao et al., 2018; Duan et al., 2011; Fan et al., 2010). Therefore, the application of S decreases the As translocation from root to shoot and finally in rice grain via GSH mediated detoxification of As (Noctor et al., 2018; Srivastava et al., 2019; Yadav and Srivastava, 2021). Application of S also reduces As translocation from soil to plant by enhancing the iron-plaque formation in rice plant and therefore, As becomes less bioavailable to plants (Hu et al., 2007). Soil microbial redox reduction of sulfate into sulfide also immobilizes As by precipitation of secondary FeS or AsS (Xu et al., 2019). Therefore, proper dose of sulfate fertilizers is important to minimize the S deficiency and As toxicity in plants. Thus, the objectives of this experiment were to demonstrate: (1) the effect of sulfate on growth and yield of rice under As stress, (2) effect of sulfate on As uptake and translocation.

6.2. Materials and methods

6.2.1. Soil sampling and its characterization

Soil sample was collected from Madhusudankati village (22°54′14.51″ N, 88°46′25.36″ E) of Gaighata block in North 24 Parganas district which is one of the worst As endemic regions in West Bengal, India (Chowdhury et al., 2020b). The sample was collected from farmland (0–15 cm depth) and air dried properly. Physico-chemical and textural properties of top soil and irrigational water were given in **Table 6.1**. The soil was mainly clay type in character (clay: 68.2%, sand: 11.2%, and silt: 20.6%) with pH, oxidation-reduction potential (ORP), and EC of 7.43 ± 0.01, 174 ± 3.1 mV, and 57.8 ± 0.2 mS/m, respectively. Soil organic carbon, available N, P, K, and S were 0.83 ± 0.06%, 49.1 ± 1.4 mg/kg, 14 ± 1.8 mg/kg, 78 ± 1.33 mg/kg, and 10.6 ± 0.22 mg/kg, respectively. Total As and Fe concentrations in soil were 11.6 ± 0.2 mg/kg and 19.4 ± 0.88 g/kg,

respectively. The soil was irrigated with treated surface water supplied through a local non-government organization (Joardar et al., 2021b) throughout the experiment. pH, sulfate, As, and Fe in the water were 7.05 ± 0.08, 3.45 ± 0.1 mg/L, < 0.003 mg/L, and 0.23 ± 0.04 mg/L, respectively.

Table 6.1: Physico-chemical and textural properties of top soil and irrigational water

Soil*	
Parameters	**Values**
pH	7.43 ± 0.01
ORP (mV)	174 ± 3.1
EC (mS/m)	57.8 ± 0.2
Organic carbon (%)	0.83 ± 0.06
Available N (mg/kg)	49 ± 1.4
Available P (mg/kg)	14 ± 1.8
Available K (mg/kg)	78 ± 1.33
Available S (mg/kg)	10.6 ± 0.22
Total Fe (g/kg)	19.4 ± 0.88
Total As (mg/kg)	11.6 ± 0.2
Soil texture (clay)	
clay (%)	68.2
sand (%)	11.2
silt (%)	20.6
Irrigation water**	
Sulfate (mg/L)	3.45 ± 0.1
Iron (mg/L)	0.23 ± 0.04
Arsenic (mg/L)	< 0.003
pH	7.05 ± 0.08

*Soil: 0-15 cm; **Irrigation water (treated surface water)

6.2.2. Seed preparation and plant growth before transplanting

The rice genotype selected for this experiment was *Ranjit (IET- 12554)*, a semi-dwarf variety with good grain yielding capacity. This rice variety was collected from the same study area as that of the soil and this rice variety is being cultivated in As contaminated areas of West Bengal. All the seeds were sterilized with 30% H_2O_2 for 15 min (Zhang et al., 2020) and washed with deionized water three times. The seeds were sown in the seed beds and maintained till the transplanting stage. After 25 days, the rice seedlings were transplanted in the individual experimental pots.

6.2.3. Pot experiment and experimental design

Bottom sealed PVC pots (length: 30 cm × diameter: 30 cm) were used in this experiment to prevent the loss of nutrient and As. Each pot was filled up with 6 kg air dried soil. The experimental set up was composed of a total number of five treatments comprising four different sulfate levels and

one control set and all the treatments were triplicated. Sulfate in soil was administered as Na_2SO_4 at 0 (S0), 20 (S20), 40 (S40), 60 (S60), and 80 (S80) mg/kg in this investigation, based on two previous studies that found the increasing dose of sulfate (up to 120 mg/kg) could significantly reduce As accumulation in plants (Hu et al., 2007; Shi et al., 2020). One week prior to the transplantation of rice seedlings, the N, P, K (urea, super phosphate, muriate of potash) were applied to soil in the ratio of 2:1:2 along with different doses of sulfate solutions. Nitrogen was applied in three split doses; 50% at the time of pot preparation and 25% each at tillering and panicle initiation stage. To stimulate the rice growth, anaerobic condition was maintained with water level up to 4 cm till the maturity stage. Arsenic-safe (<3 μg/L) water was used in this experiment to irrigate rice plants. Plants and rhizosphere soils were taken out from each treatment along with replicates at harvesting stage for further analysis.

6.2.4. Chlorophyll, oxidative stress and antioxidant in plants

Total chlorophyll, cysteine (Cys), TBARS and hydrogen peroxide (H_2O_2) content were measured according to the prescribed method of Arnon (1949); Bates et al. (1973); Gaitonde (1967); Heath and Packer (1968) and Velikova et al. (2000), respectively from 25 DAT (days after transplanting) rice plant.

6.2.5. Antioxidant enzyme assays

For the estimation of antioxidant enzymes, about 0.2 g root and leaf tissues were homogenized separately in pre-chilled mortar and pestle with 2 mL of 0.1 M potassium-phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% PVP (w/v) (Polyvinylpyrrolidone). The homogenized tissue samples were used for the estimation of antioxidant enzymes. The protein concentrations of the enzyme extracts were measured according to Lowry et al. (1951) using BSA protein (Bovine serum albumin) as standard. Superoxide dismutase (SOD; EC 1.15.1.1), Ascorbate peroxidase (APX; EC 1.11.1.11) and Catalase (CAT; EC 1.11.1.6) activity were estimated by following Beauchamp and Fridovich (1971), Nakano and Asada (1981), and Aebi (1984), respectively. The detailed methodology of enzyme assays was placed in our previous publication.

6.2.6. In vivo detection of reactive oxygen species (ROS) with dichlorofluorescein (DCF) staining and root ultra-structural study by scanning electron microscopy (SEM)

At tillering stage, root tips of rice plants from each treatment were selected and kept in 1 mL detection buffer (DB), DB (2.5 mM HEPES, pH 7.4) consisting of 10 mM DCF-2DA fluorescent

dye (Invitrogen, Carlsbad, CA, USA) for 10 min in dark. Amount of ROS in roots were measured in Floid Cell Imaging station microscope (Life Technologies) (Sarkar et al., 2020).

Plants at flowering stage were harvested and the roots of the plants were cleaned properly. Roots were cut transversely and kept in 2.5% glutaraldehyde solution for 2 h followed by dehydration through a series of different concentrations of ethanol (10, 20, 30, 40, 50, 60, and 70%). The dehydrated samples were sputter coated with platinum prior to observation under scanning electron microscope (SEM: Carl Zeiss Model Number – EVO18 Special Edition).

6.2.7. Plant harvesting

At 125 DAT, the plants were harvested and tiller number, number of panicles, number of grains per panicle, number of filled grain per panicle, test weight (1000 grain wt.), above ground biomass and plant height were recorded.

6.2.8. Estimation of total S in plant tissue

For the estimation of total S content in rice plant, the plant sample (0.5 g) was digested overnight with a diacid mixture of nitric acid and perchloric acid at 3:1. The entire digested sample was placed on hot plate for completion of digestion. The digested sample was then filtered and taken for the estimation of total S. The total S content in rice plant was estimated using the turbidimetric methodology prescribed by Tabatabai and Bremner (1970).

6.2.9. Digestion and estimation of total arsenic in plant and rhizosphere soil

About 0.02–0.2 g of dry plant parts and soil samples were digested with HNO_3 and H_2O_2 in 2:1 ratio. The estimation of total As in all digested samples was conducted in an Atomic Absorption Spectrophotometer (Varian AA140, USA) coupled with Vapour Generation Accessary (VGA-77, Agilent Technologies, Malaysia) using the HG-AAS method. The intricate information of the HG-AAS system was reported in our earlier publications (Chowdhury et al., 2020a; Joardar et al., 2021a).

6.2.10. Quality control and quality assurance

To maintain the quality control and quality assurance of the generated analytical data, As concentrations in standard reference materials (SRM) and other analytical protocols like proper standardization, blank measurements, analysing duplicates and spiked samples were measured following the methodology described in our earlier publication (Chowdhury et al., 2020b). The recovery of As in analyzed Rice flour 1568a (NIST, Gaithersburg, MD, USA), Tomato leaf 1573a

(NIST, Gaithenburg, MD, USA), and River sediment 1645 (NBS, Washington, DC) were 90.1 ± 2.78%, 97.7 ± 1.35%, and 91.2 ± 2.69%, against their certified values of 0.29 ± 0.03 mg/kg, 0.112 ± 0.004 mg/kg and 66 mg/kg, respectively.

6.2.11. Bioconcentration and translocation of arsenic in plant

Bioconcentration and translocation factor of As were evaluated according to the process described by Chowdhury et al. (2020b).

$$\text{Bioconcentration factor (BCF)} = C_{plant} / C_{soil}$$

Where, C_{plant} and C_{soil} represent the As concentration (dry weight basis) in rice plants and soils, respectively.

$$\text{Translocation factor (TF)} = C_{stem/leaf/panicle/husk/grain} / C_{root}$$

Where, $C_{stem/leaf/panicle/husk/grain}$ and C_{root} are the concentration of As in the stem, leaf, panicle, husk, grain and root, respectively. The value of TF > 1 means the plants can translocate As effectively from root to other parts of plant.

6.2.12. Statistical analysis

All the experiments were carried out in triplicates and the concentrations were expressed in terms of mean ± standard deviation. The experimental data was statistically evaluated by one-way analysis of variance (ANOVA) test in OriginPro (2019b) software and the average values were separated by Tukey-HSD test at $p \leq 0.05$ significance level.

6.3. Results

6.3.1. Effect of sulfate application on plant growth and yield

Rice plant height and biomass had changed significantly from transplanting stage to harvesting stage. The findings revealed that As stress has a substantial impact on rice growth and yield attributes. However, the difference in plant biomass and height were observed in mature plant due to different doses of sulfate application compared to S0 treatment (**Table 6.2**). In comparison to the control set (S0), an increase of 11.2% and 28.9% in plant height and above ground biomass were observed at the highest sulfate dosage (S80). Soil As also had an effect on the number of tillers and panicles, with more pronounced effects in rice plants of S0 treatment. As a result of lower number of tillers and panicles in rice plants under As stress, grain production in the S0 treatment was reduced. However, S80 treatment improved the grain test weight by 14.1% as

compared to S0 treatment. In comparison to S0 treatment, the grain yield and grain filling were increased by 26.2% and 38.7%, respectively, in S80 treatment. In addition to grain yield and grain filling loss, As toxicity has been considerably decreased test weight of rice grain. However, S80 treatment improved the grain test weight by 14.1% as compared to S0 treatment. Among different levels sulfate fertigation, S20 had the least influence on plant growth and yield attributes under As stress. The ANOVA analysis showed that results of growth and yield attributes were mostly insignificant in S40 and S60 treatment. Among all treatments, the growth and yield attributes were found to increase steadily in a sequence of S0 < S20 < S40 < S60 < S80.

Table 6.2: Effect of sulfate application on different yield attributes under arsenic stress

Treatments	No. of Tillers	No. of panicle	No. of grain/panicle	No. of filled grain/panicle	Test wt. (g)	Above ground biomass (g d.w.)	Plant height (cm)
S0	10.3±1.54c	11±1c	111±4.04d	79±4e	18.3±0.71c	30.2±2.39e	94.7±4.34d
S20	11.7±1.15b	12.7±0.57b	131±4.50c	103±4.5d	19.2±0.72bc	33.3±2.64d	97.7±3.91c
S40	12.7±0.57ab	13±1ab	141±5.50b	115±5.56bc	19.7±0.77b	35.4±2.80c	100±4.02b
S60	13±1a	13.7±0.57a	145±5.03b	121±6.02b	20.8±0.78ab	38.3±3.03b	102±4.07b
S80	13.3±0.57a	14±1.15a	151±6.55a	129±6.55a	21.3±0.8a	42.4±3.36a	107±4.27a

The same letters with in the same column indicate values are not significantly different at $P \leq 0.05$ using Tukey's HSD multiple comparison test. [S0 = No sulfate, S20 = 20 mg/kg sulfate, S40 = 40 mg/kg sulfate, S60 = 60 mg/kg sulfate and S80 = 80 mg/kg sulfate].

6.3.2. Effect of sulfate application on oxidative stress, photosynthetic activity, and anatomical structure of root

Staining of rice roots with DCF-2DA gave a qualitative estimate of in vivo ROS. The toxicity triggered by As, led to the production of ROS inside the cell. The increased ROS production inside cell ultimately increased the intensity of green fluorescent. It was evident that the gradual increase in the sulfate application decreased the intensity of green fluorescent in root tips (**Fig. 6.1a–e**). The subsequent measurement of H_2O_2 content in root and leaf showed the maximum level of H_2O_2 attained when there was no sulfate applied (**Fig. 6.2a**). The reduction in H_2O_2 levels were 26.9% and 29% in leaf and root of S80 treatment, respectively as compared to S0 treatment. The significant ($p \leq 0.05$) reduction in TBARS (thiobarbituric acid reactive substances) content was observed with the increment in sulfate application. The amount of TBARS in root (71.3 ± 5.44 nmol/g FW) and leaf (55.5 ± 4.24 nmol/g FW) of S0 set were highest among all the treatments. Increasing sulfate fertigation significantly ($p \leq 0.05$) increased Cysteine content in rice root and

shoot (**Fig. 6.2c**). Cysteine content was high in root for all the treatments as compared to leaf. Rice plant root exposed to S80 treatment had higher concentration of Cysteine (45.7%) than S0 treatment. Total chlorophyll content increased significantly ($p \leq 0.05$) with the increment in sulfate application (**Fig. 6.2d**). The total chlorophyll content increased by ~15.8%, 23.2%, 29.3%, 34.6% in S20, S40, S60, S80, respectively as compared to S0. The cross section of root was observed under SEM to analyze its anatomical structure under As stress. The SEM study revealed highest distortions of root cellular structure in S0 set (**Fig. 6.3i–j**). The distortions in epidermis, endodermis, undeveloped aerenchyma and loss of root hair were prominent in control due to toxicity triggered by As. However, sulfate application gradually improved the root anatomical structure and growth under As stress (**Fig. 6.3a–h**).

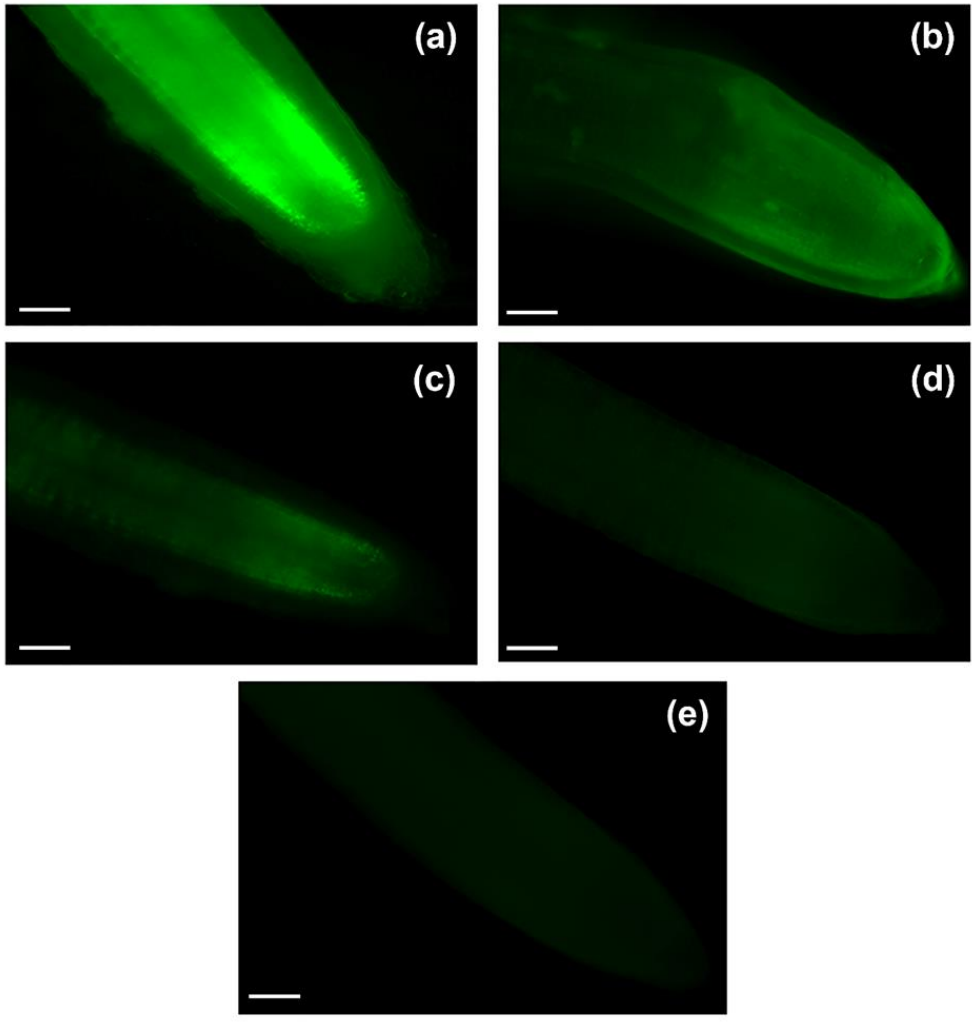


Fig. 6.1. In vivo detection of ROS in rice root, obtained through different treatments, observed under Floid's cell imaging station microscope. The white bars indicate a scale of 100 µm [S0 (a), S20 (b), S40 (c), S60 (d), and S80 (e)]

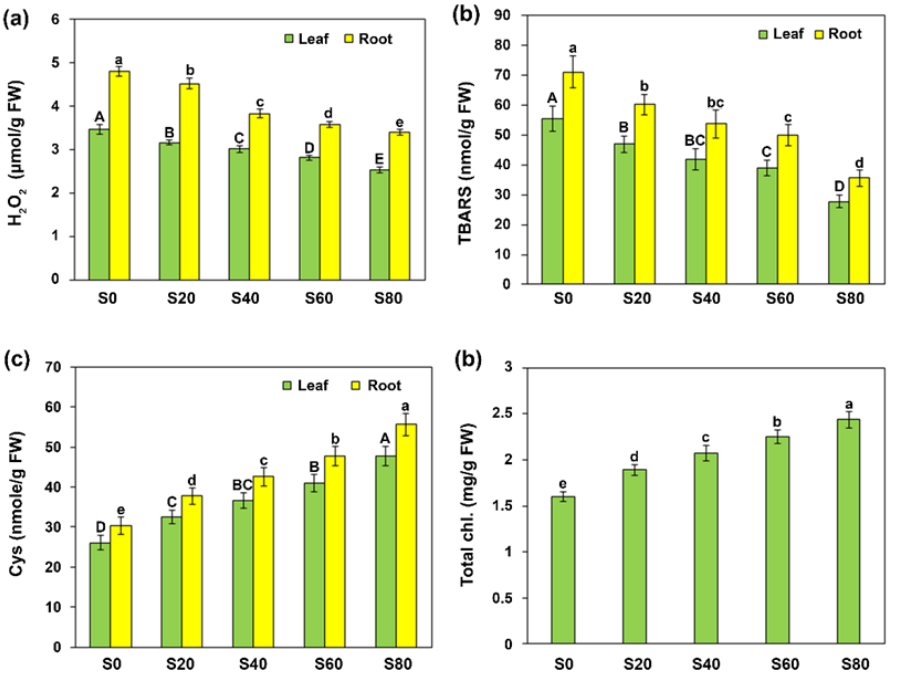


Fig. 6.2. Concentration of (a) H_2O_2, (b) TBARS, (c) Cysteine, and (d) total chlorophyll in rice plant obtained through different treatments. Vertical bars represent mean with standard deviation and same letters in the vertical bars are not significantly different (Tukey's HSD multiple comparison at $p \leq 0.05$)

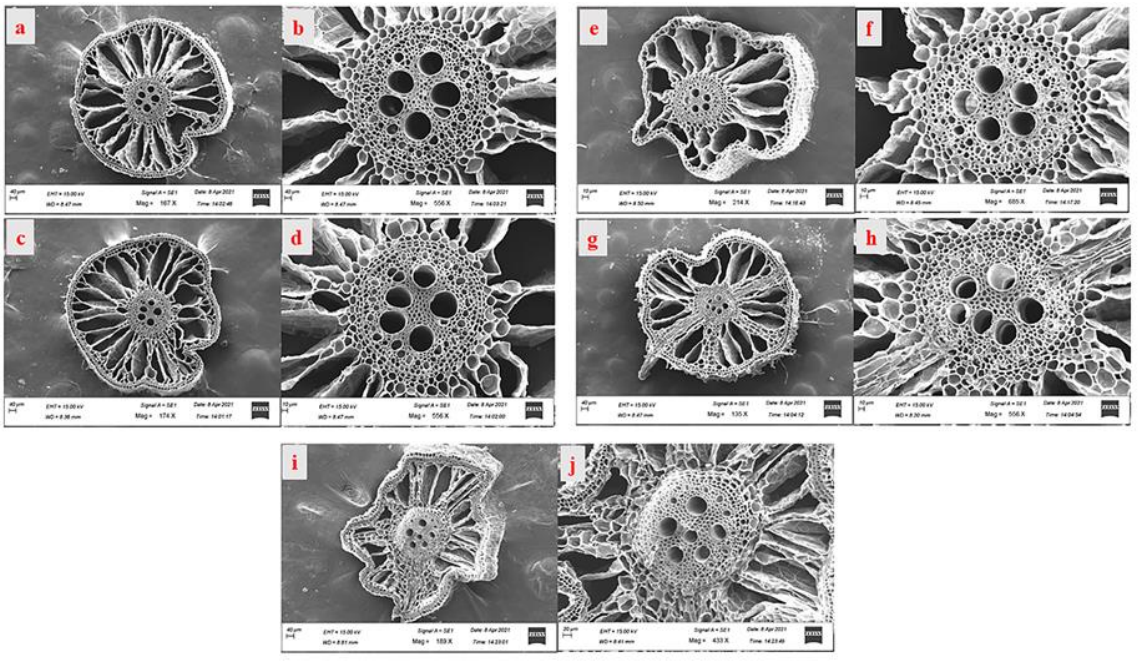


Fig. 6.3. SEM images showing anatomical features of rice root under different sulfate treatments [a-b: S80, c-d: S60, e-f: S40, g-h: S20, and i-j: S0]

6.3.3. Effect of sulfate application on antioxidant enzyme activity

It was evident that As exposure enhanced the activity of antioxidant enzymes (SOD, APX and CAT); however, as the dosage of sulfate was increased, the antioxidant enzyme activity decreased in rice plants. Under stress conditions, SOD is the key enzyme that converts oxyradicals to H_2O_2. The SOD activity in root and leaf of S0 set significantly increased ($p \leq 0.05$) upon As exposure (**Table 6.3**). The SOD activity upon sulfate treatment decreased in dose dependent manner. The SOD activity in root and leaf of S80 treatment decreased by 36.7% and 33.6%, respectively as compared to control set. Degradation of accumulated H_2O_2 into H_2O and O_2 under oxidative stress was catalyzed by APX and CAT. The activity of APX and CAT in root and leaf decreased upon increasing sulfate application as accumulation of H_2O_2 decreased consistently. Due to the high accumulation of H2O2 in root as compared to leaf, the activities of APX and CAT in root were high in all treatments. The APX and CAT activities in root of S80 treatment decreased significantly ($p \leq 0.05$) by 38.7% and 43.5%, respectively as compared to S0 set.

Table 6.3. Activity of Superoxide dismutase [SOD] (U mg^{-1} Protein), Ascorbate peroxidase [APOX] (μmol min^{-1} mg^{-1} protein) and Catalase [CAT] (μmol min^{-1} mg^{-1} protein) in root and shoot of rice plant

Treatments	SOD		APX		CAT	
	Root	Leaf	Root	Leaf	Root	Leaf
S0	115±4.53a	87.9±2.43a	1.24±0.02a	0.95±0.03a	84.2±3.93a	62.7±3.69a
S20	94.7±2.62b	81.5±3.96ab	1.04±0.03b	0.83±0.06b	77.9±4.0ab	53.5±4.2b
S40	83.5±3.39cd	73.7±2.04b	0.9±0.03c	0.73±0.03c	73.2±3.21bc	49.7±3.95bc
S60	79.6±2.66de	67.6±1.87cd	0.85±0.03cd	0.65±0.03d	63.6±3.67c	46.8±1.91c
S80	72.7±2.01e	58.3±3.74d	0.76±0.02d	0.53±0.03e	47.5±4.14d	37.3±5.85d

The same letters with in the same column indicate values are not significantly different at $P \leq 0.05$ using Tukey's HSD multiple comparison test. [S0 = No sulfate, S20 = 20 mg/kg sulfate, S40 = 40 mg/kg sulfate, S60 = 60 mg/kg sulfate and S80 = 80 mg/kg sulfate].

6.3.4. Effect of sulfate application on arsenic bioaccumulation and translocation and S uptake

The application of sulfate had a significant impact on the accumulation of As in different parts of rice plants (**Fig. 6.4a**). Arsenic uptake was further defined in terms of BCF which showed that

increment in sulfate application constantly decreased the BCF from soil to root and root to shoot (**Table 6.4**). Root As concentration (mean ± SD: 14.8 ± 0.37 mg/kg dw, range: 14.5–15.2 mg/kg) was highest in S0 set as compared to other sulfate treatments. Contrarily, the plant sulfate accumulation was found to increase with increment of sulfate level (**Fig. 6.4b**). The uptake of As and S in plant showed a negative relation (**Table 6.5**). Arsenic concentration in different plant parts varied significantly ($p \leq 0.05$) in all treatments. Throughout the treatments, accumulation of As in different parts of plants at harvesting stage was observed in order of root > stem > leaf > panicle > husk > grain (**Fig. 6.4a**). In all cases, TF value of As <1; however, translocation of As from root to grain was significantly affected by sulfate as TF value reduced significantly ($p \leq 0.05$) upon increase in sulfate dose. The TF value of As from the root to other parts of rice plants was similar in the S20 and S0 treatments, indicating that the lower dose of sulfate (S20) had no influence on As translocation. It was also found that the S20 treatment had the highest BCF and TF of all four sulfate regimens. The total S accumulation upon S60 and S80 treatment increased by 74.6% and 67.8%, respectively as compared to S0 set (**Fig. 6.4b**). Similar to As, sulfur was also mainly concentrated in roots than other aerial parts of rice plants in all treatments.

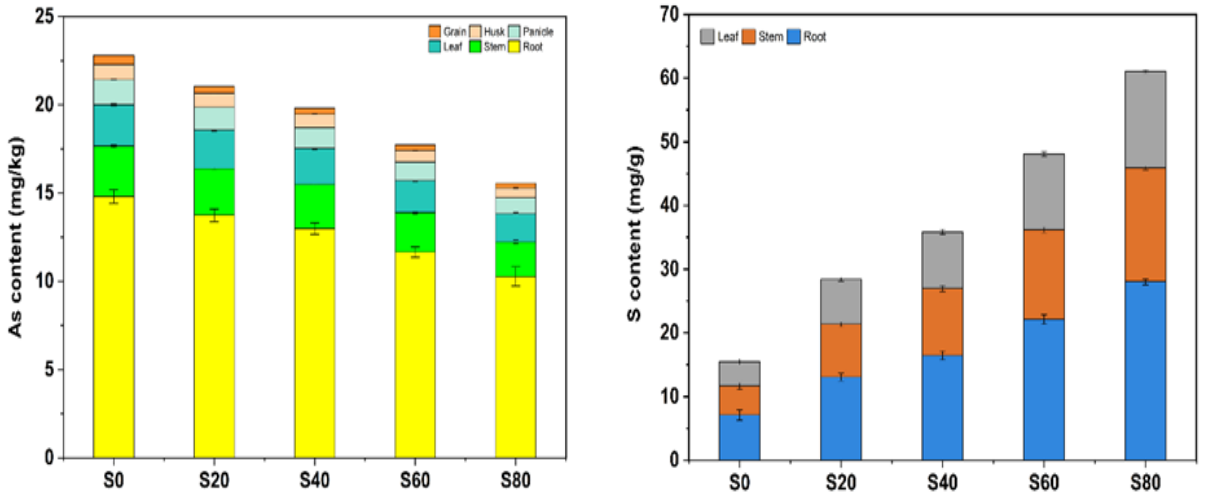


Fig. 6.4. Concentration of (a) As and (b) S in different parts of rice plants, obtained through different sulfate treatments. Vertical bars represent mean with standard deviation and same letters in the vertical bars are not significantly different (Tukey's HSD multiple comparison at $p \leq 0.05$)

Table 6.4: Trend of BCF and TF of arsenic in different plant parts through application of different doses of sulfate

Treatments	BCF	TF				
		root-stem	root-leaf	root-panicle	root-husk	root-grain
S0	1.59±0.02a	0.193±0.01a	0.158±0.001a	0.094±0.002a	0.059±0.001a	0.0349±0.0001a
S20	1.43±0.02b	0.193±0.02a	0.157±0.001a	0.094±0.002a	0.058±0.001a	0.0274±0.0002b
S40	1.35±0.02c	0.192±0.02a	0.156±0.001a	0.092±0.002b	0.057±0.001ab	0.0266±0.0001bc
S60	1.2±0.01d	0.191±0.02ab	0.155±0.002ab	0.091±0.002b	0.056±0.001b	0.0259±0.0001c
S80	1.06±0.04e	0.190±0.02b	0.153±0.001b	0.089±0.004c	0.055±0.001b	0.0251±0.0001c

The same letters with in the same column indicate values are not significantly different at $P \leq 0.05$ using Tukey's HSD multiple comparison test.

Table 6.5: Correlation between arsenic and S uptake by rice plant

	Root S content	Root As content	Stem S content	Stem As content	Leaf S content	Leaf As content
Root S content	1					
Root As content	-0.99	1				
Stem S content	1	-0.99	1			
Stem As content	-0.99	0.99	-0.99	1		
Leaf S content	1	-0.99	1	-0.99	1	
Leaf As content	-0.99	0.99	-0.99	0.99	-0.99	1

6.4. Discussion

Arsenic stress is one of the most major abiotic stress inducer metalloid that inhibits plant development and also has a negative impact on human health. Sulfur plays an important role in plant growth promotion and function. Earlier studies observed exogenous application of sulfur or sulfate compounds helps to remediate As toxicity in plants (Hu et al., 2007; Shi et al., 2020). Studies have mostly focused on reducing As concentration in rice grain; nevertheless, a considerable amount of As remains in rice grain, which may or may not be removed after the post-harvesting process; yet, only a few studies have extended their research to include rice post-harvesting (Kumarathilaka et al., 2021).

6.4.1. Application of sulfate to enhance plant growth in arsenic stressed conditions

Presence of excessive As in the growth medium can hamper plant growth and yield by increasing oxidative stress and decreasing nutrient uptake (Abbas et al., 2018). As a non-essential metalloid, As impacts plants directly or indirectly by reducing the uptake of important nutrients such as

phosphorus, selenium, zinc, silicon, calcium, manganese, potassium, and magnesium, since As may enter plant cells through essential nutrient routes (Siddiqui et al., 2020). Seed germination, growth, reproduction, and production capability of plants are all affected by the presence of As in soil. The maintenance of flooded condition during rice cultivation increases the bioavailability of AsIII which is more toxic than AsV. It also reported that both AsIII and AsV were toxic to plants; however, toxicity of AsIII on rice plant was more pronounced as compared to AsV. Sulfur is not only an integral part of amino acids and enzymes but also critical for plant's defense system against biotic and abiotic stress (Shi et al., 2020). Sulfate transporter (*OsSul1;1*) maintains the sulfur pool inside the cell under As stress, enabling the plant to withstand the stress (Kumar et al., 2019). In this study, increasing dose of sulfate application improved plant growth and yield by lowering toxicity caused by As toxicity. This observation was similar to Shi et al. (2020) where they reported increasing sulfate fertigation improved grain and biomass yield in wheat plant under As and Cd stress. In another study, sulfate application improved rice plant shoot growth and biomass by 16.3%, and 70.7–100.2%, and grain weight was also considerably improved by sulfate application as compared to control treatment (without sulfate) under As stressed condition (Tang et al., 2020). In As and Pb co-contaminated soil, Zou et al. (2018) observed that sulfate treatment significantly increased rice plant tiller numbers, height and biomass. Most importantly, sulfate application increased the number of filled grain/panicle and also improved the test weight of the grains (Table 6.1). On economical perspective, this outcome can help the farmer to overcome the loss they suffer from lower yield of rice grain due to As toxicity.

6.4.2. Effects of sulfate application on oxidative stress management, photosynthetic activity and root anatomical structure

Arsenic stress intensifies ROS accumulation, thus generating oxidative stress inside the plants. This in turn suppresses the plants' growth. The excessive production of ROS caused by the activation of NADPH oxidase (an ROS-producing enzyme) and the impairment of plant antioxidant systems induced by As exposure might have a substantial impact on plant metabolic activities (Mishra et al., 2019). Plant metabolism may be severely harmed by ROS under As stressed condition, which can cause permanent damage to vital macromolecules such as carbohydrates, proteins, DNA, chlorophyll and lipids (Abbas et al., 2018; Siddiqui et al., 2020). In-vivo, intracellular ROS detection in rice roots revealed that ROS levels and sulfate application rates are inversely related (Fig. 6.1a–e). Sulfate is well known to scavenge ROS mediated toxicity in plants. Thus, increasing sulfate nutrition might reduce the ROS accumulation under As stress

and enhance the photosynthetic activity and growth either by lowering As uptake or reducing free As (Dixit et al., 2016; Srivastava et al., 2014). The subsequent measurement of H2O2 from rice root and leaf showed that accumulation of H_2O_2 was high in root as As exposure in root was much higher than in leaf. The enhanced oxidative stress is associated to the breakdown of membrane lipid (Kaya et al., 2020). Increased levels of TBARS and H_2O_2 are well-known indicators of oxidative stress. Lowest level of lipid peroxidation was observed in S80 treatment as increasing sulfate application lowering the ROS mediated oxidative stress. The result complies with that of Dixit et al. (2015). In present study, Cysteine content in plant is enhanced with increasing sulfate fertigation (Fig. 6.2c). The assimilated sulfur gets reduced to Cysteine inside the plant that plays a key role in GSH (reduced glutathione) and PCs (phytochelatins) synthesis (Anderson, 1980). The PCs thus formed complexes with As species, that transported to vacuole and minimized the free As in cytoplasm (Meadows, 2014). Therefore, application and management of proper sulfate fertigation can reduce the As translocation from root to shoot by vacuolar compartmentalization of As in root tissue (Duan et al., 2013). Chlorophyll is the integral part of photosynthetic machinery and As toxicity severely affects the photosynthetic activity that causes decline in photosynthetic pigments. The As stress directly interferes with the plant's photosynthetic activity which leads to the reduction in plant growth and yield (Srivastava et al., 2017). Furthermore, the presence of As in shoot tissues causes a range of physiological changes, including suppression of the tetrapyrrole biosynthetic pathway, PSII photochemistry, chloroplast abundance and structure, ATP production, and enhanced oxidative stress (Jung et al., 2019). Another mechanism that induces photo-oxidative damage to chlorophyll is ROS overproduction under As toxicity (Iqbal et al., 2019). The result showed that the application of Na_2SO_4 increased the chlorophyll content (Fig. 6.2d). This finding complied with that of Yadav and Srivastava (2021). Sulfur nutrition has direct correlation with photosynthetic activity as Muneer et al. (2013) found, low S condition results in reduction of photosynthetic pigments in *Brassica napus*. The SEM study of root cross sections revealed changes in cellular integrity and root structure. The sulfate application found to improve anatomical structure of root (Fig. 6.3a–h) as compared to control (Fig. 6.3i–j). Plant root of S0 set demonstrated severe alteration in structure of epidermal, cortical cells, aerenchyma and loss of root hair. These observations were in agreement with the findings of Singh et al. (2016) and Yadav and Srivastava (2021). Kholodova et al. (2011) reported that alteration in root anatomical structure under As can hamper the water uptake efficiency and plant growth. Moreover, sulfate supplementation improved the root anatomical structure via amelioration of As and reduced the As uptake in plant.

Antioxidant enzymes such as SOD, CAT and APX are instrumental to counteract the ROS mediated toxicity under As stress (Dave et al., 2013). The antioxidant enzymes' activity were highest in the S0 set to alleviate the oxidative stress produced by As, which was comparable to what was found in earlier studies in rice (Dixit et al., 2015, 2016). The down regulation of antioxidant enzymes in sulfate application was observed due to the lower accumulation of H_2O_2. Adhikari et al. (2018) also reported that sulfate application down regulates antioxidant enzyme activities by reducing the oxidative stress in maize plant under Cd stress. The lowering of oxidative stress and antioxidant enzyme activities indicate sulfate mediated amelioration of As.

6.4.3. Arsenic and sulfur accumulation in rice plants

The results from the current study clearly indicated that As accumulation in root was highest as compared to other aerial parts of rice plant (Fig. 6.4a). Root being the primary entry point for As to get into the plant system, maximum accumulation of As was observed in root before it got transported to other parts of plants. The translocation of As from root to grain following the order of root > stem > leaf > panicle > grain, supports our previous findings of As translocation during pre- and monsoonal paddy cultivation (Chowdhury et al., 2018a, 2020b). However, the increasing sulfate application causes significant ($p \leq 0.05$) dip in As uptake and accumulation in plants. The decreased bioavailability of As to rice plants may be due to the sulfur induced formation of iron plaque in the rice root which in turn minimizes the As accumulation in grains (Hu et al., 2007). In soils and sediments, sulfur (S) plays an important function in regulating As solubility and availability. It was found that adding elemental sulfur (S0) and Na2SO4 to rice soil reduced accumulation of As in rice plants by promoting Fe root plaque formation (Hu et al., 2007), as well as lowering As mobility in soil pore water and As uptake by rice plants grown under flooded conditions with high microbial Fe reduction (Xu et al., 2019). Sulfate application also increased the S uptake and accumulation in different plant parts (Fig. 6.4b). The increased S accumulation in rice tissues enhances the synthesis of S-containing ligands, such as glutathione and phytochelatins which help to sequester As into the vacuole by chelation and restrict the flow of As from root to rice grain (Duan et al., 2011; Liu et al., 2010).

6.4.4. Arsenic concentration in raw rice

Sulfate application significantly reduced the accumulation in rice grain and this outcome was similar to the observation of Wisawapipat et al. (2021) who claimed that addition of sulfur amendments to soil decreased total As in rice grain by 69–70%. Additionally, speciation study

observed that inorganic As and DMA in rice grain was reduced by 63–71% and 56–71%, respectively upon sulfate application. Arsenic concentration in raw rice grain (dehusked grain) in this study was found above the permissible limit of inorganic As in polished rice (0.2 mg/kg) prescribed by Codex Alimentarius Commission (2014), irrespective to the level of sulfate application. However, the dehusked rice grain in this experiment was not polished and if further polishing of these dehusked (brown rice) grains were done to remove the bran, then a significant amount of As can be reduced considerably. Earlier studies found that both organic and inorganic As (iAs) tend to accumulate in the bran layer and 10% (w/w) removal of bran portion from brown rice could decrease the total As contents by 51–70% in white rice (Naito et al., 2015; Sun et al., 2008).

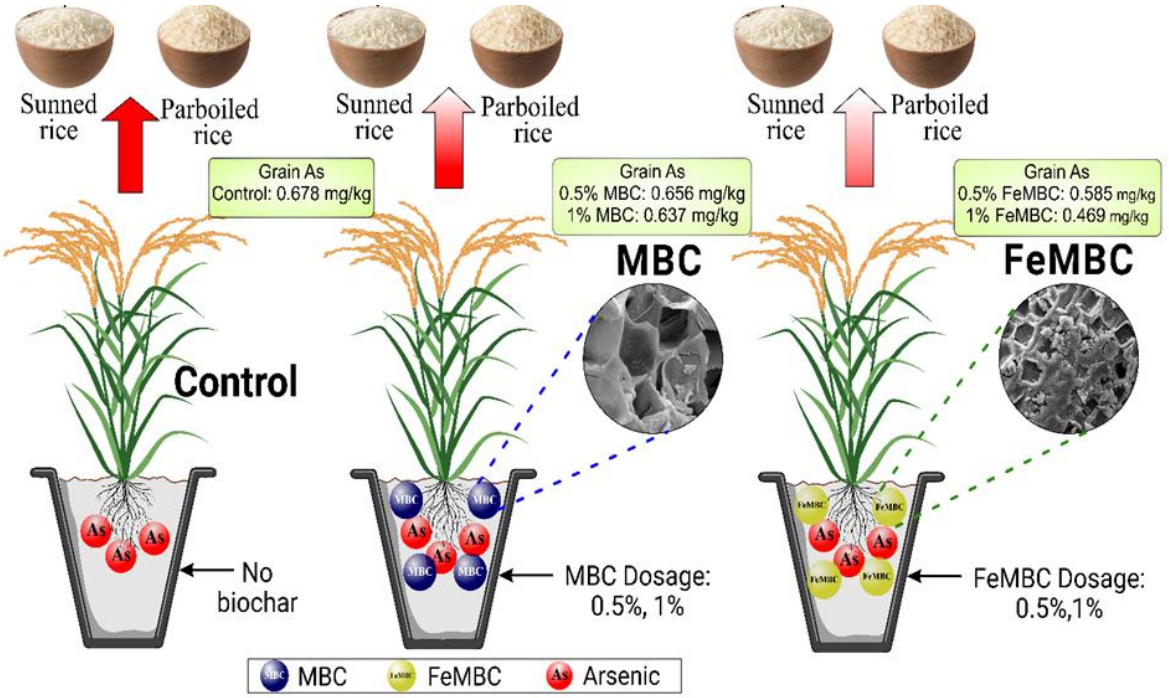
6.5. Conclusion

The present study demonstrated that application of sodium sulfate caused reduction in As concentration in rice grain and also promotes the plant growth and yield under As stressed condition. Increasing sulfate application reduced the oxidative stress in rice plant by lowering the uptake and accumulation of As from soil. However, further field study may be done to optimize the sulfur doses based on the availability of sulfur in the soil. Apart from As translocation, future studies might look at the influence of sulfate on the accumulation of various As species in rice grains.

Chapter 7

Evaluation of iron modified biochar on arsenic accumulation by rice

Graphical Abstract



7.1. Introduction

Arsenic is a toxic metalloid, and exposure to it can cause substantial damage to living organisms. The contamination of As in groundwater and agricultural soil poses a significant threat to humans. In India, a major part of Ganga-Meghna-Brahmaputra (GMB) flood plain, especially West Bengal, and the country like Bangladesh, are severely affected by elevated level of As in groundwater (Chowdhury et al., 2020). Rice, a major cereal crop and staple food source for half of the world's population, is predominantly grown in areas with As contaminated water and soil in GMB plain of India (Majumdar et al., 2020a). Arsenic present in the rhizosphere soil can enter the root and translocate into the rice grain, ultimately contaminating the food chain. Thus, the presence of As in rice grain is a matter of concern that requires a proper solution. Arsenic exists mainly in highly toxic inorganic [Arsenite: AsIII and Arsenate: AsV] and less toxic organic (DMA and MMA) forms in the environment. Under anaerobic condition, AsIII is the predominant form of As, whereas AsV is mainly present in aerobic condition (Majumdar et al., 2023a). These toxic inorganic As (iAs) species accumulate in rice plant and adversely affect their growth and yield. The amount of As in rice grain also varies among different cultivars. However, individuals living in arsenic-endemic regions of the world still consume a significant amount of As through cooked rice. Besides water, rice grains contribute significantly to As exposure to humans (Joardar et al., 2021; 2023). Based on observations from an earlier study, the ingestion of As-enriched rice may lead to an increase in the As load in the body, even when consuming As-safe water (Chowdhury et al., 2020). To meet the World Health Organization's iAs limits in brown (250 µg/kg), polished (300 µg/kg), and rough rice grains (350 µg/kg), the Food and Agricultural Organization (FAO) of the UN has issued a report urging countries to conduct studies aimed at reducing As level in rice grain below the recommended threshold value (FAO, 2014; 2016; BfR, 2016).

Until now, scientists across the world have developed various strategies to mitigate the mobility and bioavailability of As, as well as translocation and load in rice grain. The implementation of inorganic and organic amendments, nanoparticles, nanofertilizers and biochar both in laboratory and field level studies, alongside different water management practices, has been practised to reduce the toxicity of As in rice grain (Kumarathilaka et al., 2021; Majumder et al., 2023b). Biochar is a carbon-rich product derived from the slow pyrolysis of biomass under oxygen-limiting conditions. It has the potential to be used for long-term carbon sequestration due to its high carbon content and environmental stability (Majumdar et al., 2023a). Biochar is a good amendment because of its ability to immobilise toxic elements in the soil, thereby improving soil health and

plant development (Islam et al., 2021; Majumdar et al., 2023a). As a result, it has received particular interest as a possible remediating agent for As-contaminated soils due to the presence of diverse functional groups, high porosity and specific surface area (Wen et al., 2021).

Previous studies have shown that Fe-modified biochar might be used to remove As and other hazardous elements from aqueous solutions (Niazi et al., 2018; Majumdar et al., 2022). Furthermore, researchers hypothesised that Fe-(hydro)oxides might restrict the mobility of As in soil, lowering its bioavailability and propensity for leaching (Hussain et al., 2021; Irshad et al., 2022). Fe-enriched absorbents can aid in the development of root Fe-plaque, which can retain large amounts of metal(loids) (Sebastian and Prasad, 2015). Studies on the As sorption capacity of Fe-modified biochars and the accumulation of As in rice grain in As-contaminated soil under various water management regimes have been undertaken (Islam et al., 2021; Wen et al., 2021).

Rice grain consists of a variety of micronutrients; however, the effect of biochar treatment on rice grain nutritional content was unclear. Whether a soil amendment is effective or not should be justified by analyzing its efficacy and determining the flux rate of the concerned element. For As, this study has developed a novel equation to define the flux rate through the deeper soil strata allowing us to identify the efficacy of the proposed treatment compared to the control soil without any amendments. Hence, the present study aimed to investigate the (i) effect of biochars [mango leaf biochar (MBC) and Fe-modified mango leaf biochar (FeMBC)] on growth and yield of rice under As stress; (ii) effect of MBC and FeMBC on the As availability and uptake in different aboveground biomass of rice plant; and (iii) effect of MBC and FeMBC on the assimilation of selective micronutrients (Fe, Zn, Cu, Mn and Se) content in rice grain.

7.2. Materials and methods

7.2.1. Preparation and characterization of biochar

Biochar was produced from dried mango leaves, which are abundant in most parts of the world. The dried mango leaves were collected from the academic campus of Jadavpur University, Kolkata. Subsequently, the leaves were cut into small (<2 cm) pieces, and mango leaf biochar (MBC) was prepared through pyrolysis in a muffle furnace at 500 ºC for 1 h. The biochar was sieved through a 100 mesh sieve and stored in a desiccator. The Fe-modified biochar (FeMBC) was prepared following the method of Wen et al. (2021). To synthesize FeMBC, MBC was vigorously mixed with a $FeCl_3.6H_2O$ solution at a ratio of 1: 20 (Fe: biochar, w/w). The mixture underwent sonication at 25 °C for 1 h to ensure uniform mixing. The solution was then dried in an

oven at 60 °C until it reached to a constant weight. Subsequently, it was pyrolyzed at 500 °C for 1 h to improve the Fe loading. The concentrations of Se, Zn, Mn, and Cu in MBC and FeMBC were determined using Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES, Perkin Elmer, Avio 200, USA). Iron and As concentrations were determined using Atomic Absorption Spectroscopy method (AAS, Varian, AA140) after digestion with a mixture solution of HNO_3 and H_2O_2 at ratio of 2:1. The physicochemical properties of the synthesized biochars (MBC and FeMBC) were characterized using Brunauer–Emmett–Teller (BET) specific surface area (SSA), Scanning Electron Microscopy (SEM) images, Energy Dispersive X-ray (EDX) spectrometry, Fourier Transform Infrared (FTIR) spectroscopy, and X-Ray Diffraction (XRD).

7.2.2. Soil collection and characterization

Paddy soil was randomly collected from plough layer (0–20 cm) of As-contaminated paddy fields in the Gaighata Block of North 24 Parganas district, West Bengal, India (22°54′14.51″ N, 88°46′25.36″ E). The soil was dried in an oven at 60 ºC before being pulverized. For the pot trials, the soil was sieved (< 2 mm) and homogenized. The ground soil was used for analysis of soil properties like texture, pH, electrical conductivity (EC), soil organic carbon (SOC), total Se, total As, and available Cu, Zn, Fe and Mn concentrations. **Table 7.1** provides a summary of the ~~soil's~~ basic characteristics of soil.

Table 7.1: Physico-chemical properties of soil

Soil	
pH	5.67
EC (mS/cm)	1.84
SOC (g/kg)	15.7
Available N (mg/kg)	46.3
Available P (mg/kg)	7.30
Available K (mg/kg)	54.5
Total As (mg/kg)	17.1
Total Se (mg/kg)	0.23
Available Fe (mg/kg)	61.8
Available Cu (mg/kg)	1.08
Available Zn (mg/kg)	1.28
Available Mn (mg/kg)	33.4
Soil texture (%)	Clay: 55.3
	Silt: 21.1
	Sand: 23.6

7.2.3. Pot experiment

In this study, the Satabdi (Minikit, IET 4786) rice cultivar was selected. The rice seed underwent sterilized with 30% H_2O_2 for 15 min and following by washing with distilled water. The seeds were previously sown in seed beds and remained there until the transplanting stage. For the experiment, bottom-sealed PVC containers were utilized to prevent the loss of nutrients and As. Each pot received a total of 6 kg of dried soil. There were five experimental sets in total, and each set was triplicated. The experimental sets included control (C), 0.5% MBC (w/w), 0.5% FeMBC (w/w), 1% MBC (w/w) and 1% FeMBC (w/w). One week before transplanting the rice, a basal dose of N (Urea, CH_4N_2O), P (Disodium hydrogen phosphate, Na_2HPO_4) and K (Potassium chloride, KCl) was applied in the ratio of 2:1:2. Two days before the transplanting the rice, all pots were flooded with tap water (As: <3 µg/L) and anaerobic conditions were maintained till maturity stage. At the harvesting stage, both rice plants and rhizosphere soil were extracted for further analysis. Rhizospheric soil samples were collected from each treatment at 10, 30, 50, 70, 90 and 110 days after transplanting (DAT) for the analysis of pH, EC, and OC according to Chintala et al. (2014).

7.2.4. Chlorophyll, lipid peroxidation, H_2O_2 and proline

The acetone extraction technique was used to determine the chlorophyll (chl) concentration of 25 DAT rice plant (Arnon, 1949). Lipid peroxidation (TBARS: thiobarbituric acid reactive substances), H_2O_2 and proline contents in leaf of rice plants were measured according to Heath and Packer (1968), Velikova et al. (2000) and Bates et al. (1973), respectively.

7.2.5. SEM-EDX analysis of root iron plaque and iron plaque extraction

The collected root samples of rice plants were rinsed properly with water to remove soil from the root. Prior to observation of the root surface under Scanning Electron Microscope (SEM; Carl Zeiss Model Number – EVO18 Special Edition), the samples were coated with platinum. The elemental composition present on the root surface was investigated utilizing EDX (SmartEDX, Carl Zeiss).

For the extraction of root Fe plaque, fresh root samples were treated with a solution of dithionite-citrate-bicarbonate (DCB) (Lin et al., 2017). Briefly, roots from each treatment group were taken out and incubated at 25 °C for 60 min in a 30 ml mixed solution containing 0.03 M sodium citrate, 0.125 M sodium bicarbonate, and 0.8 g sodium dithionite. After extraction, the roots were rinsed

3 to 4 times with Milli-Q water (18.2 MΩ cm; Merck, IQ700) before being mixed with the 30 ml DCB extracts. The total amount of the extract was adjusted to 50 ml, and the extract solution was then filtered using a Whatman No. 42 filter before As and Fe estimation using AAS.

7.2.6. Plant growth and yield attributes

Different yield attributes (like number of tillers, filled grain per panicle, 1000 grain weight, aboveground biomass, and plant height) were recorded at the harvesting stage (110 days).

7.2.7. Analysis of soil samples

The soil pH, EC and organic carbon were measured according to the standard methods described by Jackson (1973) and Walkley and Black (1934), respectively. Cationic micronutrients (Fe, Mn, Cu, and Zn) were extracted using a 5 mM diethylenetriaminepentaacetic acid (DTPA) solution at pH 7.3. After DTPA extraction, concentrations of Fe and Mn, Zn, Cu were measured using an AAS and ICP-OES, respectively (Lindsay and Norvell, 1978). To quantify the extracted plant available As, 5 g of soil was mixed with 75 ml of 0.5 M NaH_2PO_4 and agitated for 2 h at 250 rpm on a shaker. The suspension was immediately filtered, and the concentration of As in solution was measured using AAS. For quantification of total As, about 0.1 g of soil sample was digested using a mixture solution of HNO_3 and H_2O_2 at the ratio of 2:1. The total As concentration in the soil sample was determined using AAS.

7.2.8. Estimation of plant arsenic and concentration of micronutrient in rice

The process of extraction and estimation of plant As was described in our previous studies (Chowdhury et al., 2020). A solution mixture of HNO_3 and H_2O_2 in 2:1 was used to digest 0.2 g of dry plant material. Total As concentration in plant sample was determined using AAS (Varian model AA140, USA) (Das et al., 2021a, b). The concentration of micronutrients (Mn, Zn, Se, Cu and Fe) in brown uncooked rice was determined using ICP-OES (Perkin Elmer, USA). About 0.2 g of raw rice grain was digested with HNO_3 and H_2O_2 at the ratio of 2:1. The digested samples were then diluted to 10 mL with Milli-Q water and kept for analysis. The concentrations of Mn, Se, Zn, and Cu were measured using ICP-OES and Fe concentration was measured in AAS.

7.2.9. The equation for the arsenic flux determination

The term 'flux' has been used to understand and estimate the amount of As in terms of concentrations, that are getting percolated down to the deeper soil layers or being flushed out of

the soil surface. Thus the residual As from the difference of these two conditions is termed as flux rate. The primary goal of this flux assessment is to understand the effect of considered environmental parameters in elemental concentration modulation. Majumdar et al. (2021) showed the effect of water management practices on As flux dynamics. Similarly, in this study, we elucidated the effects of variable biochars usage on As flux.

To assess the efficacy of soil amendments, here, in the form of MBC and FeMBC, it was necessary to identify the flux rate of As before and after the treatment process. A parametric equation has been developed for any pot amendments to the soil based on the analyzed data of the concerned pot setup. This equation is based on the field trials of As flux determination under differential agronomic practices, modified after Majumdar et al. (2021). This equation can be applied for any soil amendment and for any stable elemental flux assessment to understand the amount of that element getting percolated down or taken up by the associated plants within a pot culture.

$$[(d.W_c).E_b + P_b] - [(d.W_c).E_a + P_a] = (R_p \,.\, W_c).F_r$$

Where d is the active soil sampling depth within the pot; W_c is the concerned elemental concentration in the applied water; E_b and E_a are the concerned elemental concentration in the soil before and after the amendment; P_b and P_a are the concerned elemental concentration in the associated plant before and after the amendment; R_p is the rate of the percolated element in the pot down to the soil depths through water and F_r is the flux rate (mg/kg) of As in the soil. The difference between before-treatment and after-treatment would allow us to assess the flux concentration of the element.

7.3. Results and discussion

7.3.1. Characterization of synthesized biochars

The pH of FeMBC (4.69) was observed to be lower compared to the pH of MBC (8.87). This difference is attributed to the formation of Fe^{2+} during the synthesis of FeMBC, resulting in a lower pH than that of MBC (Wen et al. 2021). The SEM images of MBC revealed a distinct porous structure, while the surface of FeMBC was covered with Fe oxides (**Fig. 7.1a-b**). **Fig. 7.1c-d** and **Table 7.2** illustrate the elemental composition of the MBC and FeMBC surface measured by EDX. After the addition of Fe, the amount of Ca and K in biochar significantly decreased (**Fig. 7.1c-d**). Sun et al. (2019) also reported that during the synthesis of Fe-modified biochar, the levels of Ca, K, and Mg were substantially decreased after the addition of Fe. They concluded that the increase

in loaded Fe's molar content was caused by a greater loss of Ca, K, Mg, and Si from the Fe-modified biochar. According to Li et al. (2017), competitive ion exchange and surface precipitation of Fe during the synthesis of Fe-modified biochar may cause a rise in Fe and a reduction in Ca, Mg, K, and Si. Iron oxides might be formed as a result of the coexistence of Fe, O, C, and Cl elements, as demonstrated by EDX analysis of the FeMBC surfaces. These findings were in line with those of Wen et al. (2021), who similarly produced Fe-modified biochar (FeBC) from biochar through the pyrolysis of *Platanus orientalis* branches. Surface area and pore volume have a significant impact on loading efficiency. After Fe precipitation, pores on the MBC surface may have become clogged, resulting in a drop in specific surface area from 19.63 m^2/g for the MBC to 7.46 m^2/g for the FeMBC composites (**Table 7.2**). These results corroborated with Sun et al. (2019), who similarly reported a reduction in specific surface area consequent to the precipitation of Fe on the surface of biochar or the degradation of the pore structure was caused by pre-soaking of the biochar in an acidic $FeCl_3$ solution. The functional groups on the surfaces of the MBC and FeMBC composites were identified using the FTIR spectra (**Fig. 7.1e**). The wide spectral band at 3603 cm^{-1} and 3338 cm^{-1} in the MBC and FeMBC was linked to the existence of the -OH group. The presence of hydroxyl group in Fe-modified magnetic biochar was also noted by Cho et al. (2017). The C-H stretching vibrations are responsible for the peak at 3000-2800 cm^{-1}, whereas at 1617 cm^{-1}, an aromatic C=C band was spotted (Sun et al. 2019). However, the key bands at 1008 cm^{-1} and 680 cm^{-1} in FeMBC were attributed to the stretching of Fe-O group. Islam et al. (2021) found that appearance of peaks in that range confirmed the presence of iron oxide on the surface of Fe-modified biochar. The PXRD (Power X-Ray Diffraction) patterns of MBC and FeMBC were displayed in **Fig. 7.1f**. The PXRD pattern of MBC displays the characteristic peaks of stishovite [SiO_2; $2\theta = 30.23°$], chloromagnesite [$MgCl_2$; $2\theta = 15.04°, 38.32°$], sodalite [$Na_4Al_3ClSi_3O_{12}$; $2\theta = 24.52°$], pentlandite [$(Fe,Ni)_9S_8$; $2\theta = 15.41°, 29.55°, 44.31°$), and Fe-Ringwoodite [$Fe_2(SiO_4)$; $2\theta = 36.11°$]. Vyavahare et al. (2019) synthesized biochar from mango leaves to remove crystal violet dye from aqueous solution and they observed comparable XRD pattern to our synthesized MBC. However, no apparent peaks were observed in the PXRD pattern of FeMBC. This observation suggests that the Fe content in FeMBC is in an amorphous state and other stable components of biochar were masked by the Fe-oxide that coated the surface of the biochar (Pan et al., 2022; Zhang et al., 2020).

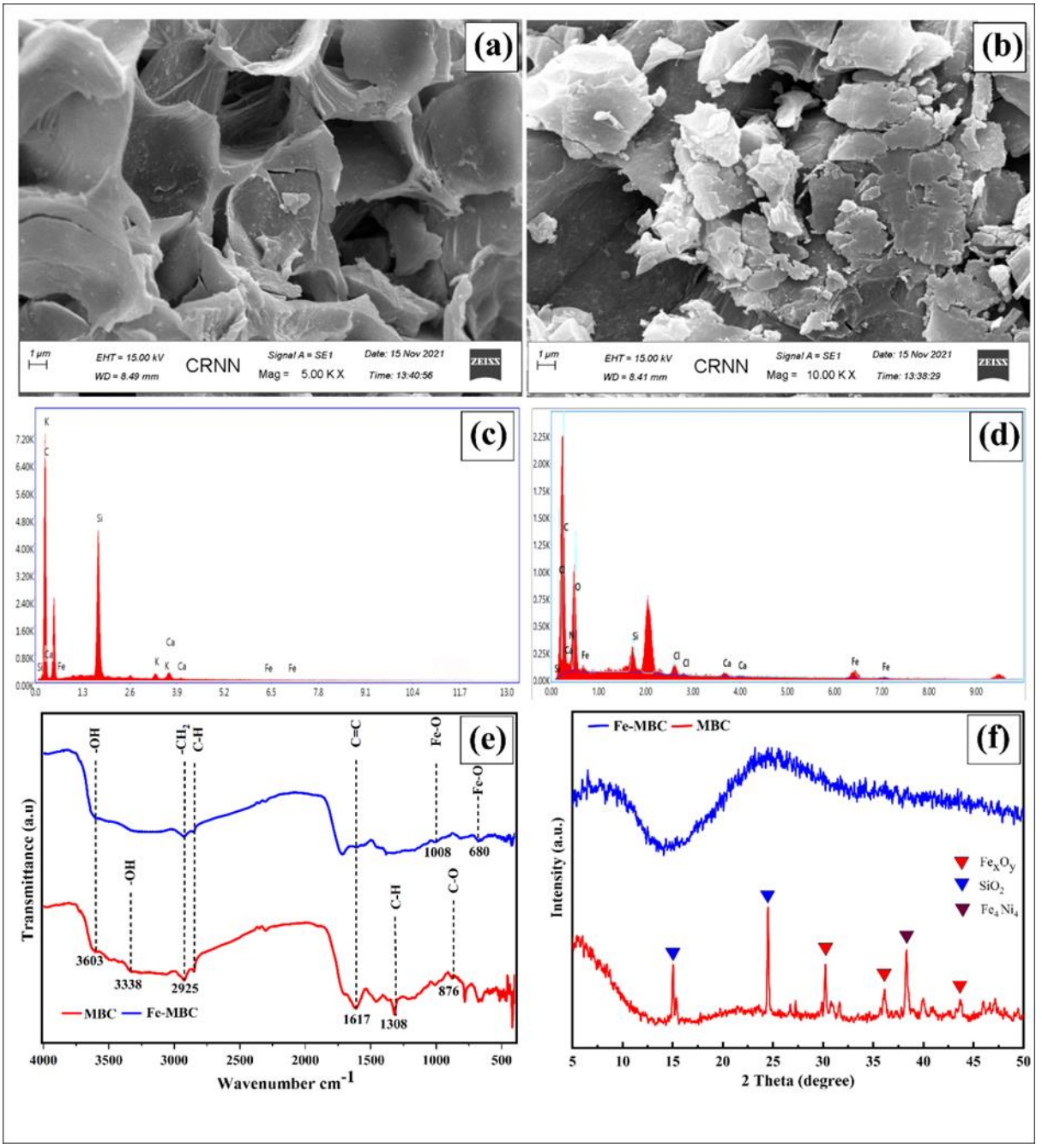


Fig. 7.1. Scanning Electron Microscopy of (a) MBC and (b) FeMBC; Energy Dispersive Spectrometry of (c) MBC and (d) FeMBC; (e) FTIR spectra of MBC and FeMBC; and (f) PXRD spectra of MBC and FeMBC

Table 7.2: Physico-chemical properties of MBC and FeMBC

	MBC	FeMBC
pH	8.87	4.69
EC (mS/cm)	0.67	2.13
SSA (m^2/g)	19.63	7.46
Pore volume (cc/g)	0.028	0.0214
Ash (%)	8.96	14.4
C (%)	42.95	53.8
N (%)	10	8.25
O (%)	34.25	13.8
Si (%)	1.93	21.28
As (mg/kg)	0.72	0.49
Se (mg/kg)	0.12	0.18
Fe (g/kg)	4.36	45.6
Cu (g/kg)	0.002	0.0039
Zn (g/kg)	0.127	0.144
Mn (g/kg)	0.093	0.107

7.3.2. Effect of biochars on soil pH, soil organic carbon (SOC) and electrical conductivity (EC)

The main factor regulating the mobilization of hazardous elements from surface precipitation is pH of the soil (Amen et al., 2020). The addition of MBC and FeMBC in soil changed the soil pH (5.67) differently ($p \leq 0.05$) (**Fig. 7.2a**). At initial phase, the addition of both 0.5% and 1% MBC in soil slightly increased the soil pH by 0.07 and 0.24 unit, respectively as compared to control. Due to the high pH of biochar (8.87; Table 7.1), the 0.5% and 1% MBC might have influenced the soil pH to rise (Wen et al., 2021). The hydrolysis of soluble alkaline minerals (Ca, Na, and K) in biochar may have caused an elevation in soil pH (Lu et al., 2014). However, application of both 0.5% and 1% FeMBC in soil initially decreased the rhizospheric soil pH by 0.12 and 0.19 unit, respectively than control. The FeMBC with lower pH (4.69; Table 7.1) than MBC produced a greater amount of H^+ ions during the hydrolysis of exogenous Fe from FeMBC, causing soil pH to drop (Yin et al., 2017). The pH of rhizospheric soil gradually increased with time being for all treatments till maturity phase (90 days) and fall again at harvesting stage. The release of Fe from Fe-plaque at maturity stage increased the acidity of the soil which causes the reduction of pH in

all the treatment groups (Yin et al., 2017). The SOC content significantly increased ($p \leq 0.05$) after MBC and FeMBC treatment compared to that in the control treatment (**Fig. 7.2b**). A higher SOC increases drainage, microbial development, soil aeration, and water retention capacity. Additionally, SOC prevents the erosion and loss of nutrients via leaching, which enhances the growth of plants (Lin et al., 2017). The SOC content in 1% MBC and 1% FeMBC were increased by more than 14% than control after 10 DAT. Several studies have found that applying biochar to soils in various agricultural practices can substantially enhance the total organic carbon content (Wang et al., 2018; Wen et al., 2021).

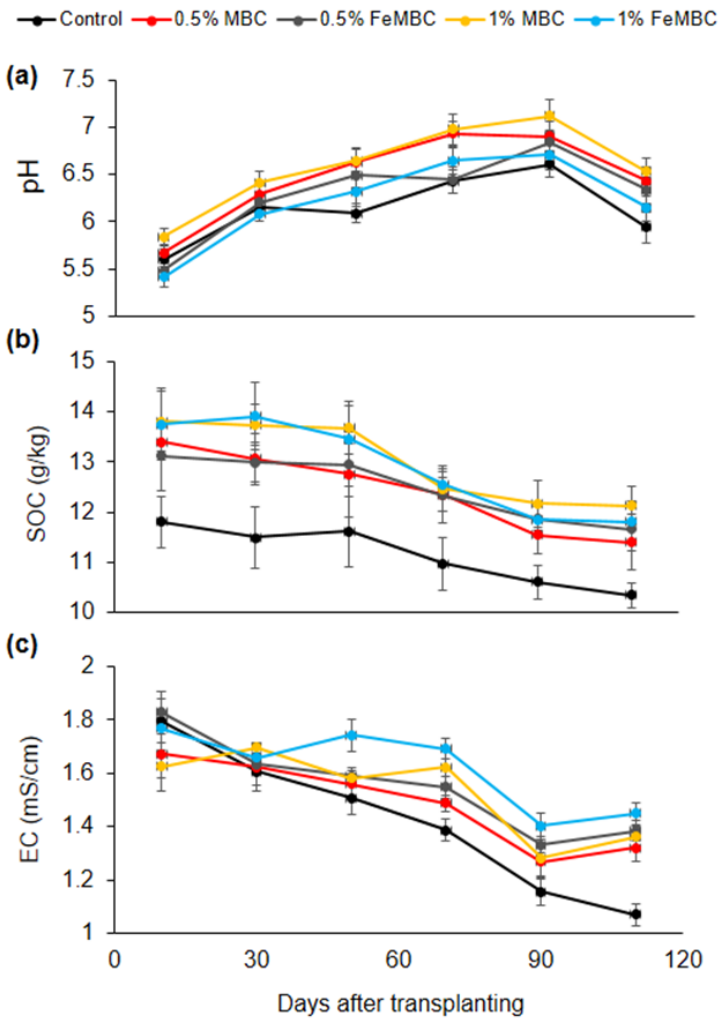


Fig. 7.2. Temporal changes in soil (a) pH, (b) SOC, and (c) EC among different treatments during the period of paddy cultivation

The SOC content of MBC and FeMBC treatments differed significantly; however, the difference between varied dose of MBC and FeMBC treatments was not significant at 70 DAT. Biochar

application provided a favorable environment for the formation of soil aggregation and prevented or retarded the breakdown of SOC (Sun et al., 2020). Like pH, soil EC changes during cultivation period. EC exhibited the opposite trend of pH during the experiment (**Fig. 7.2c**). The EC of the rhizospheric soil was, however, clearly in the following sequence till 110 days: 1% FeMBC > 0.5% FeMBC > 1% MBC > 0.5% MBC > control. This finding is also consistent with the findings of Tsai and Chang (2020), where they observed a negative relationship between pH and EC. The soil employed in this work was weakly acidic (pH = 5.67, Table 7.1), and release of weakly bound nutrients (cations and anions) from biochar into the soil solution resulted in a rise in EC (Chintala et al. 2014). The release of cationic nutrients (Ca, Mg, K, Zn, Cu, and Mn) from biochar are available to plants, resulting in increased plant growth. The ash component of biochar was also responsible for the rise in soil EC. During the pyrolysis and production of MBC and FeMBC, the ash content was changed (Table 7.1). The increased ash content in FeMBC includes easily soluble oxides of CaO, MgO, K_2O, and other anions. The release of these ions causes a rise in EC. In this experiment, FeMBC contained more ash than MBC, which might explain the higher EC in the 0.5% and 1% FeMBC treatments than MBC and control treatments.

7.3.3. Effect of biochars on chlorophyll, TBARS, H_2O_2, and proline content in plant

High oxidative stress induced by As stress causes depletion in photosynthetic activity in rice plants. The total chlorophyll content in rice plants of control was significantly reduced compared to biochar treated plants (**Fig. 7.3a**). However, the chlorophyll content of 0.5% FeMBC and 1% MBC were rather similar. The chlorophyll content of 1% FeMBC treatment was highest among all the treatments. The chlorophyll content in 1% MBC and 1% FeMBC were increased by 24.3% and 31.8%, respectively than control. Biochar can increase the soil water holding capacity and improve the effective soil moisture for crops, producing a positive effect on crop growth (Jun et al., 2020). They noticed that the addition of biochar reduced the declination rate of soil water level having a positive effect on the leaf water potential that helped to alleviate the harmful effects of metalloid stress on the photosynthesis. Lyu et al. (2016) reported that biochar could improve photosynthetic machinery function under stressed condition by increasing chlorophyll synthesis, electron transport chain processes, and photosystem (PSI and PSII) activity. The improved photosynthetic activity under FeMBC treatment helps to boost the rice plant growth and yield. Lipid peroxidation and H_2O_2 are the indicators of oxidative stress in plant. Cell membrane disruption occurs when reactive oxygen species (ROS) are produced in response to abiotic stress. Plants are damaged by oxidative stress caused by the generation of ROS, which leads to the breakdown of cell membrane

and biomolecules. Presence of toxic metalloid (As) in soil induces the production of TBARS and H_2O_2 in rice plants. In control set, TBARS and H_2O_2 content were significantly ($p \leq 0.05$) high than other biochar treated plants (**Fig. 7.3b and 7.3c**).

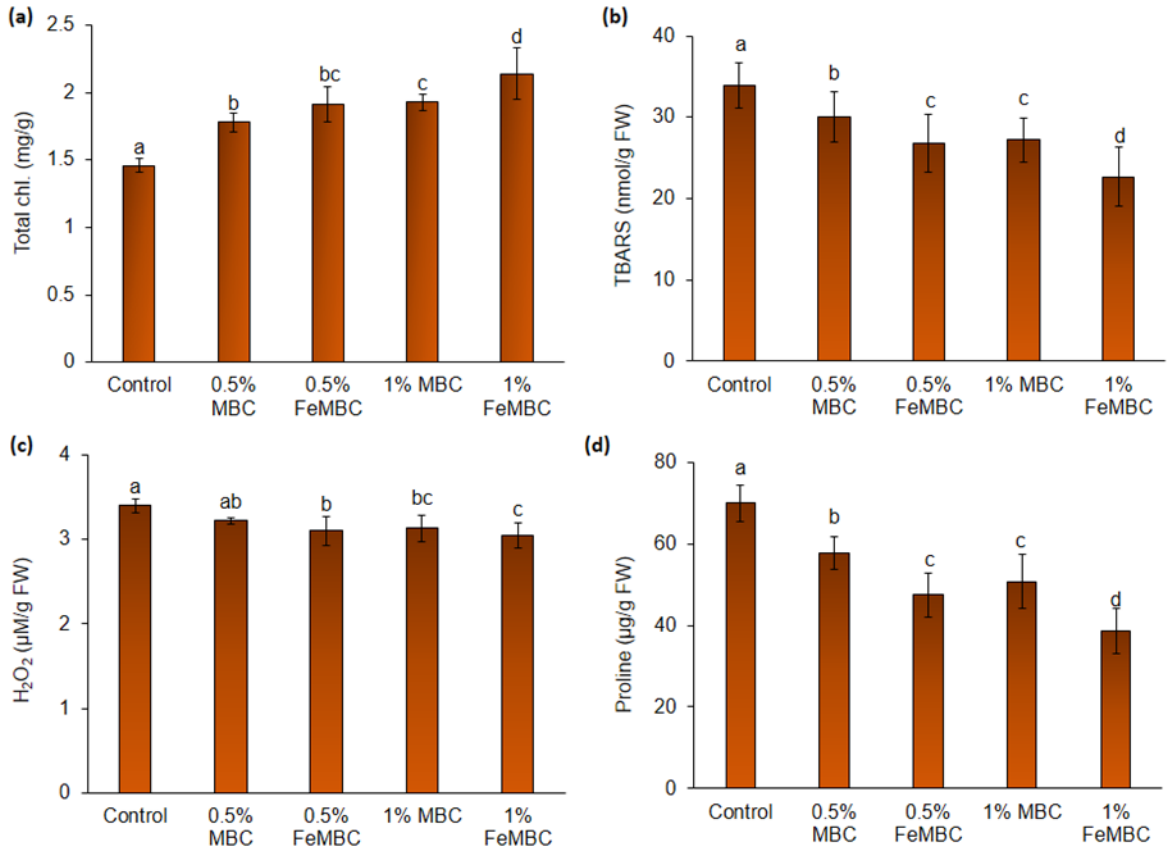


Fig. 7.3. Effect of different doses of biochars on (a) Chlorophyll, (b) TBARS, (c) H_2O_2, and (d) proline. Different letters in bar graph show significant difference among treatments ($p \leq 0.05$)

The low dose of biochar (0.5% MBC) was not that much effective in reduction of TBARS and H_2O_2 levels in rice plant than other biochar treatments. The TBARS and H_2O_2 content in plant were much improved in 0.5% FeMBC, 1% MBC and 1% FeMBC. The TBARS content in 0.5% and 1% FeMBC were reduced by 21% and 33.1% than control. However, in case of H_2O_2, 0.5% and 1% FeMBC treatment reduced its level by 8% and 10.2%, respectively as compared to control. Proline is a key osmolyte that helps to reduce the membrane damage caused by oxidative stress. The high oxidative stress in control caused due to the production of high proline content (**Fig. 7.3d**). The amount of proline measured in 0.5% FeMBC and 1% MBC was almost similar. However, the proline content in 1% FeMBC was reduced by 45.5% compared to control. Our observation supports the previous findings that biochar treatment lowered TBARS, H_2O_2, and

proline levels in plants (Majumdar et al., 2023a). This study showed that the TBARS, and H_2O_2 contents in plant leaves were significantly decreased by the application of biochar. Lyu et al. (2016) suggested that biochar enhanced active oxygen scavenging capacity in plant leaves, and reduced membrane lipid peroxidation. Another study showed that increase in oxidative stress could inhibit the electron transport activity of PS I and PS II (Wang, 2014). Therefore, addition of biochar can improve the defense and photosynthetic activity of rice leaves against metalloid stress by reducing oxidative damage. Earlier studies also claimed that biochar has been proven to work as an adsorbent to reduce As toxicity in plants (Chen et al., 2016; Jun et al., 2020). The heavy metal adsorption capacity of biochar is related to its characteristics, including carbonization, surface area, surface morphology, and surface functional groups (Wen et al., 2021). Jun et al. (2020) observed that biochar promoted the absorption and reduced the accumulation of metal(loid) in the sunflower plant, enhancing its growth.

7.3.4. Effect of biochar application on plant growth and yield

The results showed that both the MBC and FeMBC treatments improved the rice plant growth and yield compared to control (**Table 7.3**). The number of tillers in 0.5% and 1% MBC treatment have been increased by 18.8% and 25.8%, respectively as compared to control. Whereas, in case of 0.5% and 1% FeMBC treatment, increase of 32.8% and 34.8% were observed in tiller number, respectively as compared to control. The number of filled grain was also high in FeMBC treatment than both the control and MBC treatment. The 1% FeMBC treatment has highest number of filled grain (120 filled grains/panicle) among all the treatments. Biochar has been demonstrated to increase plant yield by stimulating growth and shielding plants from heavy metals through improving physical properties of soil such as increasing soil pH, cation exchange capacity, and strengthening nutrient retention capacity (Hartley et al., 2016; Islam et al., 2021; Jun et al., 2020). Arsenic toxicity lowered the test weight of the rice grains in control. Test weight of rice grain in control was reduced by 3.3-13.2% than the other treatments. The test weight of rice grains was higher in 1% FeMBC treatment by 6.2% than 1% MBC treatment. As reported by Islam et al. (2021), grain yield was improved by 14% and 24.7%, respectively, using 2% Iron (Fe)-enriched corncob biochar (FCB) and 2% Fe-enriched corncob-eggshell biochar (FCEB). Jun et al. (2020) observed that the biomass of sunflower plants and their roots, stems, leaves and seeds in the 5% biochar treatments were increased by 64.7, 20.4, 32.8, 36.5 and 230%, respectively in Cd, Pb, As polluted soil. The aboveground biomass and plant height of rice plant were in the following order of 1% FeMBC > 0.5% FeMBC > 1% MBC > 0.5% MBC > control. Lin et al. (2017) observed that

ferromanganese oxide biochar composites (FMBC) enhanced the weights of stems, leaves, and grains by increasing soil cation exchange activities and lowering harmful levels of As in rice. However, due to differences in soil type and condition, it may not be appropriate in different soils, thus adequate dosage selection for biochar is essential before application.

Table 7.3: Plant growth and yield under different treatments

Treatments	No. of Tillers	No. of filled grain/panicle	Test wt. (g)	Above ground biomass (g d.w.)	Plant height (cm)
Control	14.33±1.57a	99.66±10.6a	17.7±0.78a	36.6±4.13a	91.33±2.51a
0.5% MBC	17.66±3.05a	108.33±15.56b	18.32±0.4a	40.27±2.28a	94.33±3.05b
0.5% FeMBC	21.33±0.57c	117±12.48c	19.66±0.72b	47.3±4.58b	99±3.46c
1% MBC	19.33±2.08bc	113.33±6.5b	19.13±0.58b	43.84±4.46b	96.3±2.51bc
1% FeMBC	22±2c	120±13c	20.4±0.86c	50.34±6.26bc	102±2.64d

Different letters in column show significant difference among treatments ($p \leq 0.05$; n=3)

7.3.5. Effect of biochars on root iron plaque

The presence of Fe-plaque on the surface of rice roots acts as a critical buffer against the entry of harmful metals such as As into the root cortex. Metal(loid) uptake by rice roots and their burden in rice tissues can both be decreased by sequestering met(loid) in Fe-plaque (Yin et al., 2017; Islam et al., 2021). Fe and As concentrations of the Fe-plaque in the MBC and FeMBC amendments were substantially higher than the control. Soil enrichment with various treatments increased the Fe and As concentration of the root Fe-plaque in the following order: 1% FeMBC > 0.5% FeMBC > 1% MBC > 0.5% MBC > control (**Fig. 7.4a and 7.4b**). Iron concentration in Fe-plaque of 1% FeMBC treatment was 51.3%, 39.1%, and 38.2% higher than the control, 1% MBC, and 0.5% MBC, respectively. Similar to Fe, As concentration of 1% FeMBC was higher than control, 1% MBC, and 0.5% MBC by 82.3%, 45.8%, and 51.5%, respectively. The use of biochar and Fe-enriched biochar amendments results in increased Fe-plaque formation on rice root surfaces, which aids in the sequestration of heavy metals and metalloid (like As) and reduces their absorption in plants (Sebastian and Prasad, 2015; Wu et al., 2016). The formation of root iron plaques was observed, and the elemental composition was documented using SEM-EDX (**Fig. 7.4c-g**). **Fig. 7d-g** shows that the rate at which MBC and FeMBC treatments were administered had a direct relationship with the development of iron plaques in the root.

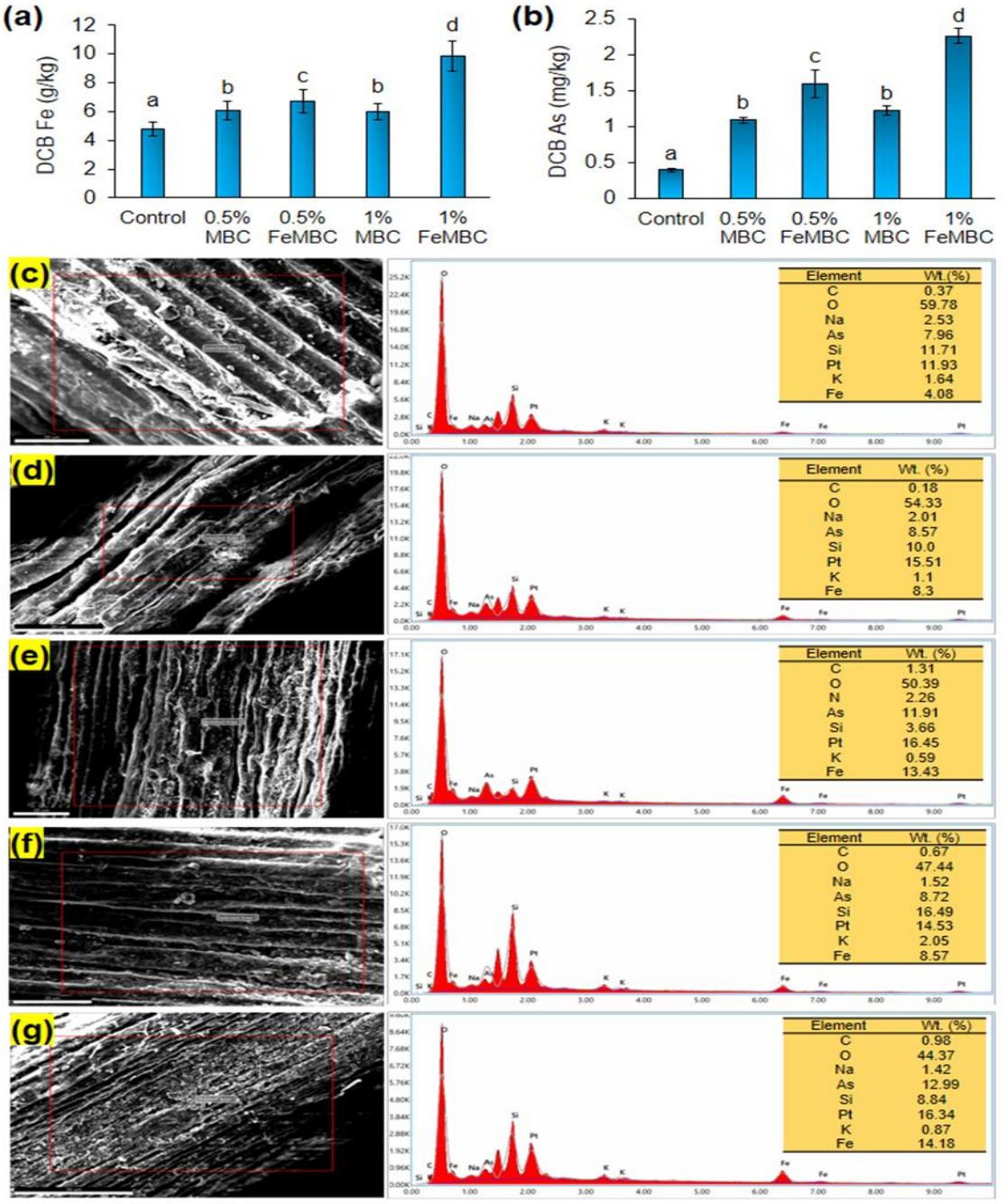


Fig. 7.4. Effects of MBC and FeMBC treatments on (a) Fe and (b) As concentration of root Fe-plaque. SEM-EDX of rice roots showing Fe-plaque formation: (c) control, (d) 0.5% MBC, (e) 0.5% FeMBC, (f) 1% MBC, and (g) 1% FeMBC. Different letters in bar graph show significant difference among treatments ($p < 0.05$)

A comparative study of the elemental composition of the surface of the Fe-plaque covered roots using an Energy Dispersive X-ray Spectrometer indicated a relative enrichment of Fe and As on the plaque surface of 1% and 0.5% FeMBC treatment, respectively, as compared to the other

treatments. The FeMBC treatment increased the level of amorphous Fe oxides in rhizosphere soil which stimulated the formation of root Fe-plaque followed by the sequestration of As in Fe-plaques. Increased Fe-plaque development on the surface of the rice root as a result of oxidation of Fe^{2+} restricted the uptake and accumulation of As in rice tissues, particularly in the grains (Chowdhury et al., 2018a; Irshad et al., 2022). The formation of amorphous Fe^{3+}-arsenate compounds might be also one of the reasons for the immobilization of As by FeMBC (Mensah et al., 2020). Hossain et al. (2009) also observed that addition of Fe^{2+} material ($FeSO_4.7H_2O$) to the soil boosted Fe-plaque formation and decreased As concentration in rice plants.

7.3.6. Available arsenic concentration in rice plant and rice grain

The application of 0.5% and 1% FeMBC considerably ($P \leq 0.05$) reduced the potentially bioavailable concentration of As in the soil by 33.3% and 47.6%, respectively, in comparison to the control (**Fig. 7.5**). The available soil As concentration was lower following application of 1% and 0.5% MBC compared to the control; however, the percentage decrease between 0.5 and 1% was not statistically significant.

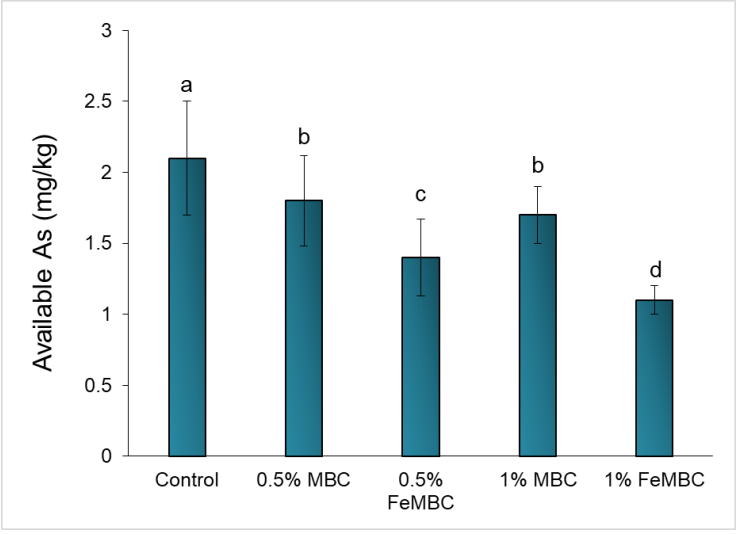


Fig. 7.5. Effect of biochar applications on soil available As. Different letters in bar graph show significant difference among treatments ($p < 0.05$)

The use of biochar to reduce As bioavailability is comparable to other study (Yin et al., 2017; Yu et al., 2017). Arsenic has also a smaller ionic radius (0.58 Å), which may encourage its diffusion into biochar micropores when biochar was amended (Qiao et al., 2018). Arsenite (AsIII) in soil solution is oxidized to less hazardous and less mobile arsenate (AsV) by donating electrons to functional groups of biochar that act as electron acceptors (e.g. C=O, semiquinone-type free

radicals, phenolic-OH) (Amen et al., 2020; Niazi et al., 2018). Arsenic species [AsIII and AsV] may undergo redox reactions in the presence of potent oxidizing and reducing agents on the surface of biochar, which might lead to As complexation with Fe on the surface of the biochar (Amen et al., 2020; Wen et al., 2021; Yuan et al., 2017). FeMBC with a higher number of Fe functional groups and a larger surface area was able to bind As firmly on its surface, reducing As availability and bioaccumulation in rice grains (Wu et al., 2018).

In As-contaminated soil, MBC and FeMBC amendments had a distinct effect on total As accumulation in rice plants ($p \leq 0.05$). Both MBC and FeMBC treatments of As-contaminated soil were observed to reduce As concentration in aboveground portions of rice plants (Fig. 6a-f). According to the observations, FeMBC was found to be more effective than MBC in reducing the As content of aboveground biomass. However, the reduction pattern of 0.5% FeMBC and 1% MBC was moreover equal. The 0.5% MBC treatment in soil was shown to be less effective in reducing As accumulation in different rice biomass. The use of 1% MBC reduced the As concentration in rice roots, straw, leaf, panicle, and grains by 24%, 21%, 19%, 27%, and 6%, respectively. On the contrary, paddy soil enrichment with 1% FeMBC reduced As concentration in roots, straw, leaf, panicle, and grains by 40%, 38%, 32%, 46%, and 31%, respectively, showing a considerable improvement in plant survival and safety when compared to the control plants.

Wu et al. (2018) found that applying Fe-modified biochar (Fe-BC) in As-contaminated soil lowered the As load in rice grains by altering the As fractions in soil. It was previously observed by Yu et al. (2017) that applying Fe-fertilizers to the soil increased the development of Fe-plaques in root and reduced the intake of As in plants (Wen et al., 2021). In comparison to FeMBC, the high pH in the soil after MBC treatment induces increased phytoavailability and uptake of As in rice. Furthermore, our findings are consistent with those of earlier research studies that found Fe-enriched biochar to be effective in reducing As build up in rice grains (Irshad et al., 2022; Islam et al., 2021; Yin et al., 2017). The 0.5% and 1% MBC treatments also reduced the As accumulation in rice plant than control (**Fig. 7.6a-f**). The EDX and XRD data revealed that biochars contain a significant quantity of inherent silica (Si), which may be the cause of the lower accumulation of As in rice plants. Herath et al. (2020) observed that Si-enriched biochar reduced As accumulation in rice straw and grain. The main Si transporters in rice are Lsi1 and Lsi2, and these proteins also tightly control the intake of AsIII. The presence of high Si in the rhizospheric zone downregulates Lsi1 and Lsi2 proteins in rice, and at low availability of Si transporter, competition between Si and AsIII increases, limiting AsIII entry into rice roots via Si transporter (Ma et al., 2008). All the

study results indicated that FeMBC are more effective than MBC in reducing As concentration of aboveground biomass. Rice parboiling during post-harvest processing reduces the As concentration in rice grain of all treatments (**Fig. 7.6f**) in comparison to sunned rice grain (**Fig. 7.6e**). Surprisingly, the highest decrease of As concentration during parboiling was recorded in 0.5% MBC (22.4%) and control (21.3%) from their respective sunned rice grain.

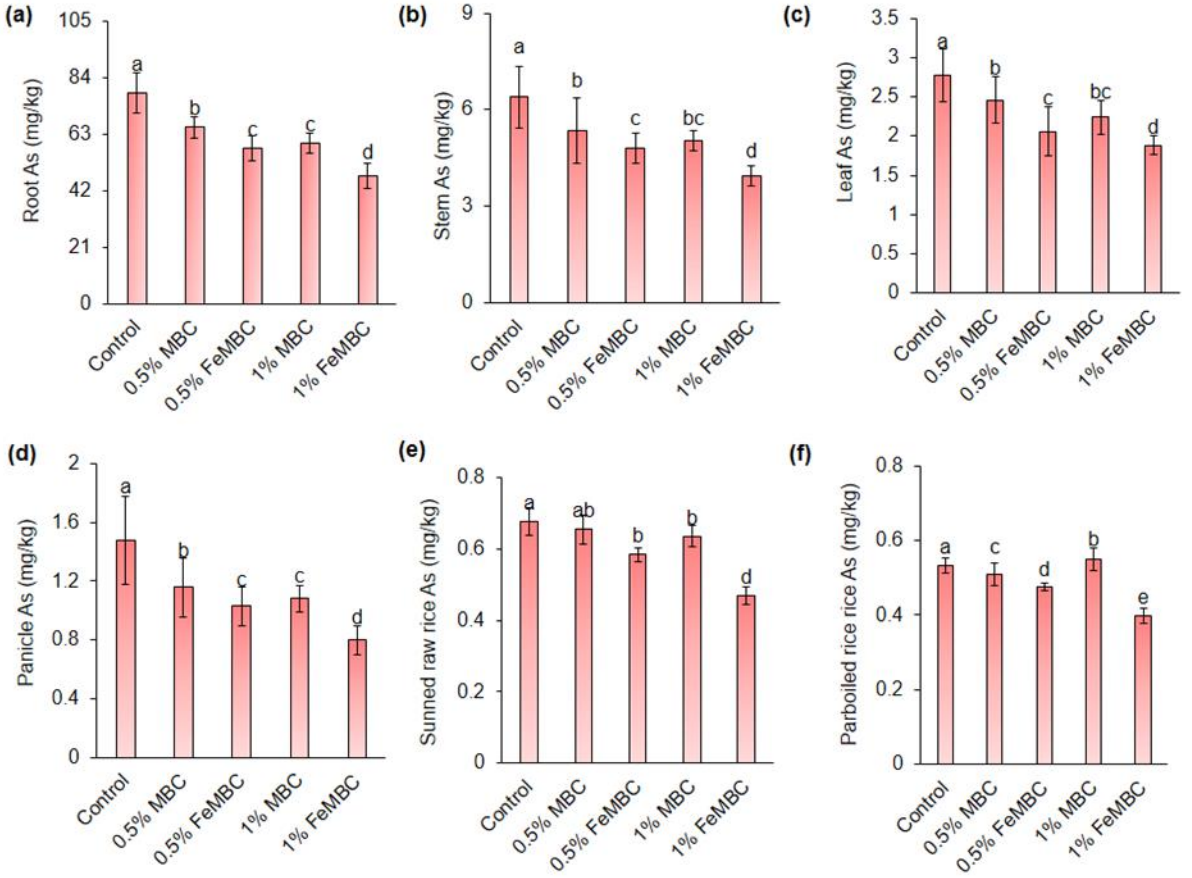


Fig. 7.6. Effects of MBC and FeMBC treatments on arsenic concentration in: (a) root, (b) stem, (c) leaf, (d) panicle, (e) sunned uncooked rice, and (f) parboiled uncooked rice. Different letters in bar graph show significant difference among treatments ($p < 0.05$)

7.3.7. Effect of biochars on micronutrient concentrations in rice grains

In both developing and developed countries, managing soil micronutrient deficiency is one of the biggest challenges (Hartley et al., 2016; Majumdar et al., 2023b). Fe, Zn, Se, Cu and Mn concentrations in uncooked and cooked rice (sunned and parboiled) were estimated (**Table 7.4**). Both 0.5% and 1% FeMBC treatments considerably increased Se and Mn concentrations as compared to control, 0.5% and 1% MBC treatments for both uncooked and cooked rice (Table

7.4). Zinc concentration in uncooked sunned rice grain of 0.5% and 1% FeMBC treatments was reduced by 9.2-12.3% and 18.7-20.8%, respectively, compared to uncooked sunned rice grain of 0.5% and 1% MBC treatments. Similar to Zn, Cu concentration in uncooked sunned rice of 0.5% and 1% FeMBC treatments was decreased by 11.8-17.6% and 18.8-24.1%, respectively, compared to uncooked sunned rice of 0.5% and 1% MBC treatments. Similar results were observed by Korai et al. (2021), where they found that application of biochars improves the micronutrients concentrations of rice and wheat grains. Biochar more efficiently binds to micronutrients and release them in the rhizosphere at slow rate (Dhaliwal et al., 2019). However, Cu concentration in uncooked rice (sunned and parboiled) was observed to be highest in control than all the biochar treatments. Cang et al. (2020) also observed that biochar application in soil reduced the Cd and Cu accumulation in rice and wheat plants. Opposite observations were also reported by other studies where biochar application improved the Zn and Cu concentration in cereals and vegetables (Gartler et al., 2013; Moreno-Jimenez et al., 2016). Iron concentration in uncooked and cooked rice of biochar treated groups was found higher as compared to control (Table 7.4). However, among different biochar treatments, the 0.5% MBC treatment showed the highest Fe content in grain. Food enriched with Fe can help to improve the health status in India, especially when the population relies on staples with very low Fe levels (Khare et al., 2022). The micronutrient deficiency is alarming for the inhabitants severely exposed to high levels of As in rural West Bengal (Chowdhury et al., 2020; Das et al., 2021b). Therefore, enhancing micronutrient content and simultaneously improving their bioavailability is crucial. However, results in this present study indicate that biochar application somewhat prevent the hidden hunger by improving the Fe, Zn, Se and Mn concentrations in rice grain. Previous studies indicate that micronutrients in raw rice grain may be negatively impacted by metal(loid) exposure; however, different biochar amendments can be used to biofortify food crops with essential micronutrients (Cang et al. 2020; Chowdhury et al. 2020; Das et al. 2021b; Hartley et al. 2016; Korai et al. 2021; Khare et al. 2022; Moreno-Jimenez et al. 2016). Although, the concentration of micronutrients in the harvested crops may vary from crop to crop, cultivation period and method, dosage and type of biochar, soil type, post-harvest processing of the crop etc. **Fig. 7.7** represents a data-based network analysis that indicates the efficacy of MBC and FeMBC separately in controlling physico-chemical parameters and bioavailability of the elements shown. Edge lengths (green) are the analyzed data from this experiment indicating the lower degree of As bioavailability in FeMBC compared to the MBC. SOC and pH are higher in MBC while EC is lower than FeMBC. Inter-related networking provides a brief summary of the primary sources or treatments (here MBC and FeMBC) and how these are

linked to the other factors of the associated system. While using the MBC and FeMBC, bioavailable As remains close to the primary nodes indicating the lesser edge values associated with the BA-As with MBC and FeMBC, proving the fact of its applicability in minimizing the As in soil while promoting other nutrients, having a greater edge values, hence at greater distance from the primary nodes. This network establishes the efficacy of MBC and preferably FeMBC for the promotion of soil nutrition while minimizing the bioavailable As.

Table 7.4: Micronutrient concentrations in raw rice grain

Type of rice	Treatments	Micronutrients concentration (mg/kg)				
		Fe	Zn	Se	Cu	Mn
Sundried brown rice	Control	8.40	3.00	0.06	1.14	7.65
	0.5% MBC	9.98	5.20	0.07	1.08	9.40
	0.5% FeMBC	9.88	4.72	0.10	0.89	12.85
	1% MBC	9.28	5.38	0.08	1.01	10.37
	1% FeMBC	9.47	4.26	0.11	0.82	14.04
Parboiled brown rice	Control	6.88	2.97	0.05	1.02	6.12
	0.5% MBC	7.95	3.83	0.05	0.96	8.82
	0.5% FeMBC	7.60	3.60	0.07	0.79	11.14
	1% MBC	7.61	3.83	0.06	0.90	9.13
	1% FeMBC	7.29	3.23	0.08	0.73	12.07

All values are mean of 3 replicates

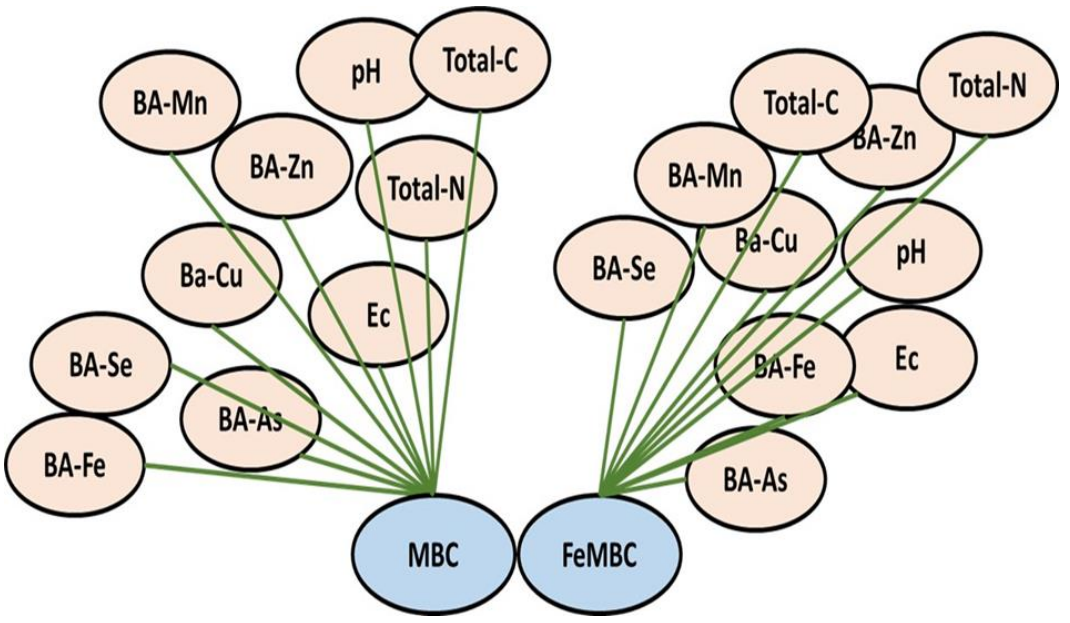


Fig. 7.7. Inter-relationship of MBC and FeMBC-controlled parameters. The subjected parameters are analyzed for a network relationship where nodes are the selected elements and factors with their respective edges as determining values

7.3.8. Effect of biochars on arsenic flux rate

While improving the nutritional status of the rice grain, As minimization was also achieved which has been proved by the experimental data obtained. However, it is essential to pin point the exact efficacy degree of any soil amendment for the abiotic stress management (Majumdar et al., 2020a; b). **Table 7.5** shows the parametric equation defining the applicability of MBC and FeMBC in minimizing the As flux compared to the control setup. Here, the flux of As indicates the difference of As content before and after treatment phase and the remaining As concentration getting percolated down to the lower soil strata. The control setup shows a lower flux rate whereas MBC has a higher flux and the FeMBC has the highest As flux rate in the pot culture. This proves the As uptake by the rice plant was higher in control soil without any amendment; however, the application of 0.5% and 1% FeMBC had the most As restriction and immobilization ability resulting in the highest flux of As and least rice plant uptake.

Table 7.5: Flux rate of arsenic determined in soil before and after the treatment phases

$[(d.W_c).E_b + P_b] - [(d.W_c).E_a + P_a] = (R_p \,.\, W_c).F_r$							
Setups	**d (μm)**	**W_c (μl)**	**E_b (μg)**	**P_b (μg)**	**E_a (μg)**	**P_a (μg)**	**R_p (μl)**
Control	5000000	45	2200	710	2100	690	650000
0.5% MBC	5000000	45	2200	690	1700	640	640000
0.5% FeMBC	5000000	45	2200	690	1400	600	660000
1% MBC	5000000	45	2200	680	1600	630	650000
1% FeMBC	5000000	45	2200	700	1300	490	640000

Setups	**$(d.W_c).E_b$**	**$(d.W_c).E_a$**	**$(R_p \,.W_c)$**
Control	4.95E+11	4.725E+11	29250000
0.5% MBC	4.95E+11	3.825E+11	28800000
0.5% FeMBC	4.95E+11	3.15E+11	29700000
1% MBC	4.95E+11	3.6E+11	29250000
1% FeMBC	4.95E+11	2.925E+11	28800000

Setups	**$[(d.W_c).E_b + P_b]$**	**$[(d.W_c).E_a + P_a]$**	**$[(d.W_c).E_b + P_b] - [(d.W_c).E_a + P_a]$**	**F_r**
Control	4.95E+11	4.725E+11	22500000020	769.2307699
0.5% MBC	4.95E+11	3.825E+11	1.125E+11	3906.250002
0.5% FeMBC	4.95E+11	3.15E+11	1.8E+11	6060.606064
1% MBC	4.95E+11	3.6E+11	1.35E+11	4615.384617
1% FeMBC	4.95E+11	2.925E+11	2.025E+11	7031.250007

Setups	**F_r if data converted in mg/kg (ppm)**
Control	0.769231
0.5% MBC	3.90625
0.5% FeMBC	6.060606
1% MBC	4.615385
1% FeMBC	7.03125

7.4. Conclusion

The present study demonstrated that both MBC and FeMBC decreased the plant-available fraction of As, which leading to decreased As concentration in the rice grain by 6.3% (0.5% MBC), 8.5% (1% MBC), 13.8% (0.5% FeMBC), and 30.8% (1% FeMBC); while maintaining the growth and yield. In comparison to their respective 0.5% and 1% MBC treatments, the 0.5% and 1% FeMBC treatments showed greater percentage of As reduction (11-28%) in grain ($P \leq 0.05$), indicating that addition of Fe to the biochar has its own advantages in production of As-safe rice grain. The increased formation of Fe-plaque in response to FeMBC treatment promoted As sequestration in Fe-plaque, preventing entry of As into the root cortex and boosting growth by lowering oxidative stress caused by As in the plant. The biochars also improved the rice grain micronutrient concentration (Fe, Mn, Se, Zn). Although the results of this pot trial are promising, the effects of MBC and FeMBC on As assimilation in rice under field conditions need to be explored further. Application of 1% FeMBC is more promising compared to the other studied biochars; however, its effect on soil beneficial microbes needs to be further investigated. Arsenic speciation is another aspect that needs to be assessed as presence of high amount inorganic As pose risk on human and animals. It is also important to look into MBC and FeMBC stability and their long-term effects on As mobility, bioavailability, and speciation in rice cropping systems.

Objective 3

Field-scale trial of amendments in arsenic-contaminated rice fields for comparative assessment of their effectiveness and impacts on enhancement of plant yield and rice grain arsenic-reduction

Chapter 8

Effect of different amendments on rice yield and inhibition of arsenic bioaccumulation in rice (Oryza sativa L.*): A two-year field study*

8.1. Introduction

Arsenic (As) is a highly toxic metalloid that have both carcinogenic and non-carcinogenic effect on human. Arsenic from agricultural soils and irrigation water have been accumulated in plants and causes food chain contamination. In addition to its detrimental effects on humans and animals, arsenic also inhibits growth and yield of plants. Compared to other crops, rice can efficiently absorb As from the soil and accumulate a higher quantity of As in the grain (Wang et al., 2021). The use of flood irrigation for rice agriculture enhances As bioavailability, resulting in increased As uptake and translocation in rice (Arao et al., 2011). Most of the rice growing fields are in Asia and a large number of Asia's rice-growing regions, especially those in China, India, and Bangladesh, have been contaminated with arsenic (Upadhyay et al., 2020). As is also present in groundwater used for irrigation (Joardar et al., 2021). In arsenic-endemic places, studies have shown that 90% of the As in rice grains are inorganic, with the remaining As species being organic (Chowdhury et al., 2020; Halder et al., 2014). Rice is an essential crop both economically and nutritionally for half of the world's population and the increase in rice grain As is a significant cause of concern these days (Pedron et al., 2019). Grain chalkiness and nutritional quality, in addition to yield loss and As, are important factors in determining the rice's overall quality. The chalkiness of rice grains makes them brittle and less palatable to consumers. Chalkiness and post-harvest loss are caused by impaired loading of starch and the development of air pockets during grain loading. Studies showed that exposure to arsenic decreased the content of vital nutrients (Williams et al., 2009) and the negative impact of other environmental stresses (apart from As stress) on the quality of rice grains (Fahad et al., 2019; Yang and Wang, 2019). However, no research has been undertaken on the quality aspect of rice grain under As stress. Bhadwal and Sharma (2022) demonstrated that arsenic exposure decreased sucrose and starch loading during the grain-filling stage. Therefore, research into how arsenic affects chalkiness and nutrition, as well as potential corrective actions to stop arsenic accumulation in rice grains, is crucial, as these factors are major contributors to economic loss and pose a danger to human health.

Owing to variations in composition, purposes, and methods of use, each of the reagents or modifications utilized in this investigation required a comparative analysis under identical environmental conditions. This research will assist us in recommending which modifications are more effective than others for increasing production and lowering rice grain As. In our prior studies, we have reduced the As toxicity from rice by using several amendments. Therefore, a comparative assessment of all the applied amendments was followed in As contaminated rice field

based on our previous work. Therefore, the objectives of this work are improvement of rice growth yield and reduction of rice grain arsenic utilizing different amendments.

8.2. Materials and methods

8.2.1. Experimental site

The experimental field was located in Madhusudankati village (22° 54' 3.366" N, 88° 46' 32.1996" E) of Gaighata block in North 24 Parganas district, which is one of West Bengal's worst As contaminated districts. Rice cultivation was carried out at two different rice cultivation season for two consecutive years: monsoon (*Aman; M1*) (2021), post-monsoon (*Boro; PS1*) (2021-2022), monsoon (*Aman; M2*) (2022), and post-monsoon (*Boro; PS2*) (2021-2023) seasons sequentially. Rice is predominantly cultivated in this particular site. Gaighata block is situated at an elevation of 39 ft from sea level, the climate is tropical monsoon. In the monsoon season (July to November), the average daily temperature and total rainfall were 29.4 °C and 1449 mm, respectively, while in the post-monsoon season (January to May), they were 28.1 °C and 181 mm. the details of the experimental site and field layout has been given in **Fig. 8.1**. The soil of the experimental field was collected at a depth of 0 - 20 cm. The collected soil was air-dried and sieved before Physio-chemical and textural properties of the soil and irrigational water were analyzed. The details of soil and irrigational water property was placed in **Table 8.1**.

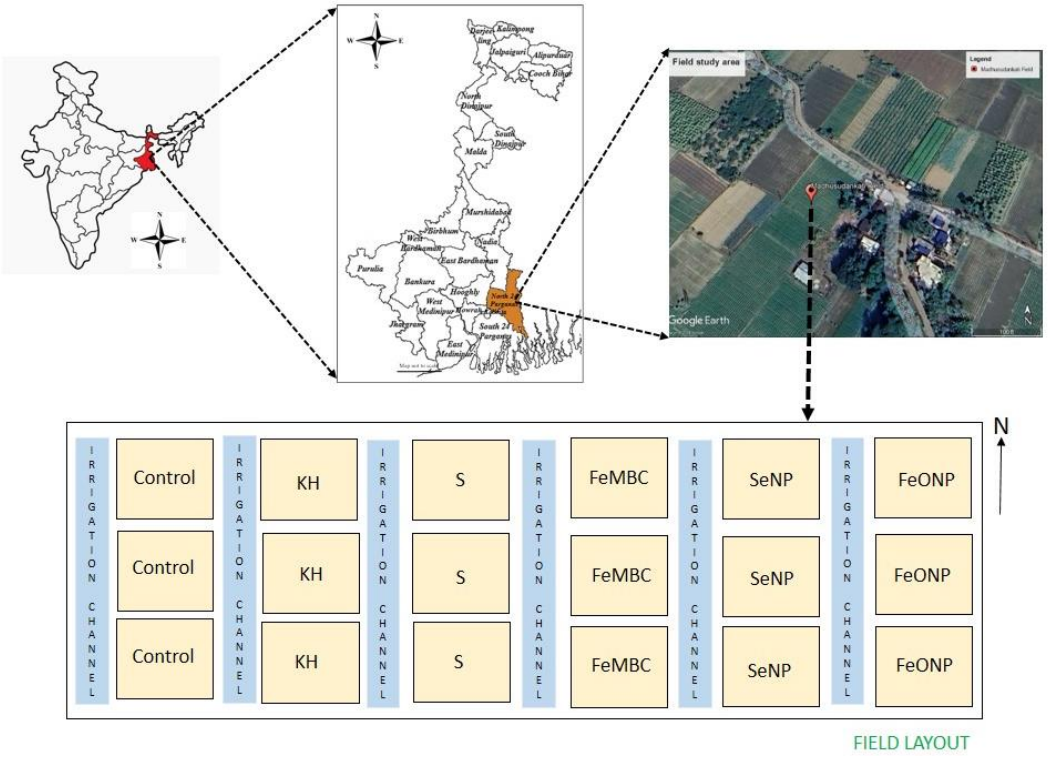


Fig. 8.1. Experimental site and field layout

Table 8.1. Physio-chemical and textural properties of soil and irrigational water

Soil		
pH		7.51
EC (mS/cm)		0.29
Organic C (%)		0.9
Sand (%)		40.1
Silt (%)		33.7
Clay (%)		26.2
Soil texture		Clay loam
Total As (mg/Kg)		18.09
Available As (mg/Kg)		1.02
XRF elemental concentration (mg/kg)		
P		1190
K		27400
Ca		7210
Mg		12500
S		244
Cu		64.6
Fe		46000
Mn		395
Zn		111
Al		86100
Si		297000
Cl		100
Na		8100
Water	**Monsoon**	**Post-monsoon**
Irrigation water applied		7200 L/plot
As added to soil by irrigation water		1202.4 mg/plot
As content in rainwater (mg/L)	BDL*	
As content in irrigation water (mg/L)		0.167
Se content in rainwater (mg/L)	BDL*	
Se content in irrigationwater (mg/L)		BDL*
pH		7.51-7.52
EC (Ms/cm)		0.524-0.53

*BDL = below detection limit; size of each plot = 400 ft^2

8.2.2. Experimental design

Arsenic sensitive rice cultivar Satabdi (IET 4786) (Mukherjee et al., 2017) was used as the rice cultivar during the monsoon and the post-monsoon season. The rice seedlings were germinated in 4 ft × 4 ft seed bed and maintained until the seedlings (around 25 days after seeding) were transferred to the respective fields. The monsoon season cultivation was carried with rain water

irrigation and during post-monsoon season, irrigation was solely dependent on shallow tube well water. Continuous flooding was maintained from transplanting to maturity phase. The plot size used for this field experiment was 20 ft × 20 ft and each plot was separated by constructed bunds to prevent inter plot lateral seepage. At transplanting, 3 seedlings per hill were planted and 15 cm ×10 cm gap in between hills were maintained.

Amendments and treatments used in experiment were: Soil application: K-humate (KH) (4 g/plot); Iron-modified biochar (FeMBC) (2.5 kg/plot), Sulfate (S) (140 g/plot), FeONPs (10 g/plot); foliar application: SeNP (30 mg/plot). Along with different treatments, a single control plot was used in this study. Every treatment was conducted in triplicate, and the amount of amendments was chosen based on previous published studies as well as our own lab-based investigations. The soil amendments were applied with the fertilizers. SeNPs were sprayed onto the leaves of the rice plant at the onset of panicle initiation and flowering stage. The prescribed N (urea), P (Single Super Phosphate), and K (Muriate of Potash) dosages were 120, 60, and 60 kg/ha, respectively. In both the seasons, Single Super Phosphate and Muriate of Potash were applied as basal dose before sowing. However, urea was applied at sowing (50%), maximum tillering (25%), and panicle initiation (25%).

8.2.3. Measurement of yield attributes

At harvesting stage, Plant root, leaf, stem, grain samples have been cleaned after harvesting to remove dirt. Root, straw, and grain samples were oven-dried for 48 h at 65 ºC. Prior to digestion, all plant samples were stored in zip lock bags. The harvested rice plants were left in the field (5-6 days) to reduce moisture content to a consistent level. To separate the grain from rice straw, a mechanical thresher was used. The yield of grain and straw were then calculated in t/ha.

8.2.4. Estimation of plant arsenic

The process of extraction and estimation of plant As was described in chapter 4. A solution mixture of HNO_3 and H_2O_2 in 2:1 was used to digest appx. 0.2 g of dry plant material. Total As concentration in plant sample was determined using AAS (Varian model AA140, USA).

8.2.5. Statistical analysis

All the experiments were carried out in triplicates and the concentrations were expressed in terms of mean ± standard deviation. The experimental data was statistically evaluated by one-way

analysis of variance (ANOVA) test in OriginPro (2019b) software and the average values were separated by Tukey-HSD test at $p \leq 0.05$ significance level.

8.3. Results and discussion

8.3.1. Effect of amendments on plant growth and yield

Rice is a staple diet for half the world's population, and arsenic poisoning can result in significant yield reductions in rice plants. Arsenic stress is one of the most major abiotic stress inducer metalloid that inhibits plant development and also has a negative impact on human health. In this study, grain and straw yields were improved as compared to control after the treatments with different amendments (**Fig. 8.2a and 8.2b**). K-humate has better grain and straw yield output among the different amendments. Among different cultivation season, post-monsoon has better yield than monsoonal cultivation period. In two years of cultivation period, second post-monsoonal season has more grain and straw yield than other three season. In an earlier investigation, Mondal et al. (2012) observed that the Satabdi rice cultivar produced more grain and biomass in the post-monsoon season compared to the monsoon season. K-humate has significantly ($p \leq 0.05$) improved the grain yield by 21.1% (M1), 18.5% (PS1), 22.8% (M2), and 22.4% (PS2) as compared to control. Similarly, straw yield as improved by 17.3% (M1), 14.7% (PS1), 16.3% (M2), and 19.2% (PS2) as compared to control after K-humate treatment. Compounds that are classified as humic acid (HA) often have comparatively greater carbon levels and aromatic ring condensation with various amino acids (Zanin et al., 2019). It has been demonstrated that HA compounds increase the permeability of membranes, respiration, and the critical micronutrient transfer via roots (Hasanuzzaman et al., 2018). Among two Fe amendment (FeMBC and FeONP), have more or less similar yields. Bidi et al. (2021) showed that Fe-compounds considerably increased the biomass and vigor of plants under As stress. Moreover, FeONPs promotes plant growth by improving hormonal (gibberellin and cytokinin) regulation, photosynthetic activity and redox process of the plants under heavy metal stress. Khan et al. (2021) found that FeO-NPs increased seed germination, fresh weight, and vigour by lowering As uptake. Islam et al. (2021), grain yield was improved by 14% and 24.7%, respectively, using 2% Iron (Fe)-enriched corncob biochar (FCB) and 2% Fe-enriched corncob-eggshell biochar (FCEB). Jun et al. (2020) observed that the biomass of sunflower plants and their roots, stems, leaves and seeds in the 5% biochar treatments were increased by 64.7, 20.4, 32.8, 36.5 and 230%, respectively in Cd, Pb, As polluted soil. Lin et al. (2017) observed that ferromanganese oxide biochar composites (FMBC) enhanced the weights of

stems, leaves, and grains by increasing soil cation exchange activities and lowering harmful levels of As in rice. Sulfur has also improved the rice grain and straw yield as compared to control. Shi et al. (2020) reported that increasing sulfate fertigation improved grain and biomass yield in wheat plant under As and Cd stress. Sulfur plays an important role in plant growth promotion and function. Earlier studies observed exogenous application of sulfur or sulfate compounds helps to remediate As toxicity in plants (Hu et al., 2007; Shi et al., 2020). Sulfur plays an important role in plant growth promotion and function. Earlier studies observed exogenous application of sulfur or sulfate compounds helps to remediate As toxicity in plants (Hu et al., 2007; Shi et al., 2020). Between the two nanoparticles, SeNPs has a somewhat higher yield than FeONPs. Earlier studies also observed similar improvement of plant growth and yield after foliar spray of SeNPs (Ding et al., 2020; Wang et al., 2023). It has been extensively documented that the use of Se can reduce oxidative stress and boost crop production (Boldrin et al., 2013).

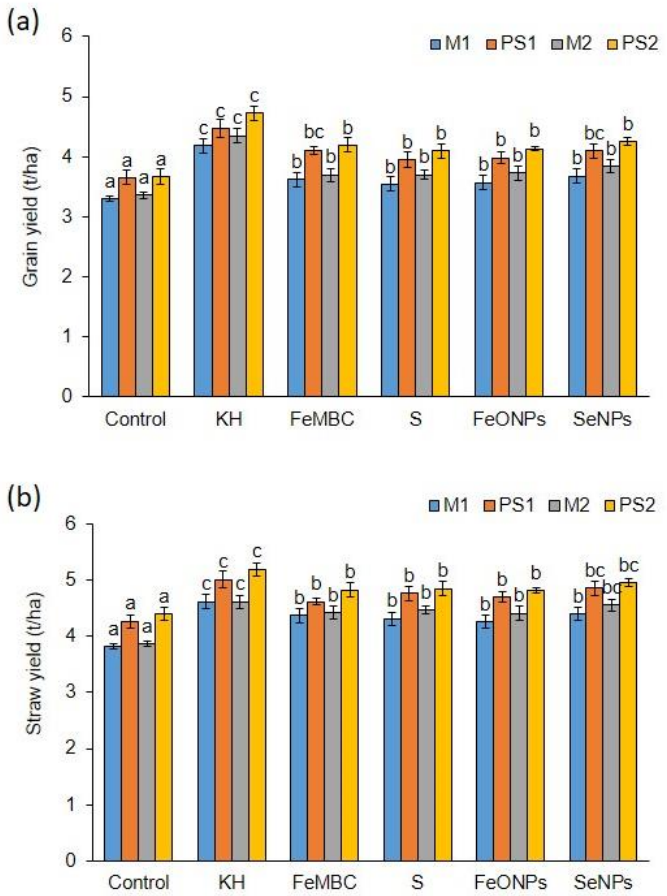


Fig. 8.2. Effects of different amendments on (a) grain (whole brown rice), and (b) straw yield. Different letters in bar graph show significant difference among treatments ($p \leq 0.05$)

8.3.2. Effect of amendments on arsenic accumulation and translocation in different plant parts

The concentration of As in different plant parts after harvesting was presented in **Fig. 8.3a-d**. Arsenic uptake and accumulation rice plants were higher in post-monsoonal cultivation (PS1 and PS2). The use of As-free rainwater during monsoonal cultivation dilutes soil As. However, using As-enriched groundwater during the post-monsoon season increased soil As load, resulting in higher As bioavailability in the soil solution. Arsenic absorption by root systems from soils, As translocation from roots to shoots, and As accumulation in grains are typically the complicated mechanisms that control the amount of As that accumulates in rice grains. Root being the primary entry point for As to get into the plant system, maximum accumulation of As was observed in root before it got transported to other parts of plants. The translocation of As from root to grain following the order of root > stem > leaf > grain, supports our previous findings of As translocation during pre- and monsoonal paddy cultivation (Chowdhury et al., 2020b). However, the application of KH, FeMBC, S, FeONPs and SeNPs application causes significant dip in As uptake and accumulation in plants. On the other hand, SeNPs exhibit root uptake of As comparable to control across all treatments. However, SeNP application ultimately reduced the translocation of As in rice grain by 39.3% (M1), 30.8% (PS1), 33.2% (M2), and 32.4% (PS2) as compared to control. Lan et al. (2023) reported that Selenium application during flowering stage significantly reduces the grain As concentration. Wang et al. (2022) found that different Se species could substantially hinder the upward transit of As in rice. Exogenous application Se may activate Glutathione peroxidase (GSH-Px) and substantially raise cysteine levels in rice plants, which helps with antioxidant defense and the GSH-Px production process that turns hazardous peroxides into non-toxic oxy-compounds (Huang et al., 2021).

Similarly, KH also reduced grain As concentration significantly by 37.4% (M1), 30.8% (PS1), 33.2% (M2), and 31.9% (PS2) as compared to control. Arsenic can bind to humic acid by forming metal bridge with the help of Fe, Ca or Al (Gong et al., 2020). However, the more probable mechanism of As-humate complexation in the present experiment may be binding of As with the functional groups like carboxyl, carbonyl, hydroxyl, methoxyl, phenolhydroxy present in humic acid. The aromatic structure of humic acid also provides certain other functional groups available for complexation. Humic acids are formed because of heteropolycondensation of phenolic substances like phloroglucinol, resorcinol, pyrogallol etc. The aliphatic side chains of such phenolic compounds may provide binding sites for As (Burges et al., 1964; Reza et al., 2012).

Thus, the provision of multiple binding sites for As-Humic acid organometallic complexation leads decrease in As accumulation in the roots and shoots. Similar reduction of As uptake, translocation, and rice grain As were also observed in FeMBC, FeONPs, and S treatments. FeMBC with a higher number of Fe functional groups and a larger surface area was able to bind As firmly on its surface, reducing As availability and bioaccumulation in rice grains. Wu et al. (2018) found that applying Fe-modified biochar (Fe-BC) in As-contaminated soil lowered the As load in rice grains by altering the As fractions in soil. It was previously observed by Yu et al. (2017) that applying Fe-fertilizers to the soil increased the development of Fe-plaques in root and reduced the intake of As in plants (Wen et al. 2021). The decreased bioavailability of As to rice plants may be due to the sulfur induced formation of iron plaque in the rice root which in turn minimizes the As accumulation in grains (Hu et al., 2007).

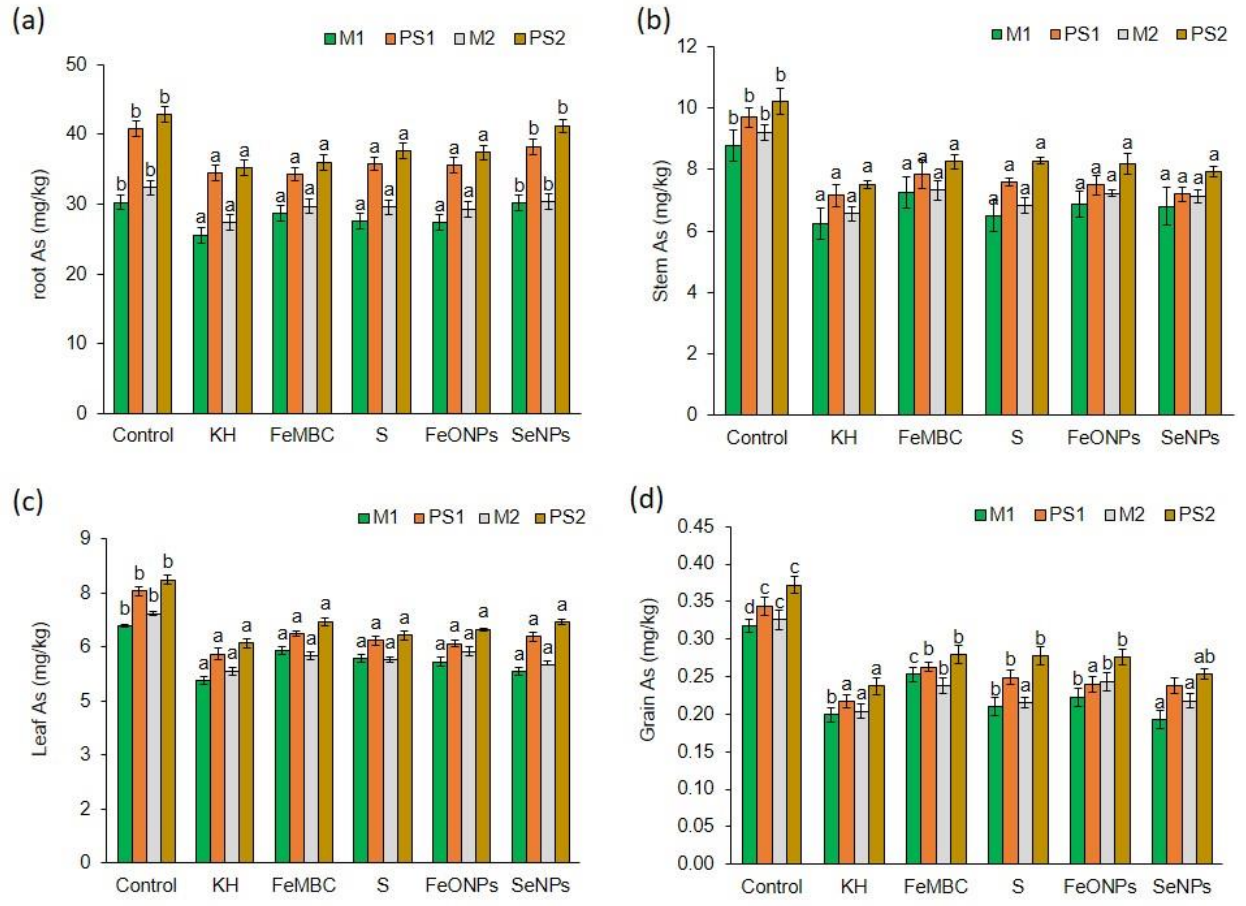


Fig. 8.3. Effects of treatments on arsenic concentration in (a) root, (b) stem, (c) leaf, (d) grain (whole brown rice). Different letters in bar graph show significant difference among treatments ($p \leq 0.05$)

In soils and sediments, sulfur plays an important function in regulating As solubility and availability. The S application enhances the synthesis of S-containing ligands, such as glutathione and phytochelatins which help to sequester As into the vacuole by chelation and restrict the flow of As from root to rice grain (Duan et al., 2011; Liu et al., 2010).

8.4. Conclusion

The two-year field study showed that plant yield and arsenic accumulation have a seasonal effect. Even though the post-monsoon season witnessed more As accumulation, the controlled groundwater irrigation technique still produced higher plant yields. All of the amendments have the ability to increase rice productivity, lessen the toxicity of As in rice plants, and lower the As content in rice grains. However, across all of the amendments, KH had a higher yield and reduced rice grain As. Long-term application of KH may be advantageous for soil health because to its lower toxicity. After analyzing all the data obtained after the use of all kind of amendments, it can be concluded that KH increases plant output and aids in the production of rice that is safe from As with respect to the other amendments.

FUTURE SCOPE OF RESEARCH

It needs to investigate further:

1. How these amendments can be used as remedial agent to alleviate the toxicity of other potentially toxic elements (PTEs).
2. Effect of these soil amendments on beneficial soil micro-biota needed to be verify.
3. Detailed study on As speciation in different rice tissues especially in rice grain is essential to provide deeper insight of As state in rice tissues and how amendments effect its speciation.
4. Effect of these amendments on soil physio-chemical properties, with special emphasis on pH, organic carbon, redox reactions.
5. Effects of these amendments on essential nutrient bioavailability and nutrient uptake in rice grain.

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RESEARCH PUBLICATIONS

First authorship

1. **Mridha, D**., Priyadarshni, P., Bhaskar, K., Gaurav, A., De, A., Das, A., Joardar, M., Chowdhury, N.R. and Roychowdhury, T., 2021. Fluoride exposure and its potential health risk assessment in drinking water and staple food in the population from fluoride endemic regions of Bihar, India. Groundwater for Sustainable Development, 13, 100558. https://doi.org/10.1016/j.gsd.2021.100558
2. **Mridha, D**., Paul, I., De, A., Ray, I., Das, A., Joardar, M., Chowdhury, N.R., Bhadoria, P.B.S. and Roychowdhury, T., 2021. Rice seed (IR64) priming with potassium humate for improvement of seed germination, seedling growth and antioxidant defense system under arsenic stress. Ecotoxicology and Environmental Safety, 219, 112313. https://doi.org/10.1016/j.ecoenv.2021.112313
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8. **Mridha, D.,** Sarkar, J., Majumdar, A., Sarkar, K., Maiti, A., Acharya, K., Das, M., Chen, H., Niazi, N.K. and Roychowdhury, T., 2024. Evaluation of iron-modified biochar on arsenic accumulation by rice: a pathway to assess human health risk from cooked rice. Environmental Science and Pollution Research, 31, 23549-23567. https://doi.org/10.1007/s11356-024-32644-z

Co-authorship

1. Chowdhury, N.R., Das, A., Mukherjee, M., Swain, S., Joardar, M., De, A., **Mridha, D.** and Roychowdhury, T., 2020. Monsoonal paddy cultivation with phase-wise arsenic distribution in exposed and control sites of West Bengal, alongside its assimilation in rice grain. Journal of Hazardous Materials. 400, 123206. https://doi.org/10.1016/j.jhazmat.2020.123206
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CONFERENCE PARTICIPATION

Participation Certificate

This is to certify that

Deepanjan Mridha

Has participated in the 8th International Congress and Exhibition on Arsenic in the Environment, Bridging Science to Practice for Sustainable Development (As2021) organized digitally at the Wageningen University and Research, Wageningen, The Netherlands from 7-9 June, 2021.

Prof. Dr. Albert van der Wal
KWR Water Research Institute Nieuwegein, The Netherlands

Dr. Arslan Ahmad
Wageningen University and Research, Wageningen, The Netherlands

Prof. Dr. Prosun Bhattacharya
KTH Royal Institute of Technology Stockholm, Sweden

Materials Research Society of India (MRSI)

(Kolkata Chapter)

Certificate of Participation

This is to certify that ***Deepanjan Mridha*** of ***School of Environmental studies, Jadavpur*** has participated at the

"Young Scientists' Colloquium 2023"

Organised by
Materials Research Society of India (MRSI), Kolkata Chapter
In Association with
School of Materials Science & Nanotechnology, Jadavpur University
In Collaboration with
Indian Institute of Engineering Science & Technology, Shibpur
On Friday, December 1, 2023 at Jadavpur University, Kolkata

Dr. Jui Chakraborty
Secretary, MRSI, Kolkata

Prof. Kalyan Kumar Chattopadhyay
Chairman, YSC 2023

Prof. N. R. Bandyopadhyay
Chairman, MRSI, Kolkata

Indian Institute of Engineering Science and Technology, Shibpur

SATEM - 2023

Certificate of Participation

This is to certify that *Deepanjan Mridha* of Jadavpur University, has participated in the

"1ST INTERNATIONAL CONFERENCE ON SUSTAINABLE ADVANCED TECHNOLOGIES FOR ENVIRONMENTAL MANAGEMENT (SATEM – 2023)"

Organized by

Department of Civil Engineering

December 20-22, 2023

22.12.2023

(Prof. D. Mazumder)

Executive Chairman, SATEM-2023

(Prof. S. Chattopadhyay)

Coordinator, CEP Cell, IIEST-S

INTERNATIONAL AWARD

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EN INDE
Liberté
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Fraternité

RAMAN-CHARPAK FELLOWSHIP-2022

This is to certify that

Mr. Deepanjan Mridha

has been awarded the Raman-Charpak Fellowship-2022 under area of **Atmospheric Sciences, Earth Sciences and Environmental Sciences.**

He carried out a part of his doctoral research on the project entitled "The effects of selenium and Silica nanoparticles on alleviation of arsenic and cadmium toxicity in rice seedlings" at University of Limoges, Limoges, France during 1st February, 2023 to 30th April, 2023.

Dr. S. K. Varshney
Adviser & Head
International Cooperation (Bilateral), Department of Science & Technology, Government of India, New Delhi

Prof. Nitin Seth
Director
Indo-French Centre for the Promotion of Advanced Research
(IFCPAR/CEFIPRA), New Delhi

Dr. Nicolas Gherardi
Deputy Counsellor for Education, Science and Culture, Embassy of France, New Delhi

MEDIA COVERAGE

বর্তমান

Dated: 20.05.2021

বীজ বপনের আগে 'সিড প্রাইমিং'-এর সুপারিশ

পটাশিয়াম হিউমেট ধানগাছে কমাচ্ছে আর্সেনিকের কুপ্রভাব

অর্পণ সেনগুপ্ত • কলকাতা: জলে আর্সেনিকের বিপদ নিয়ে কমবেশি সকলেই অবগত। তবে চাষাবাদের জলের সঙ্গে মিশে, ফসলের মাধ্যমে আর্সেনিক যেডাইনিং টেবিলেহানা দিতে পারে,সেটা অনেকের জানা নেই। ভূগর্ভস্থ আর্সেনিকমুক্ত এলাকার মানুষও সেই কারণে এই বিষের শিকার হন। তবে চালে আর্সেনিক দূষণের রক্ষাকবচ অবশ্য বের করে ফেললেন যাদবপুর বিশ্ববিদ্যালয়ের গবেষকরা। বিশ্বখ্যাত এলসভিয়ার প্রকাশনার অনলাইন জার্নালে সে গবেষণা ঠাঁই পেয়েছে।

যাদবপুর বিশ্ববিদ্যালয়ের স্কুল অব এনভায়রনমেন্টাল স্টাডিজের অধ্যাপক তড়িৎ রায়চৌধুরী এবং দীপাঞ্জন মৃধা দেখিয়ে দিয়েছেন, পটাশিয়াম হিউমেট কীভাবে আর্সেনিকের কুপ্রভাব ঠেকিয়ে দিতে পারে। তারা জানাচ্ছেন অঙ্কুরোদ্গমের আগে ধানকে ওই রাসায়নিক দ্রবণে ডুবিয়ে রেখেছিলেন তাঁরা। তারপর সেখান থেকে যখন অঙ্কুর বেরয়, তাতে দেখা গিয়েছে, আর্সেনিকের পরিমাণ বহু অংশে কম। একই পরিমাণ আর্সেনিক মিশ্রিত জলে অঙ্কুরোদ্গম হওয়া ধানে আর্সেনিকের পরিমাণ অনেক বেশি, যেটিকে আগে পটাশিয়াম হিউমেট দ্রবণে ডুবিয়ে রাখা হয়নি।

গবেষকরা বলছেন, পটাশিয়াম হিউমেট ধানের মধ্যে আর্সেনিকরোধী একটি বর্ম তৈরি করে ফেলতে সক্ষম হয়। এটি তৈরি হয় একটি অ্যান্টি-অক্সিডেন্ট মেকানিজম এর মাধ্যমে। অর্থাৎ ধানে এক ধরনের অ্যান্টি-অক্সিডেন্ট তৈরি হয়, তা আর্সেনিকের প্রভাবকে একেবারে স্তিমিত করে দিতে সক্ষম। তড়িৎবাবু বলেন, পটাশিয়াম হিউমেট দ্রবণে ধান রাখার পদ্ধতিকে কৃষি বিজ্ঞানের ভাষায় 'সিড প্রাইমিং' বলে। বলা যেতে পারে, এটি একটি টিকা, যা ভবিষ্যতে ওই ধান থেকে হওয়া গাছকে আর্সেনিকের কুপ্রভাব থেকে বাঁচিয়ে রাখতে পারে। ওই দ্রবণ বীজ তথা গাছে জিনগত কিছু ইতিবাচক পরিবর্তন ঘটিয়ে ফেলতে সক্ষম হয়। এর ফলে ফলনও অনেকটাই বৃদ্ধি পাবে বলে আমাদের আশা। কারণ দেখা গিয়েছে আর্সেনিকপ্রবণ এলাকায় ধানচাষ হলে সেখানে ফলন হয় সাধারণ এলাকার চেয়ে অর্ধেক। আর্সেনিকের বিষ ফলন বৃদ্ধিতে বাধার কাজ করে। তবে এক্ষেত্রে যেহেতু আর্সেনিক ততটা প্রভাব ফেলতে পারবে না, তাই ফলনেও তার ইতিবাচক সুফল মিলবে।

এর আগেও বিভিন্ন গবেষণায় দেখা গিয়েছে যে-পরিমাণ আর্সেনিক থাকে, তার চেয়ে অনেক বেশি আর্সেনিক থাকে ধান গাছে। সেই ধান গাছ যখন শুকিয়ে খড় বানিয়ে গবাদি পশুর খাদ্যরূপে ব্যবহার করা হয়, তখন তা পশুর শরীরে এবং দুধেও চলে আসে। মাংস, দুধ এবং দুগ্ধজাত খাবারের মাধ্যমে সেই আর্সেনিকের বিষ এসে প্রবেশ করে মানুষের শরীরে। অঙ্কুরেই আর্সেনিকের পরিমাণ কমলে খড়েও তা কম থাকবে। ফলে পাল্লা দিয়ে দূষণের ঝুঁকিও কমবে।

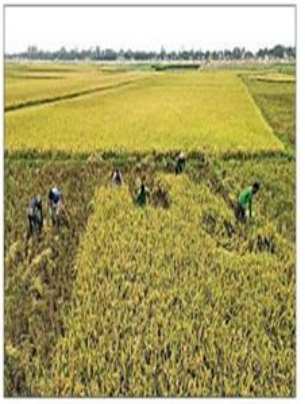
এই সময়

Dated: 19.05.2021

ধানে আর্সেনিক বিষ রুখতে দিশা বাঙালি গবেষকদের

এই সমস্যা বহু পুরোনো। তবে সমাধান মিলেছে সদ্যই। যাদবপুর বিশ্ববিদ্যালয়ের স্কুল অফ এনভায়রনমেন্টাল স্টাডিজের অধ্যাপক তড়িৎ রায়চৌধুরী এবং তাঁর ছাত্র-গবেষক দীপঙ্কর মৃধা সম্প্রতি খুঁজে পেয়েছেন এমন এক পদ্ধতি, যেটির প্রয়োগে মাটির তলে মিশে থাকা বিষাক্ত আর্সেনিক কোনও মতেই মিশতে পারবে না ধানের মধ্যে।

মাটির নীচে থাকা জলে মিশে থাকা নানা খনিজ লবণ গাছের পুষ্টি জোগায়। মূলের মধ্য দিয়ে সেই লবণ এসে ঢোকে মানব শরীরে। খনিজ লবণের পাশাপাশি ভূগর্ভস্থ জলে অনেক সময় মিশে থাকে বিষাক্ত আর্সেনিকও। বছরের পর পর ওই আর্সেনিক নানা ধরনের শস্য, বিশেষ করে চালের মধ্য দিয়ে প্রতিদিন ঢোকে শরীরে। এর ফলে ক্রমশ দুর্বল হতে শুরু করে শরীরের ইমিউনিটি। ফলে নানা অসুখও বাসা বাঁধে শরীরে।



যাদবপুর বিশ্ববিদ্যালয়ের গবেষকদের তথ্য অনুযায়ী, সমস্যা কোনও একটা নির্দিষ্ট এলাকার নয়। গঙ্গা-ব্রহ্মপুত্র-মেঘনা অববাহিকার বিস্তীর্ণ অঞ্চল জুড়েই ভূগর্ভস্থ জলে আর্সেনিকের পরিমাণ খুব বেশি। কিন্তু স্বাস্থ্য সম্মত পান এবং চাষাবাদ জন্য যতটা আর্সেনিক থাকলে মনন শরীরের পক্ষে সেটা অতিকর নয় বলে ছাড় দেয়, তার চেয়ে ঢের বেশি আর্সেনিক এই অঞ্চলের ভূগর্ভস্থ জলে রয়েছে। গবেষণা এমনই তথ্য পেয়েছেন যাদবপুর বিশ্ববিদ্যালয়ের ওই গবেষকরা।

যাঁদেরনেতৃত্বেএইগবেষণাসেই তড়িৎ রায়চৌধুরী জানান, 'আমাদের গবেষণার বিষয়টি এনভায়রনমেন্টাল জার্নাল: ইকোটক্সিকোলজি অ্যান্ড এনভায়রনমেন্টাল সেফটি-তে প্রকাশিত হয়েছে। আমরা ধানগাছকে আর্সেনিক থেকে বাঁচানোর জন্য পটাশিয়াম ফিটমাস ব্যবহার করেছি। এর ফল আমাদের হতিমধ্যেই অবাক করেছে।' তড়িতের ছাত্র-গবেষক দীপঙ্কর মৃধা বলছেন, 'ধানগাছের অঙ্কুরোদ্গমের সময়ই পটাশিয়াম ফিটমাসের প্রয়োগ করে দেখা গিয়েছে, গাছের শরীরে আর্সেনিক বিষক্রিয়ার পরিমাণ নগণ্য। স্বাভাবিক কারণেই ওই গাছে যে ধান হবে, তাতে আর্সেনিক আরও কম থাকবে।' গবেষকরা দেখেছেন, আর্সেনিক গাছের শিকড়ে সবচেয়ে বেশি থাকে এবং ক্রমশ উপরে ওঠার সময় কমতে থাকে।

তড়িতের কথায়, 'গবেষণা আরও এগোবে। ধানগাছে আর্সেনিক বিষক্রিয়ার স্থায়ী সমাধান পাওয়া গেলে তার প্রভাব কিন্তু সারা বিশ্বের উপরেই পড়বে। কারণ আমাদের দেশের যে অঞ্চল নিয়ে গবেষণা, সেখানে উৎপন্ন ধান শুধু দেশে ব্যবহার হয় না, বিদেশেও রপ্তানি করা হয়।'

গবেষকরা কাজ করার সময় দেখেছেন, গঙ্গা-মেঘনা-ব্রহ্মপুত্র অববাহিকা থেকে কিছুটা সরে গেলে ভূগর্ভস্থ জলে আর্সেনিকের পরিমাণ অনেকটা কমে যায়। যাদবপুর বিশ্ববিদ্যালয়ের গবেষকরা দেখেছেন, কয়েকটি জেলা সরে গেলেই ভূগর্ভস্থ জলে আর্সেনিকের পরিমাণও ঢোখে পড়ার মতো হারে কমে যায়। উদাহরণ হিসেবে তাঁরা দক্ষিণ ২৪ পরগনা ও পূর্ব মেদিনীপুরের কথা উল্লেখ করেছেন। তাঁদের মতে দক্ষিণ ২৪ পরগনার জলে যে পরিমাণ আর্সেনিক রয়েছে, পূর্ব মেদিনীপুরের জলে রয়েছে তার প্রায় এক-চতুর্থাংশ আর্সেনিক।

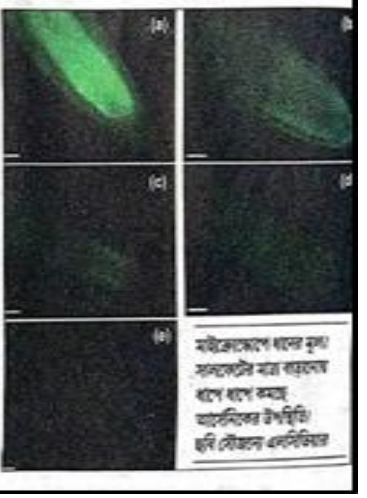
বর্তমান

Dated: 21.03.2022

কলকাতা ও শহরতলি

সালফেট সার প্রয়োগে কমে আর্সেনিকের প্রভাব

যাদবপুরের গবেষণায় আশার আলো



মাইক্রোস্কোপে ধানের মূল সালফেটের মাত্রা বাড়লেও ধাপে ধাপে কমছে আর্সেনিকের উপস্থিতি। ছবি: যৌজন্য এনভিরনমেন্ট

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Field visit at North 24 Parganas District

Field visit at Murshidabad District

Field visit at Nadia District

Field survey and experiments in Madhusudankati village, Gaighata block, Noth 24 Parganas district




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