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Biochemical alterations in arsenic stressed rice (*Oryza sativa* L.) seedlings at early growth stage

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Abstract
Arsenic (As) contamination is a major environmental issue that leads to serious health risks for living organisms. Growth, development, metabolic system, and yield of crops are drastically diminished by exposure to arsenic. Rice is the major dietary source of food for almost 3 billion people of the world. Various strategies to determine the feasible way to reduce uptake and accumulation of arsenic in paddy plants are being studied. The objective of the study was to evaluate the changes in biochemical parameters of rice genotype HUR-105 in response to the different arsenic concentration in the growing solution. Therefore, the effects of arsenic (0, 20, 50, 100, 150, 200 µM) on total chlorophyll content, total soluble sugar content, starch content and total soluble protein content with their percentage reduction in comparison with control were determined. A noticeable decrease in chlorophyll and carbohydrate content as well as protein content was observed with arsenic treatments. From the results obtained in the present study, it can be concluded that rice genotype HUR-105 is sensitive to arsenic stress.

Keywords: Rice, arsenic, chlorophyll, carbohydrates, proteins

1. Introduction
Arsenic (As) is a carcinogen and ubiquitous in the environment (Zhang *et al.* 2017) [28]. Among the metalloids arsenic is one of the important global environmental poisonous, carcinogenic and mutagenic toxicants. The presence of this metalloid, either in organic or inorganic forms in nature, causes toxicity to all forms of life including plants. The metalloid enters the environment at massive scale through the industrial application, geological processes, and the use of high arsenic contenting fertilizers and agricultural paste (Shahid *et al.*, 2019) [21]. Elevated concentrations of arsenic have been found in soil and groundwater in many countries (Wan *et al.* 2018) [26]. The intake of arsenic into the human body can occur through the food chain by consuming As-contaminated crops, vegetables, fruits, and animals and also drinking As-rich water (Abernathy *et al.*, 1999; Ghrefat *et al.*, 2016) [2, 9]. Therefore, recently arsenic toxicity has become a worldwide concern. Irrigation of arsenic contaminated groundwater, impacts of mining and smelting, and past uses of arsenic containing agrochemicals may further elevate arsenic concentration in the soil and transfer it to food crops (Liu *et al.* 2014) [16]. Long-term consumption of arsenic contaminated water and food can lead to numerous health problems, including cancer. Paddy rice (*Oryza sativa* L.) is the most widely consumed cereal in Southeast and East Asia (Wan *et al.* 2018) [26]. Unfortunately, rice is more prone to arsenic uptake than other cereals such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) (Su *et al.* 2010) [24]. Therefore, efficient control of arsenic transfer from environment to rice plants is an urgent need for food safety.
The metalloid arsenic can occur in soil in two inorganic inter-convertible oxidation forms, such as arsenate (AsV) and arsenite (AsIII). Both of them are highly toxic to humans and plants. Plants normally take up arsenic predominantly in trivalent (AsIII) and pentavalent (AsV) forms, which are known to interfere with various metabolic pathways in cells like, interaction with sulfhydryl groups and replacement of phosphate from ATP. Hence, plants not tolerant to arsenic show toxic patterns such as decrease in plant growth and crop yield (Zhang *et al.* 2017) [28]. Arsenic affects plants directly or indirectly through inhibiting the uptake of essential nutrients, such as phosphorus, selenium, zinc, silicon, calcium, manganese, potassium and magnesium because arsenic can enter cells of plants by channels of essential nutrients (Rodríguez- Ruiz *et al.*, 2019) [20]. Arsenic present in soil affects seed germination, growth, reproduction, and production capacity of plants. This metalloid inhibits extension and proliferation of root cells. However, upon translocation to shoot, arsenic can severely affect several metabolic processes in plants due to overproduced reactive oxygen species (ROS)

through the activation of NADPH oxidase (an ROS-producing enzyme) also designated as Respiratory Burst Oxidase Homolog and the impairment of antioxidant system of plants (Chen and Su, 2018) [7]. Moreover, the recent reports are not clear on how ROS are formed from the conversion of As(V) to As(III) (Abbas *et al.*, 2018) [1]. Reactive oxygen species (ROS) are produced as the earliest response to arsenic toxicity in plants, which can affect metabolism via oxidative cell damage (Venkatachalam *et al.*, 2017) [25]. The phytotoxicity of arsenic has been deeply studied, and researches showed that germination, photosynthesis and synthesis of proteins in rice seedlings were affected by arsenic treatments (Li *et al.*, 2007) [15]. Arsenic also alters photosynthetic activity by destroying cell membranes through oxidative damage, also resulting in DNA damage, particularly in As (III) treatments (Shahid *et al.*, 2019) [21]. So a sustainable method is needed to alleviate the toxicity of arsenic for effective remediation.

In this study the apparent toxic symptoms of arsenite in biochemical parameters including total chlorophyll content, total soluble sugar content, starch content and total soluble protein content were investigated. The sensitive symptoms, which could be used as indicators for arsenic toxicity, were evaluated.

2. Materials and Methods

2.1 Plant materials and growth conditions

Mature rice (*Oryza sativa* L.) seeds of genotype HUR-105 with uniform size were washed and soaked in distilled water for 24 h then surface sterilized with 1% (v/v) sodium hypochlorite (NaOCl) solution for 10 min. Fifty seeds were placed in each dish containing germination paper (Whatman paper No.40) moistened with distilled water, covered by lid and allowed to germinate in BOD incubator at 35 °C. Sodium arsenite ($NaAsO_2$) was used as source of arsenic and different arsenite concentrations (20µM 50µM, 100µM, 150µM and 200µM) were prepared. The treatments studied were expressed as (As concentration (µM): (A_1-As 20, A_2- As 50, A_3- As 100, A_4- As 150 and A_5- As 200) and Control (A_0). To analyze the changes in biochemical parameters under arsenic treatments, 7 days of old seedlings with uniform length of respective treatments, were transferred to PVC cups (12 cm diameter and 11 cm high, forty plants per cup) containing coco peat and grown in modified Hoagland's nutrient solution (Hoagland and Arnon, 1950) [12] under different arsenic concentrations. The seedlings were allowed to grow under control environment in growth chamber (Caltan-193, NSW, New Delhi) with photon flux of 400 µM m^{-2} s^{-2} PAR at 14/10 h photoperiodic settings. The temperature was set at 35 °C / 28 °C (day/night) with a relative humidity set point of 70%. The pH of the nutrient solution was adjusted to 5.8. Plants were harvested after 20 days, washed with milli-Q, separated into roots and shoots, blotted and used for the study of various parameters.

2.2 Chlorophyll content

Total Chlorophyll content was estimated in freshly harvested leaves at 30 DAG by a method described by Hiscox and Israelstam (1979) [10]. To estimate chlorophyll content 50 mg of finely chopped leaves were taken in test tube in two test tubes. Then 10 ml of dimethyl sulfoxide (DMSO) was added in each test tube and incubated at 65° C for three hours in an oven. After incubation, absorbance of DMSO containing chlorophyll was determined at 663 and 645 nm using a spectrophotometer against pure DMSO as blank. The chlorophyll content was then calculated by using following formula:

$$\text{Total Chlorophyll} = \frac{[(20.2 \times A_{645}) + (8.02 \times A_{663}) \times V]}{\text{Weight (g)} \times 1000}$$

A = Absorbance of chlorophyll extract at specific indicated wavelength, V= Final volume of the sample, W= Weight of tissue extracted on fresh weight basis.

2.3 Total Soluble Sugar and Starch content

The amount of total soluble sugars and starch was estimated using anthrone reagent as per the procedure outlined by Dubois *et al.* (1956) [8] for sugars and the procedure outlined by (Hodge and Hofreiter, 1962) [11] for starch. To estimate total soluble Sugar content 100 mg of the leaf sample was homogenized in 80% ethanol and centrifuged at 4000 rpm for 15 min. This process was repeated 3/4 times and the supernatant were collected. The final volume was made to 25 mL with 80% ethanol. 1mL aliquots were taken for analysis. The residues were utilized for starch estimation. Residues obtained was dried in oven and mixed with 6.5 mL 52% Perchloric acid and centrifuged at 4000 rpm for 15 min. The process was repeated 3-4 times and the obtained supernatant was pooled. 0.1 mL of supernatant was made up to 1 mL with distilled water and 4 mL of anthrone reagent was added and heated in boiling water bath for 8 min. After cooling, absorbance was noted at 620 nm. The starch content was calculated by multiplying total soluble sugar content with 0.9. Total soluble sugar was calculated by formula:

$$\text{Total Soluble Sugar (mg g}^{-1}\text{ FW)} = \frac{\text{Sugar value from graph (mg)}}{\text{Amount of aliquot (mL)}} X \frac{\text{Total Volume of extract (mL)}}{\text{Wt. of sample (g)}}$$

2.4 Total Soluble Protein content

The method developed by Bradford (1976) [5] was followed for estimation of total soluble protein. Fresh leaves were harvested and 100 mg of leaf samples were weighed and grinded with 10 mL of cold extraction buffer. The homogenates were centrifuged at 15,000 rpm for 15 min and the supernatant was collected and used as crude protein extract. 0.2 mL of leaf crude protein extract and 0.8 mL of distilled water were taken in a test tube and 5 mL of dye was added to it. After mixing well by vortexing (Vortexer-120209) it was kept to develop colour for at least five minutes but not longer than 30 min. The red dye turned blue when it binds to proteins. The absorbance was read at 595 nm.

3. Results and Discussion

3.1 Effect of arsenic on Total Chlorophyll

Photosynthesis is an important indicator of plant adaptability under biotic and abiotic stress and the amount of chlorophyll affects the growth rate of plants, so it can be used as an important indicator to measure the degree of stress of heavy metals on plants (Chen *et al.* 2018) [6]. The oxidative damage to plant tissues, as indicated by chlorophyll and protein

destruction, was significantly inhibited by arsenic. In this study, the changes of soluble sugar and starch content with the various concentrations of arsenic are shown in Fig.1. Chlorophyll content was negatively affected by arsenic treatments and nearly 60% reduction in chlorophyll content was observed at lower arsenic (As 20 and As 50) levels whereas almost 70% inhibition was evidenced at higher (As 100, As 150 and As 200) As levels as compared to untreated control seedlings. Hence the present study and previous literatures confirms that increasing arsenic levels severely affects chlorophyll content in leaves of rice seedlings. According to Li *et al.*, 2007 [15], chlorophyll content reduces by arsenic treatment, because arsenic enters in the leaves and get accumulated excessively and combines with -SH base of protein or substitutes for Fe^{2+}, Zn^{2+}, Mg^{2+} and destroys the structure and function of chloroplast. Arsenic enhances toxic ions and ROS production leading to breakdown and reduction of photosynthetic pigments and causes decline in chlorophyll content, carbon fixation and photosynthetic activity of plants. Generally for heavy metals the primary sites of action are the photosynthetic apparatus and its pigments and synthesis of carotenoids and chlorophyll (Rafiq *et al.*, 2014) [19] leading to reduction in chloroplast density and total chlorophyll content (Azimi *et al.*, 2021) [4]. The photosynthesis of chloroplasts is disturbed under arsenic, which causes oxygen to become its electron acceptor, and the metabolite ROS is also produced (Yan *et al.*, 2021) [27].

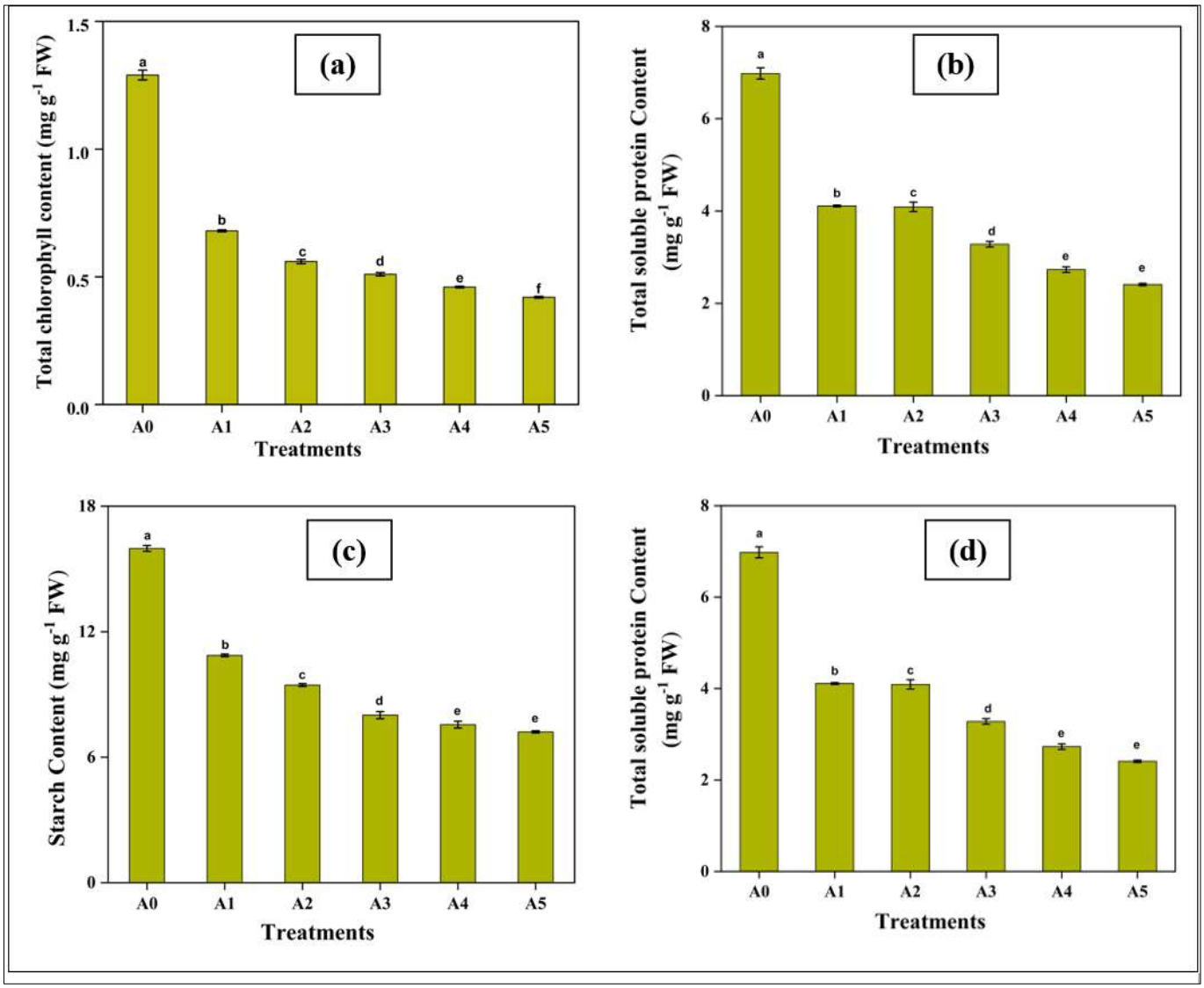

Fig 1: Effect of Sodium Arsenite (NaAsO2) on (a) Total chlorophyll content (mg g^{-1} FW), (b) Total soluble sugar content (mg g^{-1} FW), (c) Starch content (mg g^{-1} FW) and (d) Total soluble protein content (mg g^{-1} FW) in leaves of rice genotype HUR-105 at 30 days after germination.

3.2 Effect of arsenic on Carbohydrates

Environmental stress can make carbohydrate metabolism disorder. In this study, the changes of soluble sugar and starch content with the various concentrations of arsenic are shown in Fig.1. The total soluble sugars and starch content were drastically declined. Almost 40% reduction in total soluble sugar content was observed at lower arsenic (As 20 and As 50) levels and this reduction sugar content elevated with increasing arsenic concentrations and more than 50% decline in total soluble sugar content was evident at higher arsenic levels (As 100, As 150 and As 200). Further, the starch content was reduced by almost 40% at lower arsenic levels and at higher arsenic levels seedlings exhibited nearly 55% reduction over control seedlings. Toxic effects of higher concentrations of arsenic were evident from the decrease in

total soluble sugar and starch content in rice leaves. Arsenic hinders the synthesis of chlorophyll by interfering with the activity of chlorophyll synthase and after weakening the photosynthesis ultimately reduces production of carbohydrates and thereby biomass of the shoots significantly declines (Stoeva *et al.*, 2003) [23] which are consistent with present results presented in terms of soluble sugars and starch content in leaves.

3.3 Effect of arsenic on Total Soluble Proteins

The proteins play significant role in plant growth regulation by acting as storage mediums to meet the growth and nutritional demands of developing seedlings. In the present study arsenic treatments remarkably reduced the total soluble protein content in leaves of seedlings by almost 50% at lower arsenic levels (As 20 and As 50) and nearly 65% inhibition was observed at toxic arsenic levels (As 100, As 150 and As 200) over control. Arsenic encourages adverse impacts on plants and obstructs growth and protein accumulation in plants (Siddiqui *et al.*, 2020; Asgher *et al.*, 2021) [22, 3]. In present study also total soluble protein content showed similar trend. These findings are similar with the previous studies that increase/decrease in total protein content might be due to induction of stress proteins/adverse effects of ROS or increase in protease activity (Pandey *et al.*, 2015) [17]. Arsenic exposure, distorts the activities of proteases results in alteration in content of proteins and free amino acids which in turn inhibits the growth and development plants (Ismail 2012) [13]. Amino acid profiling also revealed that arsenic causes interruption in nutrient metabolism, consequently leading to loss of amino acids (Kumar *et al.* 2014) [14]. Proteins are also damaged/ modified covalently by arsenic -induced ROS, thereby delivering reactive carbonyl species (Parkhey *et al.* 2014) [18].

4. Conclusion

Arsenic toxicity has been known for centuries, and has recently received increased attention because of its chronic and epidemic effects on human health. Heavy metals including metalloid arsenic have been reported to stimulate the formation of free radicals and reactive oxygen species leading to oxidative stress. The obtained results in present study showed that seedlings of rice genotype HUR-105 exhibited negative effect of arsenic treatments. Accelerated arsenic concentration significantly decreased all the biochemical parameters studied including total chlorophyll, carbohydrates and total soluble proteins content. From the results obtained in the present study, it can be concluded that rice genotype HUR-105 is sensitive to arsenic treatment.

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