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Regulatory Role of Exogenous Melatonin in Maintaining Mineral Nutrient Homeostasis in rice (*Oryza sativa* L.) During Arsenic Stress

***Abstract*: Mineral nutrients play a crucial role in developing plant tolerance against arsenic (As)-induced phytotoxicity; however, the effect of melatonin (Mel) in the regulation of mineral nutrient homeostasis during As-stress is not yet explored. The present investigation was conducted to examine the impact of exogenous Mel on mineral nutrient status during As-stress in two different rice cultivars, viz., Khitish (As-susceptible) and Muktashri (As-tolerant). The levels of the representative macro-elements [phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S)], and micro-elements [iron (Fe), copper (Cu), zinc (Zn), manganese (Mn)], and silicon (Si) (a beneficial nutrient) have been detected through energy dispersive-X-ray fluorescence (ED-XRF) spectrometry in As-stressed rice seedlings, both in absence and presence of exogenous Mel. Mg, P and Cu level showed a decreasing trend in the stressed seedlings of both the cultivars. While Mn, K and Zn levels were lowered in the stressed Khitish seedlings, no significant changes were detected in case of Muktashri. The concentration of S, Si, Ca and particularly Fe was enhanced with As-exposure in both the cultivars. Exogenous Mel application during As-stress enhanced the level of all the mineral elements, except Fe and Cu, in Khitish, pointing to the fact that Mel largely mediated the ionome and mineral element homeostasis in the susceptible cultivar to enhance the tolerance mechanism. Overall, the present elemental analyses highlighted the fact that exogenous Mel maintains nutrient balance in rice seedlings, so as to stabilize growth and proper metabolism during As-stress.**

Key words: Arsenic stress, ED-XRF, exogenous melatonin, mineral nutrients, rice

Arsenic (As) is a widely distributed toxic metalloid, which has drawn a worldwide concern due to its potent carcinogenic ability. Unfortunately, millions of people are exposed to toxic levels of As, the long-term exposure of which causes serious health issues in humans including cancer of several organs[1]. Arsenic is ubiquitous in the environment with its absorption from the soil into the plant roots, posing detrimental effects on crop plants, thereby threatening the food availability and food security. Being a non-essential element for plant metabolism, As causes irreparable damage of important macromolecules, impede nutrient uptake, reduction in photosynthetic capacity, perturb water status and modify enzymatic activities, ultimately leading to tissue necrosis and death[2,3]. In addition, As disturbs plant metabolism by inhibiting growth and adversely influencing the nutrient uptake by competing directly with nutrients and/or altering metabolic processes, which are essential for normal physiological functioning of plants[4]. Arsenic stress causes an increased generation of reactive oxygen species (ROS), which disrupt membrane lipids and membrane permeability, consequently affecting the uptake and transport of potassium (K), calcium (Ca), magnesium (Mg) and phosphorus (P)[5,6]. Macro- and micro-elements play a pivotal role in plant growth, development and metabolic regulation as well as nutritional status, thereby determining plant durability to stress conditions[5]. The alteration in the concentration of vital elements is directly related with severe anomalies in plant system. A hydroponic experiment revealed that As-accumulation in plants may limit the accumulation of the status of minerals like K, Ca, Mg, Mn and Zn[7,8].

In recent years, melatonin (Mel) has emerged as a master regulator in plant growth, development and stress alleviator during a wide array of suboptimal conditions[9]. Melatonin is an indoleamine neurotransmitter which acts as potent antioxidant molecule to scavenge ROS in plants and enhances photosynthesis, growth, carbon fixation and defense against several biotic and abiotic stressors[10]. The basis of such tolerance is the intrinsic nature of Mel-induced stimulation of endogenous antioxidative machinery and efficiency towards inducing redox homeostasis. We earlier reported the efficacy of exogenous Mel in mitigating As- stress at the physiological and biochemical level in rice seedlings[2,3]. Although several studies have shown the detrimental effect of As in mineral nutrition in different plants, till date there are no such reports, focusing on the role of exogenous Mel in regulating the uptake and accumulation of vital minerals during As-stress. Thus, the current investigation was aimed to assess the effect of

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As-stress on various macro- and micro-nutrients and potential role of exogenous Mel in mediating As-tolerance, via controlling the accumulation of mineral nutrients in two different rice cultivars, viz., Khitish (As-susceptible) and Muktashri (As-tolerant). Khitish is a local high-yielding variety, commonly grown by the farmers. From the economic point of view, this variety is important for its higher net return and return per rupee invested during crop cultivation under both wet and dry season, because of its finer grain quality and higher market price[11]. However, Khitish is susceptible to As-stress[12]. Muktashri, on the contrary, is an As-tolerant variety, released jointly by Rice Research Station, Chinsurah, West Bengal and CSIR-National Botanical Research Institute, Lucknow, Uttar Pradesh[13]. The novel findings arising out from this investigation might disclose new avenues for the uptake and distribution of several minerals which could be utilized as biostimulating agents to improve growth and development in As-stressed rice.

Materials and Methods: **Plant Materials, Growth Conditions and Stress Imposition :** Seeds of rice cultivars, IET4094 (Khitish) and CN-1794-2-CSIR-NBRI (Muktashri) were procured from Chinsurah Rice Research Institute (Hooghly, West Bengal). Using 5% (v/v) NaOCl solution, seeds were surface-sterilized and washed extensively with deionized water. The seeds were allowed to germinate over Petri plates, lined with filter paper moistened with deionized water, and incubated at 29 ± 1 ºC for four days in dark. Following germination, the seeds were divided into non-primed (water treatment) and primed (Mel-treated) sets. The non-primed set included two subsets: (i) four day-old seedlings were continuously maintained in deionized water for 12 days; (ii) four day-old seedlings were maintained in deionized water up to 10 days, followed by treatment with 150 µM sodium arsenate for the last two days. The primed set also included two subsets: (i) four day-old seedlings were primed with 20 µM Mel for 12 days; (ii) four day-old seedlings were primed with 20 µM Mel for 10 days, followed by treatment with (150 µM sodium arsenate + 20 µM Mel) for the last two days. The seedlings of all the subsets were maintained hydroponically in the above-mentioned conditions in a plant growth chamber (N. R. Scientific, India), where the following conditions were set: temperature (32 ± 2 ºC), photoperiod (16/8 h light/dark), light intensity (700 µmol photons m^{-2} s^{-1}) and relative humidity (50%). The above mentioned solutions were replenished at every two day interval. The 16-day old seedlings were monitored for their overall growth performance and photographs taken. The seedlings were harvested for further analyses. The concentration of Mel was chosen, following Liang et al.[14], who observed that the maximum alleviation of salt stress-mediated injuries in rice plants was accomplished by exogenous supplementation of Mel at 20 µM concentration. Hence, 20 µM was selected as the final concentration of Mel for alleviating As-induced damages in rice seedlings.

Analyses of Mineral Elements: To undergo profiling of mineral elements, about 1 g of freshly harvested seedlings were freeze-dried using a freeze-dryer lyophilizer at – 80 ºC for 48 h and finely powdered. 0.2 g of each sample was converted to small pellets using a pelletizer (100 kg cm^{-2}). Each pellet was finally analysed via energy dispersive X-ray fluorescence (ED-XRF) spectrometry to estimate the level of different mineral elements. The samples were bombarded by X-ray which was emitted from X-ray tube at the anode, and the intensity of X-ray denoted the concentration of the element present. The XGT 7200, X-ray analytical microscope (Horiba Ltd., Japan) was used in our experiment.

Statistical Analyses: The experiments were carried out in a completely randomized design (CRD) with three replicates (n = 3) in each treatment with each replication containing an average of 50 seeds. Using two-way analysis of variance (ANOVA) at $p \leq 0.05$, statistical significance was calculated and the calculations were performed using XLSTAT 2018.

Results and Discussion: Arsenic is a highly toxic metalloid, which is regarded as one of the prime causes of heavy metal contamination, as well as a serious carcinogen by World Health Organization. Arsenic toxicity has been reported to modulate the accumulation of macro- and micro-elements along with other beneficial elements during abiotic stresses in order to sustain oxidative injuries[1]. In our previous communications, we reported that during exposure to As-stress, the normal metabolism of the plants gets perturbed, leading to the reduction of chlorophyll content and biomass accumulation, as well as causing stunted growth, changes in cellular water status and poor yield[2,3]. It was evident from previous studies that Mel has potential roles in counteracting heavy metal toxicity by improving the accumulation of plant osmolytes and antioxidants[4].

In the present study, As-treatment triggered the chlorosis and curling of leaf blades, as well as inhibited the overall growth in the seedlings of Khitish, while the extent of such visual damages was marginal in Muktashri. In contrast, the Mel-supplemented stressed seedlings displayed healthy-green appearance with abundant chlorophyll, in contrast to stressed-alone counterparts without Mel (Fig. 1). As-exposure decreased the Mg content by 19% and 16% in both Khitish and Muktashri

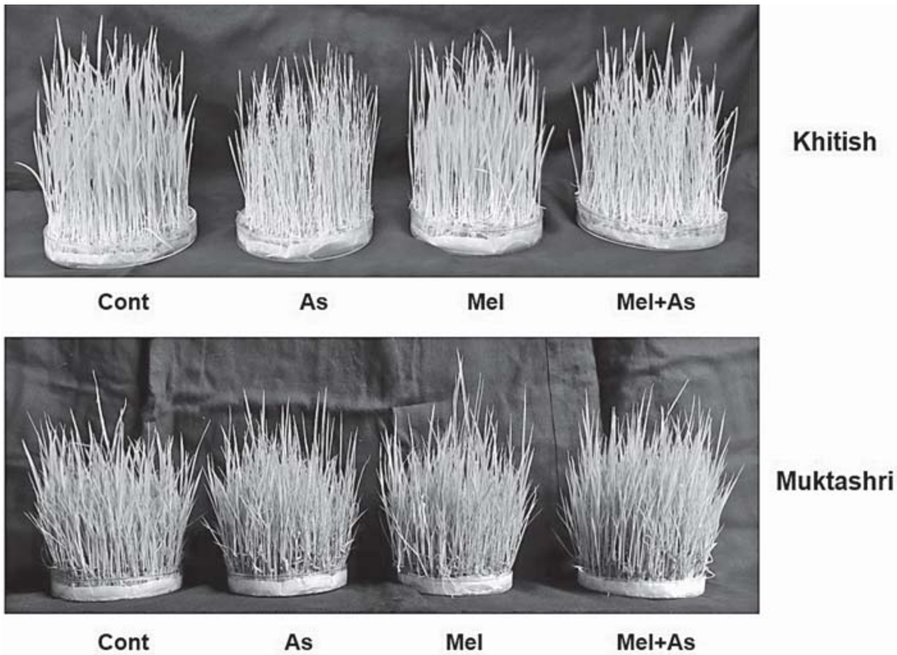

Fig. 1: Photograph illustrating the physiological growth phenotype of Khitish and Muktashri seedlings grown with deionized water, i.e., Control (Cont), 150 µM sodium arsenate (As), 20 µM melatonin (Mel) and 20 µM melatonin + 150 µM sodium arsenate (Mel+As).

seedlings, respectively, with respect to control. Mg acts as an important cofactor of enzymes involved in phosphorylation processes and also serves as central atom in the chlorophyll molecule, controlling photosynthetic machinery. As-stress however lowered the Mg level, implying negative impact on chlorophyll structure and hence the rate of photosynthesis[15,16]. This observation is consistent with the previous report by Shaibur et al.[17], although the result is in contrast to that reported by Gusman et al.[18]. Such variations in observation might be related to the fact that different plants have different adaptation mechanisms in response to stress. Exogenous treatment of Mel enhanced the Mg level by 36% and 40% in the stressed seedlings of Khitish and Muktashri, respectively, as compared to the As-treated seedlings (Table 1), meaning that Mg level was improved in presence of Mel, which in turn stabilized the chlorophyll structure. In our earlier report[3], we demonstrated that Mel supplementation enhanced total chlorophyll, chlorophyll a and b content in order to alleviate As-stress-induced chlorosis, which could be attributed to the increased uptake and accumulation of Mg, so as to maintain photosynthesis even under metalloid stress.

Arsenic exposure led to decrement in the endogenous Mn concentration by 15% in Khitish. On the contrary, Mn concentration was notably enhanced by 57% in Muktashri seedlings, compared to the respective control. Similar results were also reported by Li et al.[19] in two ryegrass species, viz., perennial ryegrass (*Lolium perenne*) and annual ryegrass (*Lolium multiflorum*). However, Mel supplementation decreased the Mn content by 19% in the stressed seedlings of Muktashri, with respect to As-alone treated seedlings, whereas a slight induction was observed in case of Khitish (Table 1). The iron (Fe) concentration was strikingly enhanced in both the cultivars during As-treatment by 286% and 172% in Khitish and Muktashri seedlings, respectively, as compared to the control. However, Fe content was decreased by 62% and 51% in the stressed seedlings of Khitish and Muktashri, respectively, following Mel supplementation during As-stress, as compared to stressed-alone counterparts (Table 1). In our earlier studies, we reported that exogenous Mel treatment up-regulated the endogenous production of Mel, which was sufficient to tackle As-stress-induced phytotoxicity[10], due to strong antioxidant properties of Mel. Mn, Fe and copper (Cu) are vital micronutrients that have several physiological roles in plants, being the structural components of several enzymatic antioxidants, particularly superoxide dismutase (SOD), which is considered as the first line of defense against (metal)loid-induced ROS[20]. Fe also acts as the cofactor of several peroxidases (POX) and catalase (CAT). The observed increase in the levels of Mn in Muktashri and that of Fe in both the cultivars could aid in appreciable up-regulation in the activity of antioxidant enzymes to cope efficiently with As-induced oxidative stress, whereas lowered levels of Fe and Mn, in presence of Mel, indicated that the antioxidants were less required during stress due to the protective nature of Mel. The reduction in Cu concentration by 42% and 28% in Khitish and Muktashri, respectively under As-stress also indicated a decline in the antioxidant capacity in both the cultivars, the susceptible variety Khitish being more affected. Exposure to this toxic metalloid could detrimentally affect the uptake of such essential micronutrients. Cu concentration was however recovered via exogenous supplementation of Mel in the stressed seedlings of Khitish and Muktashri by 85% and 34%, respectively, compared to stressed-alone counterparts (Table 1). Strong Mel-mediated activation of SOD in As-stressed set of both the cultivars, as reported

Table 1. Comparative analysis of representative mineral elements in Khitish and Muktashri seedlings, grown with deionized water (Cont), 150 µM sodium arsenate (As), 20 µM melatonin (Mel) and 20 µM melatonin + 150 µM sodium arsenate (Mel + As). All the values are means of three replicates (n = 3) ± standard error (SE). The symbols '*' (for comparison within treatments) and '#' (for comparison between varieties) represent significance at $p \leq 0.05$. The levels of all elements are represented in mg kg^{-1} dry weight (DW).

Elements	Khitish				Muktashri			
	Cont	As	Mel	Mel+As	Cont	As	Mel	Mel+As
Mg	0.376 ± 0.019	0.303 ± 0.015*	0.384 ± 0.019	0.414 ± 0.021*	0.386 ± 0.019	0.324 ± 0.016*	0.398 ± 0.020	0.454 ± 0.023*
S	0.154 ± 0.008	0.201 ± 0.010*	0.191 ± 0.009	0.298 ± 0.015*	0.293 ± 0.015#	0.423 ± 0.021*#	0.323 ± 0.016*#	0.409 ± 0.020*#
P	0.927 ± 0.046	0.783 ± 0.039*	0.963 ± 0.048*	0.859 ± 0.043*	0.731 ± 0.037#	0.618 ± 0.031*	0.754 ± 0.038#	0.716 ± 0.036*
Mn	100.8 ± 5.1	85.8 ± 4.3*	103.4 ± 5.2*	93.3 ± 4.7*	30.0 ± 1.5#	47.1 ± 2.4*#	33.6 ± 1.7#	38.1 ± 1.9*#
Fe	33.3 ± 1.7	128.4 ± 6.4*	21.8 ± 1.0*	48.4 ± 2.4*	48.2 ± 2.4#	131.4 ± 6.6*	40.2 ± 2.0*#	63.9 ± 3.2*#
Cu	4.8 ± 0.2	2.7 ± 0.1*	4.7 ± 0.2	5.1 ± 0.3*	6.5 ± 0.3#	4.7 ± 0.2*#	5.9 ± 0.3	6.3 ± 0.3
Zn	100.2 ± 5.0	83.4 ± 4.2*	151.3 ± 7.6	168.5 ± 8.4*	137.1 ± 6.9	148.8 ± 7.4#	151.9 ± 7.6	193.5 ± 9.7*
Si	7.3 ± 0.4	13.2 ± 0.7*	9.5 ± 0.5*	17.2 ± 0.9*	9.6 ± 0.5#	14.5 ± 0.7*	8.3 ± 0.4	14.0 ± 0.7*
Ca	0.052 ± 0.003	0.076 ± 0.004*	0.043 ± 0.002	0.062 ± 0.003	0.050 ± 0.003	0.053 ± 0.003#	0.042 ± 0.002	0.063 ± 0.003*
K	10.5 ± 0.5	9.4 ± 0.4	13.6 ± 0.7*	17.6 ± 0.9*	16.0 ± 0.8#	25.1 ± 1.2*#	15.3 ± 0.8*	26.0 ± 1.3*#

earlier[2], was correlated with the enhanced accumulation of Cu, which probably could serve as crucial cofactor of this enzyme, facilitating stress tolerance in rice seedlings.

Both P and K are important for energy transfer, and protein and fat metabolism[5,21] in plants. The level of P, similar to that observed for Mg, Mn and Cu, was reduced upon As-exposure by 16% and 15% in Khitish and Muktashri seedlings, respectively, than those observed in unstressed seedlings. The possible reason for decrement in P concentration under As-toxicity is due to exhibition of similar chemical speciation with arsenate (As^{V}) which arrests phosphate uptake by recognizing the phosphate transporters[22]. However, Mel supplementation restored the P content by 10% and 16% in the stressed seedlings of Khitish and Muktashri seedlings, respectively, compared to stressed-alone counterparts (Table 1). This is attributed to the fact that Mel might play a vital role in P uptake by blocking the access of phosphate transporters to As, thereby restricting As-uptake and mediating ion equilibrium under As-stress. K content was found to be differentially regulated in the two rice cultivars under As-toxicity. K content was recorded to be lowered by 11% in Khitish upon stress exposure. On the contrary, the concentration was appreciably escalated in Muktashri by 56%, when compared with that in control. It can be argued that K, being involved in stress modulation in plants[23], higher accumulation of this element under stressed condition reflects the ability of the tolerant cultivar to properly redistribute K to mitigate oxidative stress-induced injury. Supplementation of Mel more significantly enhanced K content by 88% in Khitish during As-stress, compared to As-alone treated seedlings, while no significant change was noted in case of Muktashri (Table 1). This clearly indicated that K played a significant role as a protective micronutrient in presence of Mel in the sensitive cultivar. Sulfur (S) is also of great significance, since S-containing amino acid, cysteine or S-derived metabolites such as glutathione and phytochelatin provide protection against metal(loid)-induced oxidative stress[24]. Arsenic treatment enhanced the accumulation of S content by 31% and 44% in Khitish and Muktashri, respectively, as compared to the control seedlings. Mel-supplemented stressed seedlings of Khitish, displayed further enhancement in the concentration of S by 48%, compared to stressed-alone counterparts (Table 1), pointing towards the greater involvement of S in promoting metalloid chelation to attenuate As-induced phytotoxicity in presence of Mel. However, the concentration of S remained almost unaltered by Mel in case of Muktashri.

Generally, silicon (Si) is considered as quasi-essential element for plant growth and development[25]. Rice is documented as the highest silicon accumulator among grass family, and this element primarily acts as a physical (apoplastic) barrier against different stressors, following deposition on the tissue surface[26]. Arsenic toxicity enhanced the Si content by 81% and 52% in both Khitish and Muktashri seedlings, respectively, with respect to control. Si concentration was further elevated upon Mel supplementation, particularly in the stressed seedlings of Khitish by 30%, with respect to stressed-alone counterparts (Table 1). Thus, Si visibly played a functional protective role in the susceptible cultivar. The protective role of Si during several abiotic stress conditions is well established in plants[27]. Zinc serves as the requisite cofactor for several hundreds of enzymes and is also essential for chlorophyll biosynthesis in plants[28]. Zn level was decreased in the stressed seedlings of Khitish by 17%, while remaining almost unaltered in Muktashri, with respect to the control. A striking upsurge in Zn content was noted in both the cultivars upon Mel application during As-stress, with more extent of escalation in Khitish by 102% (Table 1). Exogenous Zn was earlier reported to confer tolerance against salt stress[29]. Enhanced Zn level upon Mel supplementation in our study probably played an indirect role in the alleviation of metalloid toxicity. Under As-stress, the Ca content was increased in both the cultivars by 47% and 7% in Khitish and Muktashri seedlings, respectively, with respect to the control counterparts. The increase in Ca uptake and cytosolic Ca spike are common phenomenon during stress, because Ca acts as a second messenger in the stress-induced defense response pathways[30]. In comparison to the tolerant variety where Ca level remained unaltered, Khitish exhibited enhancement (47%) in Ca accumulation during stress which indicated Ca utilization to alleviate metalloid stress-induced injury. However, the content was reduced in the stressed seedlings of Khitish by 19%, when Mel was exogenously supplemented in conjunction with As, as compared to stressed-alone counterparts. In contrast, the Ca content was enhanced upon Mel application by 17%, as compared to stressed samples (Table 1). The improvement in Ca concentration in Mel-supplemented stressed seedlings of Khitish suggested that Mel positively influenced the Ca-dependent pathways and proteins during As-stress response. It was earlier reported that under As-stress, Mel and Ca^{2+} synergistically suppressed DNA damage, apoptosis of stomatal guard cells and ROS formation[30].

Conclusion : In a nutshell, the present study for the first time established that exogenous application of Mel during As-stress largely modulates the ionome and homeostasis of endogenous mineral elements, more prominently in the susceptible rice variety, Khitish. This presumably confers better protection of vital enzymes and biomolecules, strengthening the enzymatic antioxidants, triggering the defense signaling pathways, as apparent from our earlier studies, thereby improving the overall plant growth performances. Since Mel is ubiquitous, cheap and environmental-friendly in nature, our results prove that Mel supplementation can be an economic strategy to ensure the survival of rice plants in As-affected soil.

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