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# *Azolla pinnata* redefines its importance in rice fields as it alleviates aluminum toxicity and low pH stress

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**ABSTRACT**

Monoculture of rice seedlings or *Azolla pinnata* was challenged with different aluminum stress conditions, and both species showed significantly reduced total biomass, chlorophyll, root, and leaf length. Mixed cultures showed no stress phenotypes and notably enhanced growth parameters under low and moderate aluminum stress (10 and 30 µM). Discretely, *Azolla* plants failed to survive when grown at >30 µM aluminum treatment (pH 4.75) but sustained well when grown with rice plants. Importantly, both species accumulated less aluminum and more root exudates in mixed cultures of *Azolla* and rice plants. Furthermore, expression of *Sensitive To Proton rhizotoxicity1* (ApSTOP1 and OsART1) in both species declined significantly in mixed cultures than in monocultures. *Ammonium transporter 1* (ApAMT1 and OsAMT1.1) expressed significantly more in heterogeneous cultures, indicating that ammonium transport is unaffected. Our observations conclude that aluminum accumulation and stress effects significantly decreased in heterogeneous cultures when compared with homogenous cultures.

## 1. INTRODUCTION

Acidic soil is one of the most common issues faced by farmers in agricultural fields, where major crops are grown [1]. Acidic soil environments are stressful as well as toxic to plants [2]. When soil and surface water are acidified to a pH below 5.0, monomeric aluminum hydroxides and Al-containing minerals are dissolved to form polynuclear aluminum species and trivalent aluminum ($Al^{3+}$) that are readily absorbed by plants [3,4]. However, soluble Al like aquo/hydroxo-Al complexes have deleterious effects on plant growth and survival, primarily by hindering the uptake of nutrients from the soil [5,6]. In plant tissues, Al content ranges from 0.2 to 1000 mg/kg, and variations in Al accumulation are regulated by different factors such as plant species, soil type, and soil nutrients. It has been reported that excess Al is found in those plants that grow in acidic soils mainly [7]. Al exists in soil in the form of various ions, among which $Al^{3+}$ is the most toxic [6,8]. Al also forms insoluble oxides and hydroxides at pH 5.0 to 6.2, as well as polynuclear dissolved species below pH 5.0, that determine Al toxicity [5,9].

In Arabidopsis, $Al^{3+}$ ions are recognized and regulated by several genes, transcription factors, and transporters [10]. The principal functional tool for plant tolerance to $Al^{3+}$ includes $Al^{3+}$-induced stimulation of transporters that accelerates the discharge of organic acids from the roots. When the amount of $Al^{3+}$ increases in the soil, it is either chelated by organic acids that are excreted from the roots into the rhizosphere or Al enters the plant cells through the roots via absorption [11]. *Multidrug and Toxic compound Extrusion* [MATE] and *aluminum activated Malate Transporters* [ALMT1] are key transporters involved in the release of citrate and malate, respectively, in most plants. These transporters are regulated by the STOP1 system [12-14]. Rice plants displayed a high level of Al-tolerance when compared with other crop plants [15]. In particular, *sensitive to aluminum rhizotoxicity 1* [STAR1] and *sensitive to aluminum rhizotoxicity 2* [STAR2], which encode domains of a *bacterial-type ATP-binding cassette* [ABC] transporter, and *Al-resistance transcription factor 1* [ART1], a homologue of STOP1, are key genes involved in Al tolerance. These genes are mainly expressed in roots and regulate multiple genes for Al tolerance in rice plants [16]. The *Azolla* plant is a heterosporous aquatic fern characterized by high rates of multiplication and biological nitrogen fixation potential due to the presence of cyanobacterial symbionts. The cyanobacterial symbiont in *Azolla* can fix a significant amount of nitrogen [0.15–0.17 mg $Nh^{-1}g^{-1}$ of dry biomass] compared with *Rhizobia,* which fixes only 0.08 mg $Nh^{-1}g^{-1}$ of dry biomass in *Glycine max* [17]. In addition, studies have found that under stress conditions, *Azolla* can also increase the nitrogen use efficiency (NUE) of rice plants, thereby increasing grain yield [18].

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The peripheral zone of cavities in each dorsal frond of *Azolla* is filled with a mucilaginous fibrillar network in which $N_2$-fixing cyanobacteria reside and release ammonium for utilization by their host [19,20]. *Azolla* plants can survive in various pH ranges [3.5 to 10] [21] and can also accumulate different metal ions [22-24]. Furthermore, *Azolla* is a plant frequently intercropped with rice to take advantage of nitrogen fixed by the *Azolla-Anabaena* symbiosis [25]. Apart from these, no other major advantages were recorded for intercropping rice with *Azolla*. Therefore, this study focuses on the effects of Al on the growth and development of *Azolla* and rice plants under acidic aquatic environmental conditions. Since soil acidity is increasing due to global warming and other factors [26], we selected Al toxicity studies in the rice-*Azolla* intercropping system. Likewise, the effects of Al stress on the growth and development of *Azolla pinnata* plants have not been studied. We also investigated the effects of Al stress on the expression profiles of ammonium transporters [AMTs] and STOP1 genes in both species. STOP1 regulates Al tolerance levels by controlling organic acid exudation, whereas AMT1 controls ammonium uptake in both plant species [12, 27].

Several intercropping methods have achieved remarkable results in rice improvement programs. Recent reports clearly demonstrated that the intercropping of rice with different plant species uncovered several advantages, like increased yield, reduced heavy metal accumulation in the plant, confined N fertilizer application, etc. [28,29]. In particular, intercropping of water spinach and rice plants can control pests, increase yields [30], reduce cadmium [28] and arsenic accumulation [31], and increase silicon absorption [29]. Reduced pest attack and increased yield parameters were also recorded in the rice-*Pontederia cordata* intercropping methods [32]. Interestingly, intercropping upland rice with forage grasses produces a higher yield, biomass, and N content [33]. In addition, polyculture of rice and water mimosa can help reduce the application of N fertilizer and reduce the loss of N from the agriculture field [34]. Studies that utilized fresh *Azolla pinnata* or its compost powder in rice fields, with or without inorganic nitrogen application, reported increased yield and N uptake of rice plants, improved nutrient content of soil, as well as reductions in weeds and ammonia volatilization [35-40]. Intriguingly, the impact of Al stress on the growth and development of rice plants is well-researched [41-46], but the effects induced by Al on *Azolla* and rice plants under mixed growth conditions have not been studied yet. Hence, the aim of this study was to characterize the potential of *Azolla pinnata* as an Al toxicity mitigator in the rice-*Azolla pinnata* intercropping system. We therefore investigated *Azolla pinnata* and IR64 rice seeds in individual cultures as well as in mixed cultures to study the effect of Al toxicity in an acidic medium on both plants in relation to phenotype, microscopic, physiological, and biochemical responses. We further studied the expression profiles of the STOP1 and AMT1 genes of both plants in both cultures. Lastly, aluminum uptake by both species was determined and discussed in relation to the application of *Azolla pinnata* in rice fields for alleviating Al toxicity in acid soil.

## 2. MATERIALS AND METHODS

### 2.1. Plant Collection and Stress Treatment

For this study, *Azolla pinnata* plants were collected from Acharya Jagadish Chandra Bose Indian Botanical Garden, Howrah, and washed several times in tap water to remove algae and other plants. The tiny plants were then washed five times with distilled water to wash away other contaminants. *A. pinnata* was then cultured in Molecular Genetics Research Laboratory [MGRL] nutrient solution and sub-cultured several times for its growth and maintenance. MGRL hydroponics media contain the following nutrients: 1.5 mM $MgSO_4.7H_2O$, 2.0 mM $Ca(NO_3)_2.4H_2O$, 3.0 mM $KNO_3$, 10.3 µM $MnSO_4.H_2O$, 30 µM $H_3BO_3$, 1.0 µM $ZnSO_4.7H_2O$, 24 nM $(NH_4)_6Mo_7O_{24}$, 130 nM $CoCl_2.6H_2O$, 1.0 µM $CuSO_4.5H_2O$, 8.6 µM $FeSO_4.7H_2O$, 67 µM $Na_2EDTA$, and 200 µM $CaCl_2$ [12, 47]. After reaching the optimum growth rate of these plants, 50 plants were randomly selected and used for the stress treatments. For Al tolerance studies, different concentrations of $Al^{3+}$ ranging from 10 to 50 µM were prepared using 500 mM of $AlCl_3$ stock solution. The pH of the medium was adjusted to 4.75-4.8 using 1 N HCl. Then, surface-sterilized rice seeds (IR64) were placed on moistened filter paper on multiple layers of cotton and germinated under dark conditions. After 3 days of incubation, healthy, uniformly germinated seedlings were subjected to Al stress treatment as per the experimental setup. Ten rice plants and 50 *A. pinnata* were selected for both monoculture and intercropping experiments. Monocropping experiments were performed in smaller containers with 500 ml of media, while for the intercropping experiment, rice and *Azolla* plants were cultured together in the above media (4000 ml) in 5 L glass containers. All three culture groups (*Azolla pinnata*, *Oryza sativa* and mixed cultures of both species) were incubated at 28 ± 1°C, illuminated for 16 h/day with a light intensity of 50 ± 5 µmol $m^{-2}s^{-1}$ and maintained for 15 days. Further, monoculture experiments were repeated five times, whereas intercropping experiments were repeated three times.

### 2.2. Phenotypic and Microscopic Analysis

Both rice and *Azolla* plants, exposed to different concentrations of Al, were evaluated for morphological parameters using a ruler and ImageJ tools. In addition, fresh and dry weights were measured. For microscopic analysis, plant roots were treated with Evans blue staining [48], prepared by dissolving 0.25 g of Evan's blue in 100 ml of a 0.1 M $CaCl_2$ solution (pH 5.6). The *Azolla* roots were transferred to 2 ml centrifuge tubes, and 2 ml of dye was added. The tubes were shaken for 20 oscillations/ min for 15 min. After the incubation, the samples were washed with 0.1 M $CaCl_2$ (pH 5.6), and the roots were observed under a Dewinter Biological Binocular Microscope to evaluate variations. Finally, ImageJ software version 1.53e was used to study the root hair length and other important root traits.

### 2.3. Lipid Peroxidation Measurement

Fresh roots and leaves (~0.5 to 1 g) were collected from the control cultures and Al treated rice seedlings or *Azolla* plants, which were grown in presence and absence of each other. The plant parts were grounded in 5 mL of 5% trichloroacetic acid (TCA) and the homogenate was centrifuged at 12000 rpm for 10 min. Then, 2 mL of supernatant was mixed with 2 mL of 2-thiobarbituric acid (TBA; 0.67%), boiled for 30 min, and cooled on ice, followed by centrifugation to recover the supernatant. The absorbance of the supernatant was measured at 440, 532, and 600 nm. Malondialdehyde (MDA) content ($C=[6.45*(A_{532}-A_{600})-0.56*A_{440}]*5/0.5$, in mol/g) was calculated [49].

### 2.4. Peroxidase Enzyme Activity Assay

To assess the peroxidase enzymatic activity, fresh rice shoots or *Azolla* plants [1g] were collected from the treatments grown in absence and presence of the other under different Al stress conditions. Samples were macerated for 1 min with 3 mL of extraction buffer (50 mM potassium phosphate buffer [1.5 mL, pH 7], 1 mM EDTA [0.03 mL] and 2% [w/v] polyvinyl polypyrrolidone [PVPP] [1.47 mL]). The extracted solution was then centrifuged at 14,000 rpm for 15 min at 5°C and the collected supernatant was used to measure peroxidase enzyme activity by measuring absorbance at 436 nm [50, 51].

### 2.5. Analysis of Photosynthetic Pigments from Al-Stressed Plants

The photosynthetic pigments were measured from both rice and *Azolla* plants after 15 days of Al treatment under hydroponic, low pH conditions. Approximately, 500 mg of rice leaves and whole *Azolla* plants from different concentrations of Al stress conditions were weighed and transferred to 50 ml centrifuge tubes containing 25 ml of methanol. Before transferring the plants to the methanol solution, they were blotted with sterile paper towels or filter papers to remove the water droplets. The centrifuge tubes were then shaken at 120 rpm for 30 min, and chlorophyll a and b were estimated using a Shimadzu UV-Vis spectrophotometer UV-2600 and calculated according to the formula given by Ritchie 2008 [52].

### 2.6. Collection of Root Exudates and Quantification of Organic Acids

After 48 h of Al stress treatment, the hydroponic solution (1/50$^{th}$ strength MGRL nutrient solution containing 200 mM $CaCl_2$ and 1% sucrose [pH 5.5]) of each experimental unit was collected under aseptic conditions. In these solutions, the total malate released from the seedlings in the hydroponics medium was quantified by an enzyme reaction method [53]. Citrate was measured by chemical reaction [citrate lyase] for the citrate-coupled NADH/NAD+cycling technique with minor changes. The uptake of aluminum by both plant species in monocropping and intercropping studies was also evaluated using Atomic Absorption Spectrometry (AAS) technique [12].

### 2.7. Expression Profile Analysis

The expression profiles of ammonium transporters (AMT) genes and STOP1 genes were studied in Al-stressed plants. *OsAMT1.1* and *OsART1* of rice, and *ApAMT1* and *ApSTOP1* of *Azolla* were selected for our study. For total RNA isolation, 0.5 g of fresh roots from each Al-treated and control Rice or *Azolla* plants were collected and homogenized using liquid nitrogen in sterile mortar. The total RNA was isolated using the RNA isolation kit, Nucleospin, Takara. After RNA isolation, oligo dT primers were used for the first strand of cDNA synthesis (PrimeScript™ 1$^{st}$ strand cDNA synthesis kit, Takara). The reverse transcribed cDNA was used as a template for RT-PCR (Biorad). To check the expression level, 4 µl of template DNA, 5 µl of Pmax polymerase (Takara, Japan), and 1.5 µl of each of the forward and reverse primers were added to the PCR tubes. The quality and quantity of RNA were quantified using the methods described in Kundu and Ganesan, 2020 [13]. β-actin was used in both plant samples and as internal control. In *Azolla* plants, based on our initial experiments, β-actin is the most stable housekeeping gene in response to Al stress when compared with the Ubiquitin gene. Hence, we selected the β-actin gene as the internal reference. The amplified products were loaded on an agarose gel, and electrophoresis was carried out. The gel was stained with EtBr and imaged using the Gel documentation system.

### 2.8. Statistical Analyses

Mean values with standard errors were interpreted in all the figures. The values were calculated by a parametric mood median test. Duncan's multiple range test (DMRT) was used to determine the significance between the two treatments ($p = 0.05$, SPSS statistical program version 12.0).

## 3. RESULTS AND DISCUSSION

### 3.1. Phenotypic Variations of *Azolla* Plants and Rice Seedlings Under Al Stress

We developed three different experimental setups to examine the effects of *Azolla* plants on the growth and development of rice plants under acid soil stress conditions. In the first and second setups, only rice or *Azolla* plants were subjected to different concentrations of Al stress treatments. In the third setup, the effects of Al stress in a mixed culture (1:5 ratio) of rice (10 rice seedlings) and *Azolla* plants (50 plants) were investigated.

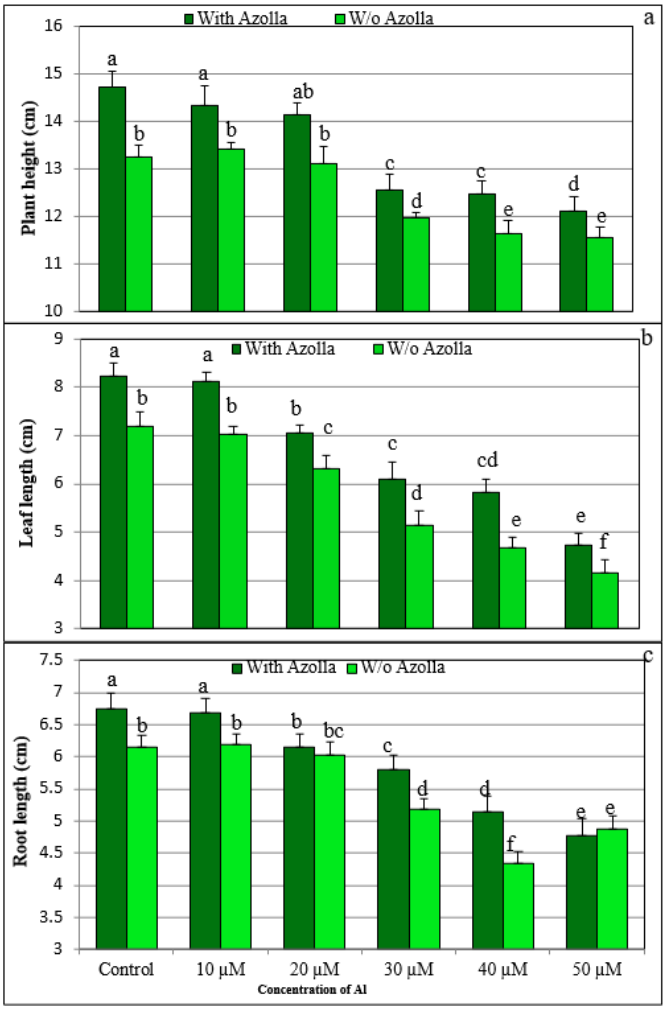


**Figure 1:** Aluminum stress effects on the total plant height **(a)** leaf length **(b)** and root length **(c)** of 15 days old rice seedlings grown with or without *Azolla* plants under different Al stress (pH 4.75) condition. Each value indicates the mean ± SE (n = 12). Th e lower-case alphabets indicate the significance at P = 0.05 according to DMRT between mono-cultured and intercropped plants.

After 15 days, rice plants, when grown alone at a 50 µM concentration of Al, attained a height of 11.5 cm. Under the same Al concentration, when *Azolla* is planted together with rice, the growth effect of rice is significantly higher (5%). Rice plants treated with moderate Al stress (30 µM) resulted in being significantly taller (5-7%) than untreated plants [Figure 1a]. The height of rice plants decreased significantly under 20 µM Al treatment, but the incorporation of *Azolla* plants

significantly increased the length of rice plants by 7%. Further, Al stress significantly reduced the leaf length of rice seedlings compared with the control treatment [Figure 1b]. In all the Al treatments, the addition of *Azolla* plants increased the leaf length of rice plants significantly by 10-17% including the controls. In respect to root traits, the root length of rice seedlings decreased significantly by 38.7% with the increase in Al concentration compared with the control treatment. Furthermore, after 15 days of continuous exposure to Al, the higher Al-stress cultures (40 and 50 µM) showed reduced root length [Figure 1c] and no root hair formation (data not shown). Under the 40 µM Al stress condition, rice roots became shorter, weaker, and were easily falling off. Interestingly, when planted with *Azolla*, the roots showed active growth and significantly increased in length (20%) [Figures 1c, 2a–h].

However, under homogeneous conditions, aluminum stress also significantly affected the growth of *Azolla* plants [Figure 3a]. At 20 µM of Al stress, the total biomass reduced significantly, and root abscission occurred. *Azolla pinnata*, when grown at concentrations above 30 µM Al, did not survive for more than 14 days. With a gradual increase in Al concentration, *Azolla's* total biomass reduced significantly by 85% (50 µM Al) [Figures 6 b–g]. Interestingly, *Azolla pinnata* survived well in mixed culture, showing a growth rate of more than 40% even at very high Al concentrations (50 µM). Also, root growth was significantly affected in *Azolla* cultures treated with 40 and 50 µM Al, and after 12 days of culture, the plants showed Al toxicity [Figures 4b–h]. The root hairs increase the surface area of primary or lateral roots and possibly enhance the nutrient uptake from the soil solution [54]. Several studies have indicated the high potential of roots and root hairs to contribute to enhanced NUE and stress acclimatization and adaptation [55]. Therefore, we evaluated the growth of root hairs in both species in monocropping and intercropping systems. The root hairs of *Azolla* plants were severely affected by Al stress under monoculture conditions [Figure 4a]. In particular, the root hair growth was stopped in 30 to 50 µM Al treated cultures. Interestingly, under mixed culture conditions, both plant species displayed no stress phenotypes. The root hairs showed no growth variation up to 50 µM Al treated cultures [Figures 4i–n]. The results of several studies have shown that the growth, development, and total biomass of *Azolla* plants are severely affected during adverse conditions [56,57]. In our study, we demonstrated that *Azolla* plants were significantly affected by Al stress and acid soil [low pH] stress conditions. Furthermore, we also noticed that the rice plants were severely affected by Al stress [Figures 3d, e]. Similarly, rice plants also displayed reduced biomass and growth rate under Al stress conditions [58-60]. Our study also demonstrated that the heterogeneous culture of rice and *Azolla* plants exhibited significant growth enhancement compared with monoculture.

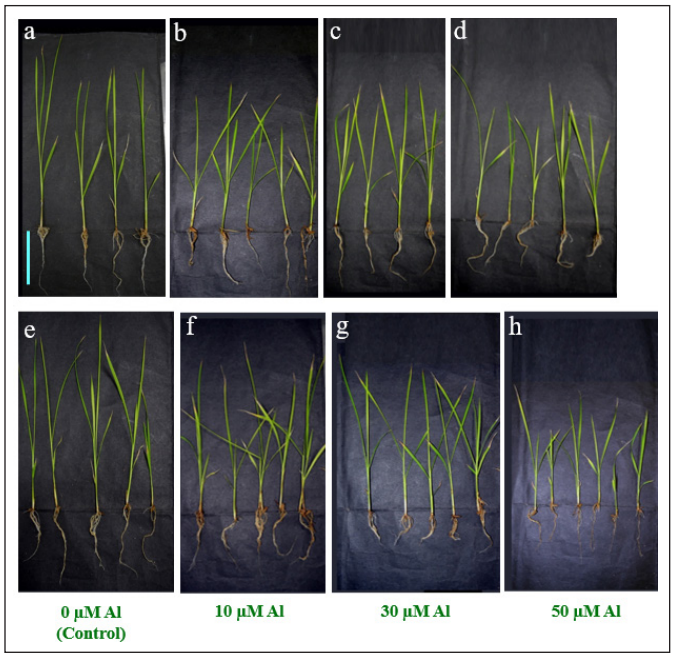


**Figure 2:** Morphological variation in the rice seedlings grown with *Azolla* **(a–d)** and without *Azolla* plants **(e–h)** in different Al stress conditions. The phenotypes were analyzed after 15 days of growth under hydroponic conditions. *(bar = 3cms).*

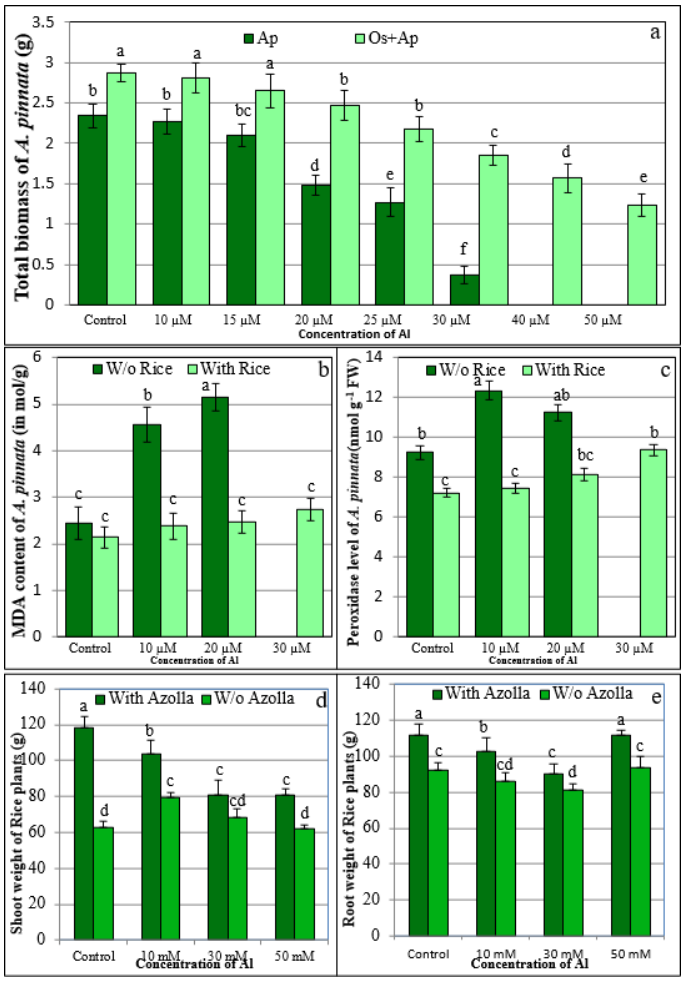


**Figure 3:** Variations in the total biomass **(a)**, MDA content **(b)** and peroxidase level **(c)** of 14 days old *Azolla* plants grown with or without rice plants under different Al stress and control condition. Each value indicates the mean ± SE (n = 15). Variations in shoot weight **(d)** and root weight **(e)** of 15 days old Rice seedlings grown with or without *Azolla* plants under different Al stress and control condition. Each value indicates the mean ± SE (n = 12). The lower-case alphabets indicate the significance at P = 0.05 according to DMRT between mono-cultured and intercropped plants. [Ap denotes Total biomass of *Azolla pinnata* in monoculture and Os+Ap denotes total biomass of *Azolla* when intercropped with rice].

### 3.2. Biochemical Characterization of *Azolla* Plants and Rice Seedlings Under Al Stress

#### *3.2.1. Variations in the total MDA content and peroxidase activity*

To understand the Al stress tolerance level of *Azolla* and rice plants, we measured the variations in total MDA content and peroxidase activity [Figures 3,5]. MDA measurement helps in determining the peroxidation level of membrane lipids in plants occurring due to abiotic stresses [61,62]. Rice seedlings produced excessive MDA [nearly 41%] in response to high Al concentrations, suggesting that $Al^{3+}$ can induce oxidative stress in the plants [Figure 5a]. When *Azolla* was grown in combination with rice plants, the MDA production showed a remarkable decline (up to 42%). In addition, when *Azolla* plants alone were treated with Al, they showed increased MDA content up to 20 µM (~2-fold increase). Treatments with Al concentrations >30 µM caused the death of the *Azolla* plants within 14 days of growth. Under mixed culture conditions, the MDA content of *Azolla* plants showed no variations up to 30 µM Al stress [Figure 3b]. Moreover, >30 µM Al treatments showed only a 20-25% increase in the total MDA content of *Azolla* plants. Our data showed that the MDA content in rice plant culture increased linearly with each stress treatment, possibly due to damage to the cell membrane caused by increased Al concentration [63].

Furthermore, we measured the peroxidase content of both species under heterogenous and homogenous culture conditions. In all the Al treatments, significant increases in peroxidase activities were recorded in rice seedlings compared to the control treatments [Figure 5b]. Importantly, the incorporation of *Azolla* plants significantly reduced Al-induced peroxidase activity. Specifically, 40 µM Al treatment increased the peroxidase activity as high as 2-fold compared to the untreated control plants. However, the introduction of *Azolla* plants decreased the peroxidase activity by approximately 4%. Moreover, homogenous cultures of *Azolla* plants did not survive when exposed to 30 - 50 µM of Al [Figure 3c]. However, when *Azolla* plants are co-cultured with rice plants, the oxidative stress of the plant is reduced. $H_2O_2$ content of the cell is mainly downgraded by peroxidases, and under stress conditions, the amount of peroxidase production increases exponentially [64,65]. In our study, we found that the peroxidase level decreased significantly in the intercropping of rice-*Azolla*. These results may indicate that rice-*Azolla* intercropped cultures were not significantly affected by Al stress.

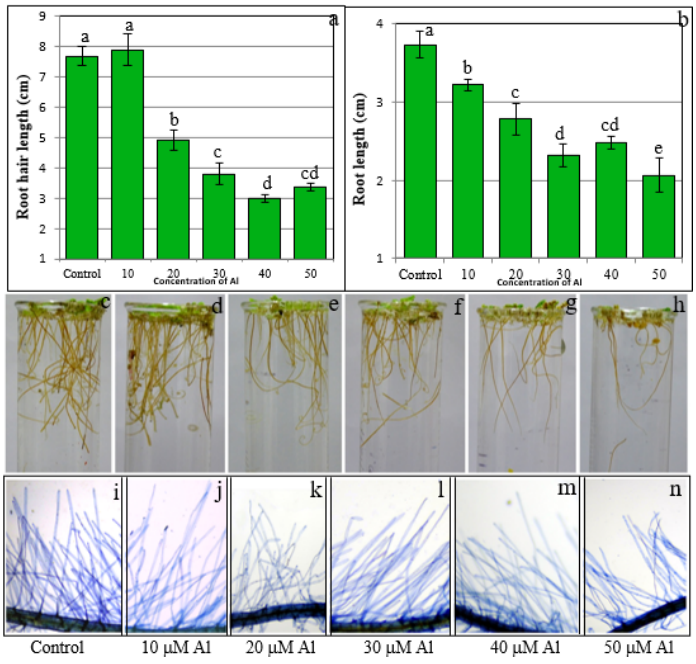


**Figure 4:** Variations in root hair length **(a, i–n)** and root length **(b, c–h)** of *Azolla* plants under different Al stress conditions in intercropped cultures. Al stress specific phenotypes of *Azolla* plants were captured by using test tubes **(c–h)**. The root hair length variations were captured after staining the roots with Evans blue **(i–n)**. Each value indicates the mean ± SE (n = 10-15). The lower-case alphabets indicate the significance at P = 0.05 according to DMRT in intercropped *Azolla* plants.

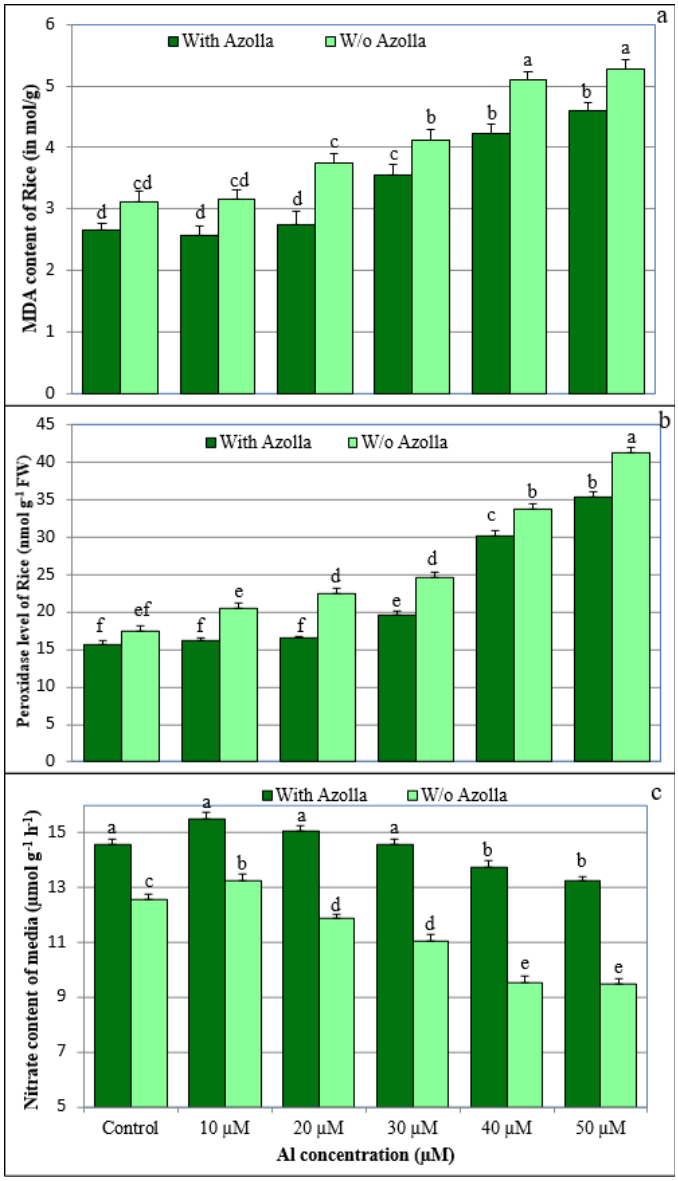


**Figure 5:** Variations in the total MDA content **(a)** and peroxidase level **(b)** of rice seedlings grown with or without *Azolla* plants under different Al stress condition after 15 days of treatment. Further, total nitrate content **(c)** was measured from the hydroponics media. Each value indicates the mean ± SE (n = 5). The lower-case alphabets indicate the significance at P = 0.05 according to DMRT between mono-cultured and intercropped plants.

### 3.2.2. Variations in the chlorophyll a and chlorophyll b content

At the highest Al concentration, the amount of chlorophyll was reduced significantly by 50% in rice plants under 50 µM Al stress. However, in rice-*Azolla* co-culture, this decrease was only 30% [Figure 6a]. When grown alone, the chlorophyll a of *Azolla* plants decreased by 50% at 30 µM, whereas in intercropped cultivation, the reduction in chlorophyll a was only about 13%. Chlorophyll b content also showed a similar trend [Supplementary Figure 1]. Interestingly, under monoculture conditions, in *Azolla pinnata*, at 30 µM Al stress, total chlorophyll b content decreased by 75%, but the decrease in chlorophyll b content was significantly reduced when grown under mixed culture conditions [Figure 6a].

In 2019, Neenu and Karthika described a reduction in chlorophyll content due to Al toxicity [5]. We assume that this decrease in chlorophyll concentration occurs because the biosynthesis of chlorophyll is severely affected during Al stress in acid soils. This is evident from the morphological variation in leaves of *Azolla pinnata* under stress [Figures 6b–g]. Apostolova et al. [66] suggested that the total pigment ratio is related to light harvesting complex II and the degree of thylakoid stacking. We also noted that the effect of Al stress is alleviated in mixed culture conditions, probably due to suppression of lipid peroxidation and ROS accumulation. Our biochemical data also supports our inference that *Azolla pinnata* interacts with Al and reduces its accumulation in rice plants.

### 3.2.3. Variations in root exudates

Many mechanisms are found in plants for tolerating Al stress, which include the secretion of organic acids to neutralize the toxic Al ions present in the rhizosphere [67,68]. Under Al stress conditions, both rice and *Azolla* plants displayed a significant reduction in all the root traits, which include root length, root numbers, and total root biomass. Further, we measured the amount of organic acid released, and these results were correlated with the root biomass. In the soil, high Al concentrations and low pH induce the release of both malate and citrate in rice plants to form Al-malate and Al-citrate [Figure 7]. In the presence of *Azolla* plants, the release of malic acid decreased by a triple factor at a 30 µM Al concentration. Malate was secreted more in the absence of *Azolla*, while citrate became dominant when *Azolla* was present. In the absence of *Azolla*, the root exudates showed a clear decrease, and hence, the rice plants displayed less tolerance to the increasing Al toxicity. The highest malate concentration was produced at a 30 µM Al concentration by the rice plants, suggesting this could be the $Al^{3+}$ concentration tolerated by rice alone in acidic soils. At 50 µM, malate produced by rice plants was found to be three times higher compared to mixed culture treatments. Since *Azolla* plants chelate Al ions by releasing organic acids (at pH < 5), they can reduce the overall stress on rice if cultured together.

Citrate secretion in rice increased when the plants were co-cultured with *Azolla* [Figure 7]. Our results are in line with Zeng et al. [69], who reported that rice plants secrete organic acids like malate, citrate, and oxalate through the roots in the rhizosphere during chromium stress to enhance the accumulation of the metal. Ma et al. [41] studied the response of rice plants to Al stress and reported Al-induced citrate secretion from the roots, while no citrate was found in the absence of Al.

### 3.2.4. Expression analysis of AMT1 and STOP1 genes

Under homogeneous conditions, Al toxicity increased the expression of ApSTOP1 [Figure 8c] and OsART1 [Figure 8a] genes by 2-fold in *Azolla* and rice plants, respectively. Interestingly, in mixed cultures, the expression of the above genes was significantly decreased in both plants [Figures 8a, c]. Likewise, under mixed culture conditions, the rice ART1 gene showed ~2-fold increased expression, and the *Azolla* STOP1 gene displayed no variation in comparison to control plants. These results clearly show that under mixed culture conditions, the ART1 of rice plants is playing an important role in Al sensing when compared with the STOP1 of *Azolla* plants. STOP1 transcription factor regulates the secretion of organic acids to neutralize toxic Al ions present in the rhizosphere or aquatic environment [9,12,13]. Hence, we investigated the variations in the root exudates of *Azolla* and rice

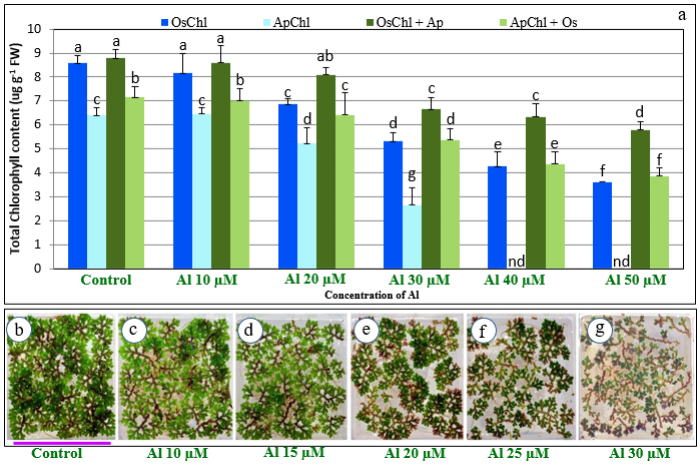


**Figure 6: (a)** Variations in the total chlorophyll (chl a and chl b) contents of rice seedlings or *Azolla* plants grown under mixed or monoculture conditions at different Al concentrations (pH 4.75). Each value indicates the mean ± SE (n = 12). **(b–g)** Stress phenotypes of *Azolla* plants under different Al stress conditions *(bar = 6.5cms)*. The lower-case alphabets indicate the significance at P = 0.05 according to DMRT between mono-cultured and intercropped plants. [OsChl: Total chlorophyll a&b of Rice in monoculture (dark blue); ApChl: Total chlorophyll a&b of *Azolla* in monoculture (light blue); OsChl+Ap: Total chlorophyll a&b of Rice in mixed culture (dark green); ApChl+Os: Total chlorophyll a&b of *Azolla* in mixed culture (light green).] *nd*-not determined.

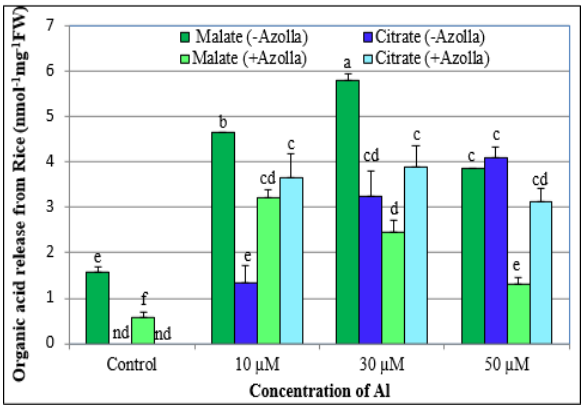


**Figure 7:** Variations in the organic acid release from rice with and without *Azolla* plants under different Al stress conditions. Each value indicates the mean ± SE (n = 3). The lower-case alphabets indicate the significance at P = 0.05 according to DMRT between mono-cultured and intercropped plants. *nd*-not determined.

**Table 1:** Variations in aluminum content of individual and mixed cultures of rice seedlings and *Azolla pinnata.*

| Aluminum content (µg/mg) | Aluminum concentration | | | | |
|---|---|---|---|---|---|
| | 10 µM | 20µM | 30µM | 40µM | 50µM |
| Mono-Cultures | | | | | |
| Rice seedlings | 0.2 ± 0.023 | 0.23 ± 0.03 | 0.4 ± 0.021 | 0.21 ± 0.02 | 0.12 ± 0.013 |
| *Azolla* | 0.27 ± 0.011 | 0.32 ± 0.012 | 0.38 ± 0.013 | 0.27 ± 0.017 | nd |
| Intercropped-cultures | | | | | |
| Rice | 0.12 ± 0.011 | 0.14 ± 0.032 | 0.21 ± 0.01 | 0.19 ± 0.021 | 0.20 ± 0.017 |
| *Azolla* | 0.16 ± 0.023 | 0.18 ± 0.021 | 0.24 ± 0.031 | 0.23 ± 0.02 | 0.23 ± 0.015 |

Mean values are calculated by using two different sets of plants (n = 4) (nd - not determined).

plants under heterogenous and homogenous conditions [Figure 7]. STOP1 is known to regulate several important agronomic traits, such as salt and drought tolerance [41,70], cellular nutrient management [71,72] etc. Thus, it is necessary to study the expression of STOP1/ART1 genes in plants under Al stress conditions.

Ammonium transporters are used by plants for ammonium uptake and transport, as well as reported to decrease ammonia toxicity under different stress conditions [73]. In our experiments, AMT1 expression was significantly reduced throughout Al-treated cultures in both rice and *Azolla* plants under homogeneous growth conditions [Figures 8b, d]. Importantly, at higher Al concentrations (30 and 50 µM), in the presence of *Azolla* plants, the AMT1 expression increased by ~ 2.2 folds in rice plants. Similar results were also recorded in the AMT1 expression of *Azolla* plants when they were cultured with rice plants [Figures 8b, d]. In mixed culture conditions, AMT1 expression in both species was significantly enhanced under low Al stress (10 µM) compared with control cultures (plants without Al treatment). These results clearly indicate that the introduction of *Azolla* plants significantly reduces the impact of Al stress on AMT1 transporters. To our knowledge, AMT1 expression has not yet been investigated in any plant species under Al stress conditions.

The STOP1 gene in *Arabidopsis* modulates the expression of CBL-Interacting Protein Kinase 23 (CIPK23) during salt and drought stress, which further helps in the transport of potassium, nitrate, and iron [70]. CIPK23 is known to phosphorylate ammonium transporters AMT1;1 and AMT1;2 in *Arabidopsis* and regulate ammonium uptake [74]. Interestingly, in plants, the interaction among STOP1, CIPK23 and

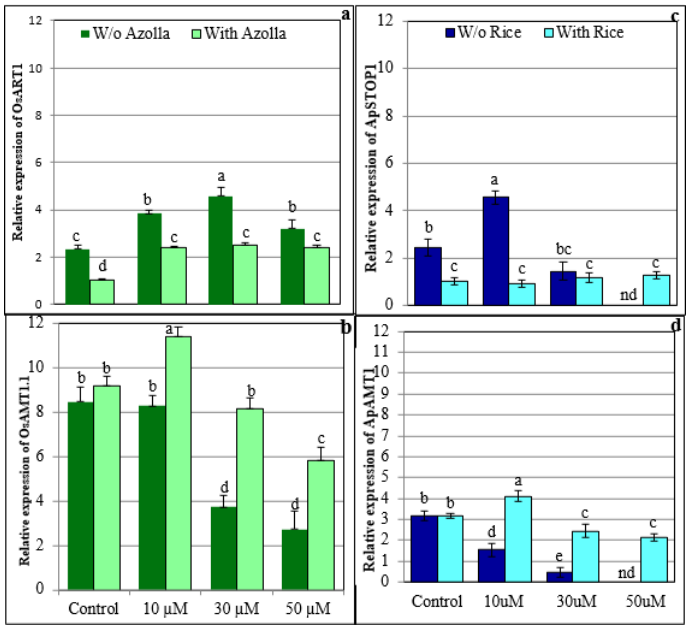


**Figure 8:** Variations in the Al stress induced expression profile of STOP1 and AMT1 genes of Rice [OsART1 **(a)** and OsAMT1.1 **(b)**] and *Azolla* plants [ApSTOP1 **(c)** and ApAMT1 **(d)**] under heterogenous and homogenous culture conditions. Each value indicates the mean ± SE (n = 3 – 5). For each gene, all the mean values from both the treatments were used for the DMRT based significance analysis. The lower-case alphabets indicate the significance at P = 0.05 according to DMRT between mono-cultured and intercropped plants. *nd*-not determined.

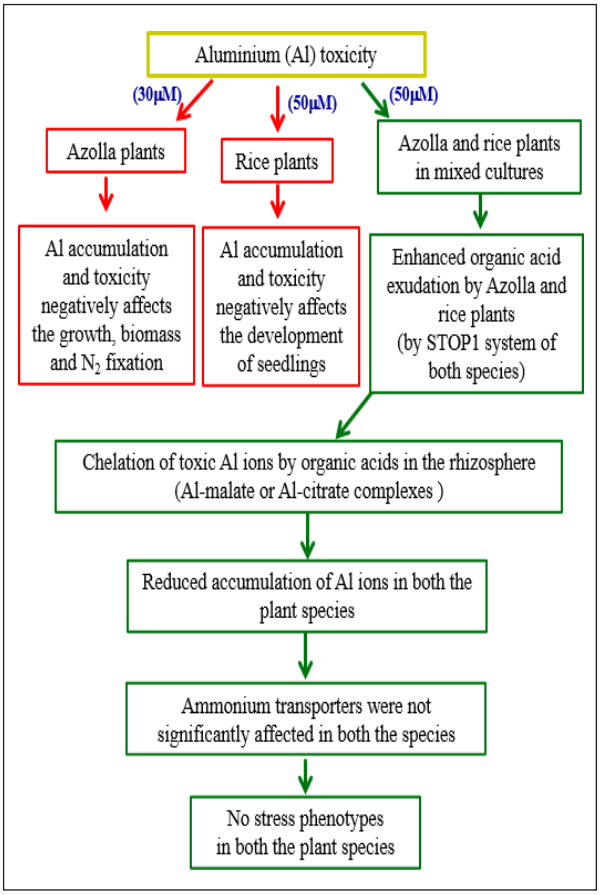


**Figure 9:** The advantages of mixed culture growth of *Azolla* and rice plants with special reference to Al stress and acid soil stress condition.

AMT1 is not yet characterized under Al stress conditions. In addition, the AMTs are involved in several agronomically important roles, like crown root formation [75], controlling manganese toxicity [76], enhancing growth and yield under low N environments [77], sheath blight resistance in rice plants [78] etc.

### *3.2.5. Aluminum accumulation*

Both rice [79] and *Azolla* [80] plants are known for sequestering Al in their leaf vacuoles, or cavities. Atomic absorption spectrometry (AAS) analysis revealed significant differences in the Al accumulation pattern between individual and mixed culture conditions. A significant reduction in Al uptake was noted in both rice and *Azolla* plants under mixed culture conditions [Table 1]. Particularly, under low and moderate Al stress conditions (10 and 30 µM), Al accumulation in rice plants grown in mixed cultures decreased by approximately 40-50 % compared with that in homogenous cultures. This may be due to the enhanced secretion of organic acids by both plants. Typically, organic acids released by plants chelate $Al^{3+}$ ions present in the rhizosphere and limit the entry of Al into the plant system [12,81,82]. Moreover, *Azolla* plants accumulated higher amounts of Al under mono or mixed culture conditions than in rice plants. Furthermore, under high Al stress conditions (50 µM), Al accumulation was higher in rice plants grown under mixed culture conditions than rice plants grown under monoculture. Possibly due to the increased Al accumulation, these plants showed a weaker phenotype with a reduced growth rate.

## 4. CONCLUSIONS

Homogenous cultures of *Azolla* and rice plants showed a significant reduction in growth rate and several stress phenotypes (reduction in leaf length, total biomass, root length, root hair length, etc.). After the introduction of *Azolla* plants to rice cultures, i.e., the mixed cultures of *Azolla* and rice plants, Al stress effects were significantly reduced in both plants. Biochemical studies (total MDA content and peroxidase levels) also demonstrated that the heterogeneous cultures of *Azolla* and rice plants are not significantly affected by Al stress under low and moderate stress conditions. In addition, expression profile analysis showed that the expression level of the rice ART1 gene (homologue of STOP1) was significantly higher than *Azolla* STOP1 gene. The Al accumulation results indicated that under low and moderate stress conditions, Al entry was inhibited, possibly due to the enhanced exudation of organic acids [Figure 9]. This study suggests that *Azolla* plants can be rationally utilized in rice fields under acidic soil conditions to achieve Al tolerance and reduce Al accumulation in Al-sensitive rice varieties.

## 5. LIST OF ABBREVIATIONS

°C: degree centigrade; ABC: ATP-binding cassette; Al: Aluminum; $AlCl_3$: Aluminum chloride; ALMT1: Aluminum activated Malate Transporter 1; AMT1: Ammonium transporter 1; Ap: *Azolla pinnata*; ART1: Al resistance transcription factor 1; β-actin: Beta actin; $Ca(NO_3)_2.4H_2O$: Calcium nitrate tetrahydrate; $CaCl_2$: Calcium chloride; CBL: Calcineurin B-like; cDNA: complementary Deoxy ribonucleic acid; Chl: Chlorophyll; CIPK23: CBL-Interacting Protein Kinase 23; cm: Centimetres; $CoCl_2.6H_2O$: Cobalt chloride hexahydrate; $CuSO_4.5H_2O$: Copper sulphate pentahydrate; DMRT: Duncan's multiple range test; dT: deoxy Thymidine; EDTA: Ethylenediamine tetraacetic acid; $FeSO_4.7H_2O$: Ferrous sulphate heptahydrate; g: Grams; h/day: Hour per day; $h^{-1}g^{-1}$: Per hectare per gram; $H_2O_2$: Hydrogen peroxide; $H_3BO_3$: Boric acid; hrs: Hours; IR: International rice; kg: Kilograms; $KNO_3$: Potassium nitrate; M: Molar; MATE: Multidrug and Toxic compound Extrusion; MDA: Malondialdehyde; mg: Milligrams; $MgSO_4.7H_2O$: Magnesium Sulphate heptahydrate; min: Minutes; ml / mL: Millilitres; mM: Millimolar; $MnSO_4.H_2O$: Manganese sulphate monohydrate; mol/g: Mole per gram; N / $N_2$: Nitrogen; $Na_2EDTA$: Sodium Ethylenediamine tetraacetic acid; $NADH/NAD^+$: Nicotinamide adenine dinucleotide + hydrogen / Nicotinamide adenine dinucleotide; $(NH_4)_6Mo_7O_{24}$: Ammonium molybdate; nm: Nanometres; NUE: Nitrogen use efficiency; Os: *Oryza sativa*; P: Probability; pH: Potential of hydrogen; PVPP: Polyvinyl polypyrrolidone; RNA: Ribonucleic acid; ROS: Reactive oxygen species; rpm: Rotations per minute; SPSS: Statistical package for the Social Sciences; STAR: Sensitive to aluminum rhizotoxicity; STOP1: Sensitive To Proton rhizotoxicity1; TBA: 2-Thiobarbituric acid; TCA: Trichloroacetic acid; µmol $m^{-2}s^{-1}$: Micromoles per metre square per second; µM: Micromolar; w/v: Weight by volume; $ZnSO_4.7H_2O$: Zinc sulphate heptahydrate.

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## 7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

## 8. CONFLICTS OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

## 9. ETHICS APPROVAL

This study does not involve experiments on animals or human subjects.

## 10. CONSENT FOR PUBLICATION

The authors have given their consent for publication of this manuscript by Journal of Applied Biology & Biotechnology, if accepted.

## 11. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

## 12. SUPPLEMENTARY MATERIAL

The supplementary material can be accessed at the journal's website: https://jabonline.in/admin/php/uploadss/1265_pdf.pdf.

## 13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

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