Differential response to acidic pH in rice seedlings

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ABSTRACT

Acidic soil is a serious harmful problem for rice crop productivity. Approximately 50% of the world's potentially arable soils are acidic, whereas in North East (NE) India 80% of arable soils are affected. In nature, it exists synergistically with other metal stresses. Hence most of the studies to date were performed in combinations. This paper highlights the detrimental effect of acidity on plants to differentiate between the effect of acidity on plant growth to that of stress in combinations. We depict it through a cascade of morphological and physiological assays, including growth, reactive oxygen species (ROS), and photosynthesis-related parameters under acidic and non-acidic rhizospheric conditions in rice seedlings of Disang and Joymati. Up to 31% root length reduction was observed in Joymati, and up to 17% reduction in Disang variety; whereas, root-relative water content was observed to reduce by 3% in Disang and 9% was recorded in Joymati cultivars. Overall, we observed limited effect on morphometric parameters like root length, biomass, and chlorophyll content irrespective of variety analyzed. On the contrary, ROS accumulation was observed to be significantly increased; more in Joymati (sensitive variety) when compared to Disang (tolerant variety). Although there was not much decrease in chlorophyll content, photosynthesis was affected immensely as depicted from chlorophyll fluorescence parameters. Hence through this study, we hypothesize that the response of plants to acid stress is rather slow.

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1. INTRODUCTION

Soil acidification is a serious environmental problem worldwide that limits crop productivity at commercial level (Mattiello et al., 2010). In North-East India approximately 80% of soil is acidic (Saikia et al., 2018). Approximately 30% of the world's total land area consists of acid soils, and it has been evaluated that over 50% of the world's potential arable lands have a pH below 5.0 (Ai et al., 2015). The soil acidity causes the presence and abundance of acidic cations like hydrogen (H+), aluminium (Al3+), and manganese (Mn2+), etc.; compared to the alkaline cations like calcium (Ca2+), magnesium (Mg2+), iron (Fe2+), potassium (K+), and sodium (Na+). The former group of elements are more soluble at low pH. The acid soil syndrome includes toxic levels of aluminum (Al), manganese (Mn), and iron (Fe), and deficiencies of numerous essential mineral elements, with phosphorus (P) being the major limiting nutrient on acid soils (Kochian et al., 2015). Though toxic elements like Al and Mn are considered to be the major causes for crop failure in acid soils; the effect of acidity, in singularity, on crop production and health has not been dealt with in detail.

Rice is one of the world’s most important crops, supplying food for nearly half of population in the world (IRRI, 2016). Rice (Oryza sativa) has been reported to be the most Al-tolerant cereal crop (Famoso et al., 2010). Al toxicity due to acidic soil, induces reactive oxygen species (ROS) or lipid peroxidation, a noxious process in plants (Awasthi et al.,...
The chlorophyll fluorescence is a powerful non-destructive technique to measure photosynthesis to have a full picture of the response of plants to their environment. Chlorophyll fluorescence is used as an indicator of photosynthetic energy conversion in higher plants. As these processes are complementary, chlorophyll fluorescence analysis is an important tool in plant research with a wide spectrum of applications (Murchie & Lawson, 2013). The singular effect of acidity on photosynthesis has been studied rarely but the acidity in combination with aluminum toxicity has been found to have drastic consequences on chlorophyll content, net photosynthetic rate, and light use efficiency (Devi et al., 2020; Lazarevic et al., 2014; Saha et al., 2020). This study focus on the impact of acidity on physiology and ROS homeostasis in rice seedling of contrasting varieties.

2. MATERIAL AND METHODS

2.1. Plant materials and growth condition

Contrasting rice genotypes were selected based on our previous studies (Awasthi et al., 2021; Awasthi et al., 2017), tolerant (Disang), and sensitive (Joymati) genotypes. An adequate amount of viable rice seeds was taken and surface sterilized with 0.1% HgCl$_2$ solution for 3-5 minutes with continuous shaking. HgCl$_2$ solution was then discarded and seeds were thoroughly rinsed with distilled water 2-3 times. The seeds were then placed properly in Petri plates with a substratum of moistened filter paper and allowed to germinate at 28±2°C for 3 days. Germinated seeds with more or less similar morphometric traits were transferred in the plastic pot (400ml) containing Hoagland nutrient medium. Seedlings were grown for 5 days in a growth chamber under white light with a photon flux density of 220µmol m$^{-2}$ s$^{-1}$ (PAR) with a 14h photoperiod. The first two days the medium was changed for healthy growth (Awasthi et al., 2017). 7-day old rice seedlings were given acidic treatment (pH 4.0, 4.2, 4.5, 4.7, 5.0, 5.2, 5.5, 7.0) for 48h; acidity of the nutrient media was adjusted using HCl/NaOH as per requirement.

2.2. Morphometric traits under pH stress

Growth was measured in terms of root length and root fresh weight and dry weight (Awasthi et al., 2017). Ten randomly selected plants were measured in centimeter-scale for root length at pH (4.0, 4.2, 4.5, 4.7, 5.0, 5.2, 5.5, 7.0) treatment after 48h of exposure.

2.3. Measurement of RWC and chlorophyll content

Relative Water Content (RWC) was determined by weighing the root and floating it on deionized water for 6h at constant temperature in diffused light. Fully turgid, root tissue samples were reweighed, and then dried in a hot air oven at 70°C for dry weight determination (Arndt et al., 2015). For photosynthetic pigment (total chlorophyll) measurement was done following Saha et al. (2016). Leaf tissues were homogenized with acetone, transferred into tubes, and sealed with parafilm to avoid evaporation until quantitated through a spectrophotometer. Acetone (95.5%) was used as a blank and concentrations were expressed in µmols g$^{-1}$ FW.

2.4. Histochemical detection of lipid peroxidation and loss of membrane integrity and ROS

For histochemical detection of lipid peroxidation, roots were stained with 10% Schiff’s reagent for 20 mins, which detects aldehydes that originate from lipid peroxides. After the reaction with Schiff’s reagent, roots were rinsed with a sulfite solution (0.5% [w/v] K$_2$S$_2$O$_3$ in 0.05M HCl) for 10 min. The stained roots were kept in the sulfite solution to retain the staining color. Photographs were taken with a stereoscopic microscope (Awasthi, Saha, et al., 2018). The loss of plasma membrane integrity was evaluated using Evans blue staining method (Kariya et al., 2013). Roots of intact seedlings were stained with 0.25% (w/v) Evans blue in 100µM CaCl$_2$ (pH 5.6) for 30 min, the stained root samples were then washed with 100µM CaCl$_2$ for 15 min. After rinsing with CaCl$_2$, root tips were cut for microscopic observation.

Detection of hydrogen peroxide (H$_2$O$_2$) was done by 3, 3-diaminobenzidine (DAB) staining (Awasthi, Paraste, et al., 2018) and superoxide radical (O$_2^-$) by nitro-blue tetrazolium (NBT) staining (Chowdhurara et al., 2019) in leaf segments of both stressed (pH 4.5) and unstressed rice leaf segments. The leaf segments were immersed and infiltrated under vacuum with 1.25mg/mL DAB staining solution, pH 7.8, dissolved in H$_2$O for 6h, and 3mg/mL NBT staining solution in 10 mM potassium phosphate buffer (pH 7.0). Stained leaves were bleached in acetic acid:glycerol:ethanol (1:1:3 v/v) solution at 100°C for 5min, and stored in glycerol:ethanol (1:4 v/v) solution until photographed.

2.5. Total ROS accumulation

The Total ROS accumulation in root apexes was labeled by using 2’,7-dichlorofluorescein diacetate (H2DCF-DA), a ROS-specific fluorescent probe (Lin et al., 2021). Briefly, root apexes were incubated with 10mM HEPES - NaOH buffer (pH 7.5) containing 10 µM H2DCF-DA for 30 min in dark. The root apexes were then washed with fresh buffer prior to detection using an epifluorescence microscope (Nikon, Tokyo, Japan).

2.6. Chlorophyll fluorescence

Chlorophyll fluorescence measurements were performed using a Junior-PAM chlorophyll fluorimeter (Walz, Germany). The following parameters were derived from the final measurements obtained after the 30 mins dark adaptation and light adaptation Fo, Fm, Fv/Fm, and Fo’, Fm’, Fv/Fm’ (Yll), qP, qN, qL, NPQ, Y(NO), Y(NPQ) and ETR. All parameters were calculated as defined previously (Murchie & Lawson, 2013; Ruban & Murchie, 2012). Six measurements were obtained for each parameter.

2.7. Statistical analysis

Each experiment was repeated thrice and the data presented are mean ± standard error (SE). The results were subjected to one-way ANOVA. Tukey test was performed for comparison between a set of experiments. The data analysis was carried out using Microsoft Office Excel 2013, statistical software SPSS 20, and Origin Lab 8.5.
3. RESULTS

3.1. Effect of acidity on morphometric attributes

pH stress was observed to have a significant effect on the growth and development of rice seedlings; mainly, pH 4.5, 4.2, and 4.0 highly reduced seedling length as observed in sensitive variety (Fig. 1). Seedling growth was found to be indirectly proportional to pH stress and duration. The relative root length of rice varieties at low pH was significantly decreased (Fig. 1). Relative water content is an indicator of the state of water balance of a plant essentially because it expresses the absolute amount of water that the plant requires to reach an artificial full solution. As plants were subjected to pH stress, a decline in root-relative water content was observed (Fig. 1) in case of both root and shoot tissues. Our result showed that the RWC content was observed to be quite significantly decreased at pH 4.5 in both varieties at 48h duration.

3.2. Total ROS accumulation

Total ROS accumulation was more under stress conditions as revealed by staining with H$_2$DCF-DA dye. In Joymati varieties total ROS accumulation was higher in response to pH 4.5 when compared to Disang (Fig. 2).

3.3. Histochemical studies for detection of lipid peroxidation, loss of membrane integrity, H$_2$O$_2$ and O$_2$ content

The lipid peroxidation and loss of membrane integrity were analyzed by Schiff’s reagents and Evans blue respectively. At lower pH, intense coloration depicted more lipid peroxidation and loss of membrane integrity in Joymati root tissue when compared to Disang. The superoxide (O$_2^−$) and hydrogen peroxide (H$_2$O$_2$) content when observed in response to pH stress in both root and shoot showed more coloration in Joymati after 48hr of stress (Fig. 3).

3.4. Chlorophyll fluorescence assay

The study of chlorophyll fluorescence parameters such as Fo, Fm, Fv/Fm, Fo’, Fm’, Fv’/Fm’, Y(II), qP, qN, qL, NPQ, Y(NO), Y(NPQ), and ETR was conducted in two rice cultivars under acidic condition. The chlorophyll degradation in both cultivars was progressively correlated with the maximum quantum yield of PSII (Fv/Fm) as well as the photon yield of PSII [Y(II)] leading...
Table 1: Chlorophyll fluorescence studies in both rice varieties under pH stress for 48 hr. For control populations and treated populations, \( n = 6 \) for each genotype Data shown are representative of two separate experiments. (*) represent Correlation is significant (\( p<0.05 \)) level.

|        | \( F_0 \) | \( F_m \) | \( F_{v/Fm} \) | \( F_{m'} \) | \( Y \ (II) \) | \( ETR \) | \( F_{0'} \) | \( F_{v'/Fm'} \) | \( q_P \) | \( q_N \) | \( q_L \) | \( NPQ \) | \( Y \ (NO) \) | \( Y \ (NPQ) \) |
|--------|-----------|-----------|----------------|-----------|---------------|--------|------------|----------------|--------|-------|-------|--------|----------|-----------|
| \( F_0 \) | 1         |           |                |           |               |        |            |                |        |       |       |        |          |           |
| \( F_m \) | 0.869     | 1         |                |           |               |        |            |                |        |       |       |        |          |           |
| \( F_{v/Fm} \) | 0.733     | 0.742     | 1              |           |               |        |            |                |        |       |       |        |          |           |
| \( F_{m'} \) | 0.886     | 0.928     | 0.501          | 1         |               |        |            |                |        |       |       |        |          |           |
| \( Y \ (II) \) | 0.692     | 0.917     | 0.858          | 0.704     | 1             |        |            |                |        |       |       |        |          |           |
| \( ETR \) | 0.584     | 0.232     | -0.068         | 0.537     | -0.149        | 1      |            |                |        |       |       |        |          |           |
| \( F_{0'} \) | 0.957     | 0.762     | 0.510          | 0.887     | 0.487         | 0.786  | 1          |                |        |       |       |        |          |           |
| \( F_{v'/Fm'} \) | 0.883     | 0.999     | 0.749          | 0.931     | 0.912         | 0.252  | 0.778      | 1             |        |       |       |        |          |           |
| \( q_P \) | -0.536    | -0.750    | -0.115         | -0.866    | -0.532        | -0.355 | -0.586     | -0.741        | 1      |       |       |        |          |           |
| \( q_N \) | -0.235    | -0.081    | 0.486          | -0.443    | 0.311         | -0.829 | -0.496     | -0.087        | 0.562  | 1     |       |        |          |           |
| \( q_L \) | -0.723    | -0.947    | -0.506         | -0.929    | -0.83         | -0.207 | -0.662     | -0.940        | 0.911  | 0.240 | 1     |        |          |           |
| \( NPQ \) | -0.429    | -0.235    | 0.298          | -0.579    | 0.169         | -0.912 | -0.666     | -0.245        | 0.600  | 0.977 | 0.342 | 1      |          |           |
| \( Y \ (NO) \) | 0.161     | -0.296    | -0.348         | 0.007     | -0.599        | 0.845  | 0.390      | -0.27         | 0.153  | -0.669 | 0.346 | -0.690 | 1        |           |
| \( Y \ (NPQ) \) | -0.356    | -0.204    | 0.371          | -0.552    | 0.194         | -0.862 | -0.600     | -0.211        | 0.629  | 0.991 | 0.344 | 0.993  | -0.643   | 1         |

- \( F_0 \): Minimal fluorescence from dark-adapted condition
- \( F_m \): Maximal fluorescence from dark-adapted condition
- \( F_{v/Fm} \): The maximum quantum efficiency of photosystem II/ Maximum quantum yield of PS II
- \( F_{m'} \): Maximal fluorescence from light-adapted condition
- \( Y \ (II) \): Quantum yield of PS II
- \( ETR \): Electron transport rate derived from \( Y \ (II) \) and PAR
- \( F_{0'} \): Minimal fluorescence from light-adapted condition
- \( F_{v'/Fm'} \): The estimate of the maximum efficiency of PSI
- \( q_P \): The proportion of open PS II / photochemical fluorescence quenching coefficient
- \( q_N \): Coefficient Non photochemical quenching
- \( q_L \): Fraction of PS II center that are open/ coefficient of photochemical fluorescence quenching assuming interconnected PS II antennae.
- \( NPQ \): Non Photochemical fluorescence quenching
- \( Y \ (NO) \): non-regulated energy dissipation (quantum yield of non-light induced non photochemical fluorescence quenching)
- \( Y \ (NPQ) \): Quantum yield of non-photochemical fluorescence quenching due to downregulation of light-harvesting function. (Quantum yield of light-induced non-photochemical fluorescence quenching).
to growth reduction. The relationship between all chlorophyll fluorescence parameters is presented in Table 1. The Fm, Fv/Fm, Y(II), Fo', Fm', Fo, Fv'/Fm', ETR, showed positive correlations while qP, qN, qL, NPQ, Y(NPQ) Y(NO) were negatively related in both cultivars under acidic environment. The close correlation between the decline in Fv/Fm and stress suggests that the rapid changes in Fv/Fm are a useful indicator of loss of photosynthetic viability on exposure to acidic stress.

4. DISCUSSION

Acid soil is a menace to world crop production which effects crops plants in synergy with other heavy metal stress like Al³⁺, Fe²⁺, Mn²⁺, etc (Awasthi et al., 2019; Bojórquez-Quintal et al., 2017; Regon et al., 2021). As such the studies conducted to date are also synergistic (Krstić et al., 2012). Through this study, we tried to bring to the forefront the effect of acidity on morphology, redox status and photosynthesis of rice seedling. The morphological aspects as measured by root length, chlorophyll content, RWC didn’t show drastic effect after 48h acid treatment (Fig. 1). Total ROS accumulation was qualitatively estimated by H2DCF-DA dye, which showed a vast change in contrast (Fig. 2). Not so significant difference in morphometric attributes whereas large qualitative accumulation of ROS might be due to slow response to acidity by plants. Hence, the morphological changes might be visible after long-term exposure (probably more than 48h of treatment). The histochemical observation was also made for H₂O₂ and O₂⁻ accumulation by NBT and DAB staining respectively. Disang showed excessive accumulation in comparison to Joymati (Fig. 3). A similar observation was made in our previous studies in response to aluminum toxicity in acidic soil (Awasthi et al., 2021; Awasthi et al., 2019; Awasthi et al., 2017). ROS is generated due to cellular metabolic activities in plants, generally, a homeostasis is maintained between the ROS generating and scavenging machinery but when in stress the homeostasis stands disturbed (Song et al., 2010). When exposed to low pH, plants showed high superoxide accumulation (Fig. 2) in shoot and root samples. A significant increment of H₂O₂ was observed after 48h of the acidic environment when compared to control (Fig. 2). H₂O₂ works as a signaling molecule to activate the response mechanism leading to tolerance, but on excess accumulation, the entire machinery succumbs (Wu et al., 2012). This might justify excess ROS production but no effect on external morphology. We also observed increased lipid peroxidation and cell death as depicted from tonality difference after staining with Schiff’s reagent and Evans blue respectively (Fig. 3). This observation indicates that the threshold limit of loss of ROS homeostasis has been reached, hence the damage.

Analysis of chlorophyll fluorescence has become one of the most powerful and widely used techniques in plant stress biology (Murchie & Lawson, 2013). Chlorophyll fluorescence studies were conducted in both the rice cultivars. The correlation analysis of all the chlorophyll fluorescence parameters [Fo, Fm, Fv/Fm, Fo', Fm', Fv'/Fm', Y(II), qP, qN, qL, NPQ, Y(NPQ) Y(NO), Y(NPQ) and ETR] was performed (Table 1). Our studies revealed that the Fv/Fm, Y(II), Fv'/Fm' decreased under acidic environment conditions. The Fv/Fm values are typically very consistent between lines and individual plants; as such, any small decline is easily noticeable and signifies clearly the loss of viability. The Fv/Fm is a prominent indicator of the study of maximum quantum yield of PSII photochemistry. Broadly, Fv/Fm, Fv'/Fm', qPSII, and qP have been called photochemical quenching parameters, and NPQ is a non-photochemical quenching parameter (Borgohain et al., 2020). Since there was not much decline in chlorophyll content but a decrease in photosynthetic efficiency indicates an early stage of stress response.

5. CONCLUSION

The study brings to the forefront for the first time the impact of only acidic stress on rice seedlings. Acidity had stress-responsive manifestations in rice seedlings but it is slow as depicted from the experiments. After 48 hrs of stress treatment there were not so significant changes in morphometric parameters whereas ROS accumulation was
considerably high, so was cell death and lipid peroxidation. Similarly, there was less decrease in pigment content, whereas photosynthetic efficiency as measured by fluorescence was significantly affected in both the varieties Disang and Joymati.

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Declaration of Competing Interest

The authors declare no competing financial or personal interests that may appear and influence the work reported in this paper.

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