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# Insight of Oxidative Stress, Ultrastructural Transformations, and Elemental Variations in Rice Seedlings Exposed to Boron Toxicity: Unraveling the Role of Fe-SOD in Boron Tolerance

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.*

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## ABSTRACT

Rice (Oryza sativa L.) seedlings differing in boron tolerance were grown in hydroponics containing varying boron concentration. (as boric acid) for 8 days. Yoshida Nutrient Solution (Yoshida et al.1976) served as control, whereas 0.5 mM boron (moderately toxic) and 1.5 mM boron (highly toxic). Boron excess (B) caused marked reduction in length, biomass and relative water content of the seedlings, with more reductions in B-sensitive cv. Malaviya-36 as compared with B-tolerant cv. Brown Gora. B-sensitive seedlings showed higher B uptake in roots and shoots compared to the tolerant. Scanning electron microscopy (SEM) revealed ultrastructural damage to the guard cells with excess boron. Energy dispersive X-ray analysis (EDXA) of rice leaves showed decline in concentration of P, S, Ca and Mg in seedlings on B treatment. Increased production of reactive

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oxygen species $O_2^{\cdot-}$, $H_2O_2$, lipid peroxides, alteration in activity of antioxidative enzymes and increased membrane permeability was observed in B treated seedlings compared to controls. Real time PCR analysis of stress regulatory genes indicated differential expression of SOD isoforms in the two sets of cultivars with B treatment. Interestingly seedlings of B-tolerant cultivar were characterized by higher level of expression of Fe-SOD and its further increased expression on B treatment. Results suggest that B toxicity involves ultrastructural and elemental changes, increased generation of ROS and altered antioxidative enzyme activities in rice seedlings and increased expression Fe-SOD isoform appears to be associated with B tolerance.

*Keywords: Rice; boron toxicity; reactive oxygen species; antioxidative defense system; EDAX; real time PCR.*

## 1. INTRODUCTION

Plants react to various environmental stresses by employing several innate defensive mechanisms [1]. Such defensive mechanisms may or may not produce visible morphological and physiological symptoms. Most of the plant's defense mechanisms are mediated by several inter-linked pathways involving numerous stress-responsive genes, enzymes and associated cofactors [1]. Boron is required by the plants for their growth in quantities ranging between 0.2-800 ppm [2], but when present in excess within the tissues, it can cause various physiological disorders leading to decline in crop productivity.

Boron is an essential micronutrient for plants [3]. It is involved in the maintenance of integrity and rigidity of cell wall, RNA and carbohydrate metabolism, transport of sugars and other molecules (Blevins et al. [4] Reid et al. [5] and in the transportation of molecules across the membrane [4]. Boron application to the soil is usually avoided as this element is normally present in sufficient amount in the soil. Boron is however many times added to cultivation areas in irrigation water [6]. Plants take up the insoluble boron in the form of boric acid [7]. Being a micronutrient, plants require only 3 ppm of boron. Following evaporation of soil water, boric acid accumulates in the soil and often forms a part of the soil crust [8]. This high concentration of boron becomes toxic to plants, particularly in arid regions and near volcanic zones [6]. Boron when taken up in excess, leads to chlorosis, leaf burn, reduced growth and ultimately reduction in yield Rossener et al. [9] Brdar-Jokanovic [3].

Boron is involved in plasma membrane trafficking of ions and molecules, hence any change in its concentration causes changes in the membrane redox potential [10]. Increased boron level in the plant tissues causes cell wall damage [11] increased production of reactive oxygen species (ROS) and such excessively produced ROS is counteracted with antioxidants as well as antioxidative enzymes such as superoxide dismutase (SOD) in order to keep the level of ROS under control [12]. Excess boron in plant tissues causes expression of stress related genes [13], lipid peroxidation [12]. cellular oxidative damage and induces expression of antioxidative enzymes. To combat excessive boron level within in the tissues, plants employ several detoxifying inter-linked cascades. Although numerous reports suggest various adverse effects of excess boron in plants, boron toxicity on various physiological functions of plants still remains elusive [5]. A wide range of proteins/enzymes have been identified the expressions of which are induced under abiotic stresses and many of these proteins/enzymes are associated with stress tolerance [13]. Crop plants differ in their capacity for boron tolerance and it has been difficult to ameliorate boron toxicity in fields, thus growing boron tolerant variety remains the only choice for boron-excess soils [3]. Attempts are in the way by various workers to identify the physiological as well as genetics characteristics associated with boron tolerance [3]. In order to study the physiological dysfunctions and cellular damages caused by excess boron to rice plants, and to identify the components associated with boron tolerance, we conducted studies to examine growth, ultrastructural changes, elemental composition, level of ROS in the tissues, activities of antioxidative enzymes and expression of genes associated with boron tolerance in seedlings of rice grown in hydroponics under toxic levels of boron. The study was conducted using rice cultivars differing in boron tolerance to further investigate the defensive mechanisms of rice plants associated with boron tolerance.

## 2. MATERIALS AND METHODS

### 2.1 Rice Seeds, Growth of Seedlings and Boron Treatment

Indica rice cultivar (*Oryza sativa* L.) seeds of cultivars Malviya-36, Brown Gora, HUR-105,

Vandana, Sahbhagi and Pant, collected from different locations in India were used. These were used to screen one boron sensitive (Malviya-36) and one boron tolerant (Brown Gora) cultivar on the basis of results obtained from preliminary experiments for growth parameters in hydroponics culture. Sensitivity and tolerance of these cultivars towards boron was evaluated based on germination as well as growth parameters such as lengths and weights of roots/shoots of seedlings. Seeds were germinated for 5 days in a BOD cum humidity incubator Maheshwari and Dubey [14] and then transferred to 200 ml nutrient solution [15] contained in plastic pots. This served as control, whereas 0.5 mM boron (moderately toxic) and 1.5 mM boron (highly toxic) added to the nutrient solutions served as boron treatment solutions. Seedlings were further raised for 8 days by placing the pots in a green house maintained at 80 % relative humidity, 28 ± 1ºC temperature, 12 h light/dark photoperiod and irradiance of 190-200 µmol $m^{-2}s^{-1}$. At $4^{th}$ and $8^{th}$ day of growth, seedlings were uprooted, roots and shoots were separated and all analyses were performed in three replicates.

## 2.2 Growth of Seedlings, Boron Uptake and Relative Water Content

To determine the effect of boron on germination percent of seeds, 50 seeds were uniformly distributed on moist filter paper in petri dishes and kept for germination in BOD cum humidity incubator [14]. The effect of boron on growth of rice seedlings was examined by placing 5d germinated seeds in Yoshida nutrient solution, which served as control and nutrient solutions with 0.5 mM and 1.5 mM boron that served as treatment solutions. Root/shoot length of the seedlings and their fresh biomass were determined at day 4 and day 8 of boron treatment. To determine dry weights, plant samples were placed in oven for 3 days at 70°C and subsequently weighed. Boron concentration in growing seedlings was determined with ICP-OES (Inductively coupled plasma-optical emission spectrometer, Optima 7000 DV, Perkin Elmer) following Moore and Chapman [16]. Samples, after washing with Milli-Q (ion free) water, were dried in oven and boron was quantified in dried samples, after digestion in nitric acid: perchloric acid (1:1) mixture. Comparable boron standards supplied by the manufacturers were used. In control and treated seedlings determination of relative water content (RWC) was done as RWC= (FW-DW) / (TW-DW) × 100 where FW, DW and TW represent fresh weight, dry weight and turgid weight respectively, following the method of Srivastava et al [17].

## 2.3 Ultrastructure Studies on Stomata Using SEM and Dispersive X-ray

The effect of excess boron on ultra-structural changes in the stomata was examined using leaves of 8 day grown control and boron treated seedlings. A thin slice of each leaf was fixed in glutaraldehyde and serially dehydrated as described earlier [17]. Gold coated samples were examined at 20 KV using HR-SEM (F.E.I. Quanta FEG 200) at Indian Institute of Technology, Madras, India. Similarly EDAX (energy dispersive X-ray analysis) of the samples was done to determine the composition of elements following the standard protocols (Nylese et al. [18] Scimeca et al. [19].

## 2.4 Detection of $O_2^{\bullet -}$, $H_2O_2$, Lipid Peroxides in the Tissues and Loss of Membrane Integrity

Histochemical detection of superoxide anions ($O_2^{\bullet -}$) in the leaves was done using the dye nitrobluetetrazolium (NBT) according to Srivastava et al [17]. Excised leaf pieces were immersed in 6 mM NBT solution prepared in 10 mM sodium-citrate (pH 6.0). After staining for 8 h in light, leaves were placed in boiling ethanol (90%) for decolourization for 10 min. Dark blue formazan deposits appeared on leaves which were visualized under light microscope. Hydrogen peroxide ($H_2O_2$) localization in excised leaves were detected using DAB (3,3′-diaminobenzidine, Amresco, USA) according to Srivastava et al [14]. Leaf samples were incubated for 8 h in 1 mg $ml^{-1}$ acidified DAB (pH 3.8) solution. Stained leaves were decolourized by placing for 10 min in 90 % boiling ethanol. After placing in saturated solution of chloral hydrate for bleaching, reddish-brown spots showing localization of $H_2O_2$ appeared on leaves which were visualized in microscope.

Lipid peroxidation products in roots were histochemically detected using Schiff's reagent according to Srivastava et al. [14]. Root tips measuring 8-10 mm were stained with Schiff's reagent for 20 min. Aldehydes produced as lipid peroxidation products took stain with Schiff's reagent. Stained root tips were then placed for 10 min in acidified sulfite solution (0.5% $K_2S_2O_5$ in 0.05 M HCl) and thereafter observed under microscope. To examine loss of membrane integrity due to boron treatment, root tips were

stained in 0.25 % aqueous solution of Evan's blue for 30 min following the method of Schutzendubel et al. [20]. Stained root tips were washed in water and observed under microscope.

## 2.5 Quantitative Detection of ROS and Lipid Peroxides

Superoxide anion was quantified in the seedlings according to Mishra and Fridovich (1972), in terms of rate of its production. Oxidation of epinephrine was recorded in terms of adrenochrome formation by measuring absorbance at 480 nm at the interval of 30 s up to 5 min using spectrophotometer (ELICO India Ltd, SL 177). Rate of $O_2^{\bullet -}$ production was expressed in terms of absorbance change at 480 nm $g^{-1}$ tissues $min^{-1}$. The level of $H_2O_2$ was measured in the tissues according to Jana and Choudhuri [21] using titanium sulfate. Absorbance of yellow colour developed was measured at 410 nm using spectrophotometer. $H_2O_2$ level was calculated (extinction coefficient = 0.28 $\mu M^{-1}cm^{-1}$) and expressed as nmol $g^{-1}$ fresh weight of tissues. The products of lipid peroxidation were determined according to Heath and Packer [22] using thiobarbituric acid and were expressed as TBARS (thiobarbituric acid reactive substances). Absorbance was measured using spectrophotometer at 532 and 600 nm and TBARS contents were calculated (extinction coefficient = 155 $mM^{-1}cm^{-1}$) and expressed as nmol $g^{-1}$ tissue fresh weight.

## 2.6 Determination of Activities of Antioxidative Enzymes

Superoxide dismutase activity was assayed according to Beauchamp and Fridovich (1974) by measuring oxidation of epinephrine to adrenochrome. Fresh samples (200 mg) were homogenized in 2 ml chilled extraction medium consisting of 100 mM potassium-phosphate buffer (pH 7.8), 0.1 mM EDTA, 2 % (w/v) polyvinyl pyrrolidone (PVP) and 0.1 % (v/v) Triton-X-100. After centrifugation at 22,000 × *g* for 10 min in cold, the supernatant was dialyzed in cellophane membranes and enzyme activity was determined. Formation of adrenochrome was measured at 475 nm in a UV-Vis spectrophotometer (Cary 50 Bio, Varian, Australia). One unit of SOD activity corresponded to the enzyme causing 50 % inhibition in the oxidation of epinephrine. The activity of catalase was determined in fresh root/shoot samples according to Beers and Sizer (1952). Extraction medium consisted of Tris-HCl (50 mM, pH 8.0), EDTA (0.5 mM) and 2 % PVP. After centrifugation, followed by dialysis of the supernatant, enzyme activity was assayed in a medium consisting of potassium-phosphate buffer (100 mM, pH 7.0), $H_2O_2$ (200 mM) and enzyme. Decrease in absorbance due to decomposition of $H_2O_2$ was measured at 240 nm using UV-Vis spectrophotometer. Using extinction coefficient 0.036 $mM^{-1}$ $cm^{-1}$, $H_2O_2$ was calculated and enzyme activity units were expressed as µmol $H_2O_2$ oxidized $mg^{-1}$ protein $min^{-1}$.

## 2.7 Expression Analysis of SOD Isoforms

RNA was extracted from fresh root/shoot samples employing *RNeasy* Mini *Kit* (Qiagen) following protocol of manufacturer. After verification of its integrity on agarose gel (1%), concentration of RNA was calculated using nano-drop spectrophotometer. To 5 µg of total RNA was added recombinant DNase I (5 units, RNase-free, Qiagen). Contents were incubated for 15 min at 37ºC. With addition of 2 µL EDTA (0.2 M, pH 8.0) reaction was terminated and contents were heated at 65º C for 10 min. mRNA was purified using mRNA purification kit (Qiagen). With the use of RT-PCR Kit (PrimeScript™, Bio-Rad) and following protocol of manufacturer cDNA first strand was synthesized. Using thermal cycler (Bio-Rad) and following the steps: 42ºC, 30 min; 95ºC, 5 min and finally 4ºC, reverse transcription was completed. Contents were placed at -20ºC for further use. Quantitative amplification of genes related to antioxidative enzymes and boron excess tolerance gene was performed using real time PCR primers as described in Table 1. Agarose gel electrophoresis was performed to run amplified products, which were then visualized in Gel-Doc (Model: BioRad Gel/chemidoc CFW-1312M-S/N13005971) fitted with Grey Scale Digital Camera.

## 2.8 Protein Determinations

In all preparations determination of protein was done using Bradford reagent [23].

## 2.9 Statistical Analysis

Each experimental analysis was carried out in triplicate using samples from independent biological replicates. Data represent mean ± S.D. on the basis of three observations. Differences between treatments and controls were analysed using software ANOVA and Tukey's multiple range test. $*p<0.05$ and $**p<0.01$ represent the level of significance of the difference between controls and treatments.

**Table 1. Forward and reverse primers used for CuZn SOD, MnSOD and FeSOD in real time PCR**

| Sr. No. | Gene | Primer Sequence | Tm (MeltingTemperature) |
|---|---|---|---|
| *1* | *CuZnSOD* | (Forward) GCACCAGAAGCCTGAAACTC | 59.4ºC |
| *2* | *CuZnSOD* | (Reverse) CGAGCGAACAGATGTAACGA | 57.3ºC |
| *3* | *MnSOD* | (Forward) GGCAAAGAAGCTTTCAGTGG | 57.3ºC |
| *4* | *MnSOD* | (Reverse) CAAGCAGTCGCATTTTCGTA | 55.3ºC |
| *5* | *Fe SOD* | (Forward) AGAACAAAGGCAGGGCTGTA | 57.3ºC |
| *6* | *Fe SOD* | (Reverse) ATGGGTTGCCGTTGTTGTAT | 55.3ºC |

## 3. RESULTS

### 3.1 Boron Treatment and Growth of Rice Seedlings

Boron concentration of 1.5 mM caused a marked reduction in germination of seeds (Fig.1). In rice *cv.* Malviya-36, the reduction was 70 % *(p<0.01)* while in *cv.* Brown Gora only 30 % *(p<0.05)* decline in percent germination of seeds could be seen (Fig. 1). The germination percent at 0.5 mM boron was almost unaffected compared to the controls. Boron treatment of 1.5 mM resulted into marked inhibition in growth of rice seedlings (Fig.1). With 1.5 mM boron treatment for 8 days, in *cv.* Malviya-36 seedlings root length was reduced by 46 % *(p<0.01)* and shoot length by 50 % *(p<0.01)* whereas in *cv.* Brown Gora 20 % *(p<0.05)* decline in the length of roots and 18 % *(p<0.05)* decline in length of shoots was observed under similar boron treatment levels. With 1.5 mM boron treatment to the seedlings for 8 days a significant reduction in fresh weight as well as dry weight was observed with more reductions in *cv.* Malviya-26 than *cv.* Brown Gora. In rice *cv.* Malviya-36, fresh weight of roots declined by 40 % *(p<0.01)* and shoots fresh weight declined by 27 % *(p<0.5),* whereas in *cv*. Brown Gora with similar level of boron treatment root fresh weight declined by 12 % and shoot by 15 % compared to controls. Similarly, 20 % *(p<0.05)* decline in the dry weight of roots and 40 % *(p<0.01)* decline in shoots dry weight was noticed in *cv.* Malviya-36, whereas under similar boron treatment level of 1.5 mM for 8 days root dry weight declined by 12 % and shoot by 20 % *(p<0.05)* in *cv.* Brown Gora (Fig. 2).

### 3.2 Uptake of Boron from the Medium and Relative Water Content

Boron concentration was determined in roots and shoots of the seedlings with Inductive Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Optima 7000 DV, Perkin Elmer, USA). The concentration of absorbed boron increased in both roots as well as shoots with increase in time as well as dose of boron treatment (Fig. 3). Roots showed higher level of absorbed boron than shoots. With 1.5 mM boron treatment for 8 days, roots of *cv*. Malviya-36 seedlings showed 24 fold *(p<0.01)* higher boron level and shoots showed 20 fold *(p<0.01)* higher boron level in comparison to the levels in controls, whereas under similar level and duration of boron treatment in *cv*. Brown Gora seedlings 20 fold *(p<0.01)* higher boron level was observed in roots and 16 fold *(p<0.01)* higher level in shoots in comparison to controls. We observed that the seedlings of *cv*. Brown Gora maintained better growth in comparison to the seedlings of *cv*. Malviya-36 under similar level of boron treatment. Seedlings grown in presence of 1.5 mM showed a marked decline in RWC compared to controls, with greater decline in *cv.* Malviya-36 than *cv.* Brown Gora (Fig. 3). With boron treatment of 0.5 and 1.5 mM for 8 days, the root RWC declined by 54 % *(p<0.01)* and 63 % *(p<0.01)* and shoot RWC by 56 % *(p<0.01)* and 73 % *(p<0.01)* respectively in the seedlings of *cv.* Malviya-36, whereas in *cv.* Brown Gora seedlings root RWC declined by 19 % and 20 % *(p<0.05)* and shoot RWC by 16 % and 21 % *(p<0.05)* respectively under similar treatment levels.

### 3.3 Effect of Boron on Ultra-Structural Changes in Stomata and Multi-Elemental Concentration in Rice Leaves

SEM analysis of leaves from control and boron treated seedlings revealed the effect of excess boron on the structure of guard cells (Fig. 4A). The extent of boron induced damage was more prominent in *cv.* Malviya-36 seedlings than *cv.* Brown Gora. Energy dispersive X-ray diffraction analysis (EDXA) of rice leaves was carried out to examine the alteration in multi-elemental concentration in the leaves of seedlings due to boron treatment (Fig. 4B). The EDXA peaks showed a decrease in concentration of important

elements which are constituents of biomolecules such as phosphorus (DNA), sulfur (proteins), calcium (plasmodesmata and cell wall) and magnesium (for cellular reactions).

### 3.4 ROS detection in the Tissues and Membrane Damage

In our histochemical studies, increased formation of superoxide, $H_2O_2$ and lipid peroxides was noted in the tissues with boron treatment. The intensity of formation was higher in *cv*. Malviya-36 than *cv*. Brown Gora (Fig. 4 C, D). Boron treatment thus causes increased ROS production in the tissues with greater production in sensitive variety than the tolerant. These overproduced ROS would then damage oxidatively biomolecules of the cell like lipids, proteins, etc. with greater damage in *cv.* Malviya-36 than *cv*. Brown Gora.

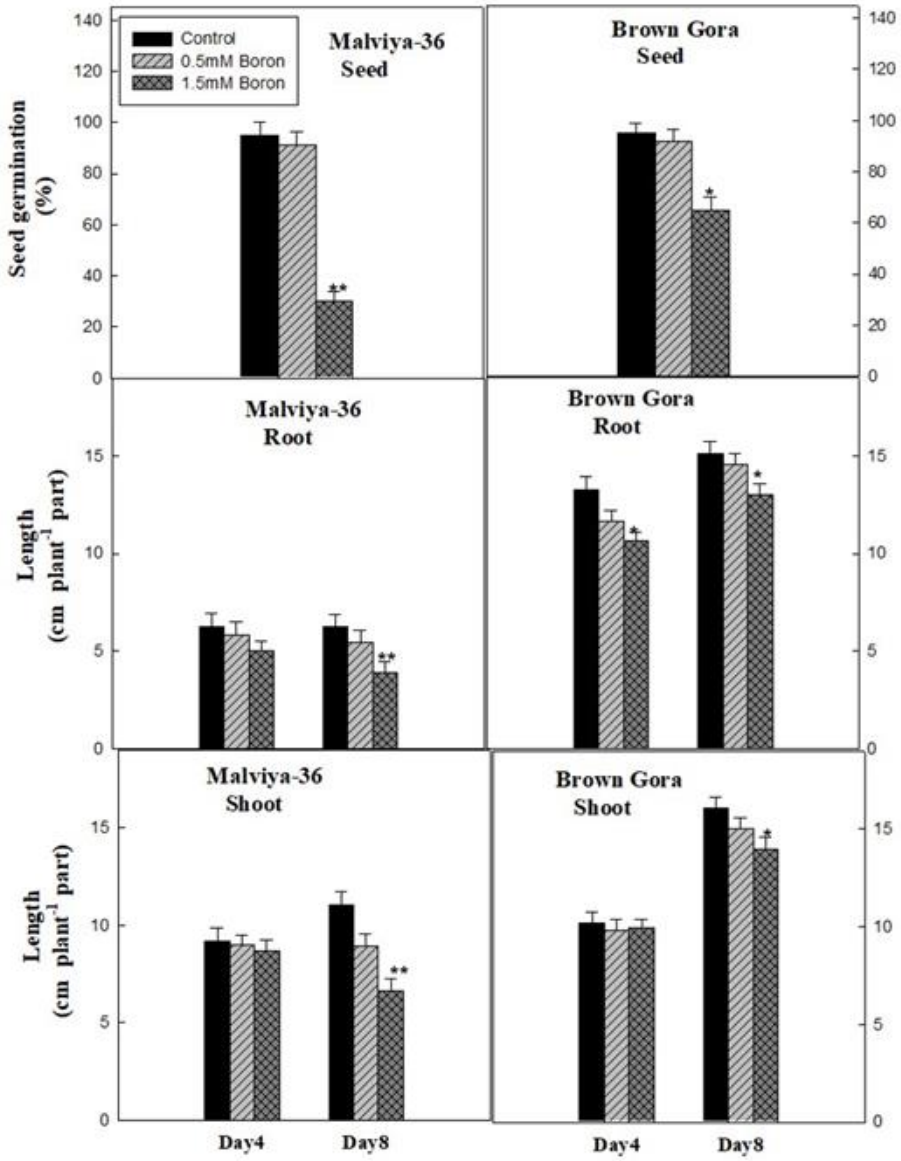



**Fig. 1. Effect of boron on germination of seeds and lengths of roots and shoots of the seedlings of rice *cvs.* Malviya-36 and Brown Gora**

*To determine germination percent, seeds were germinated for 5 days under control (0.05 mM boron), 0.5 mM boron and 1.5 mM boron, whereas effects of boron on growth of seedlings were examined by growing the seedlings for 8 days in hydroponics in Yoshida nutrient solution containing 0.05 mM boron (control), 0.5 mM boron and 1.5 mM boron. Values represent means of three independent experiments and bars indicate standard deviations. Values differing significantly from controls have been represented as asterisks (*) and (**) at p<0.05 and p<0.01 respectively following Tukey's multiple range test.*

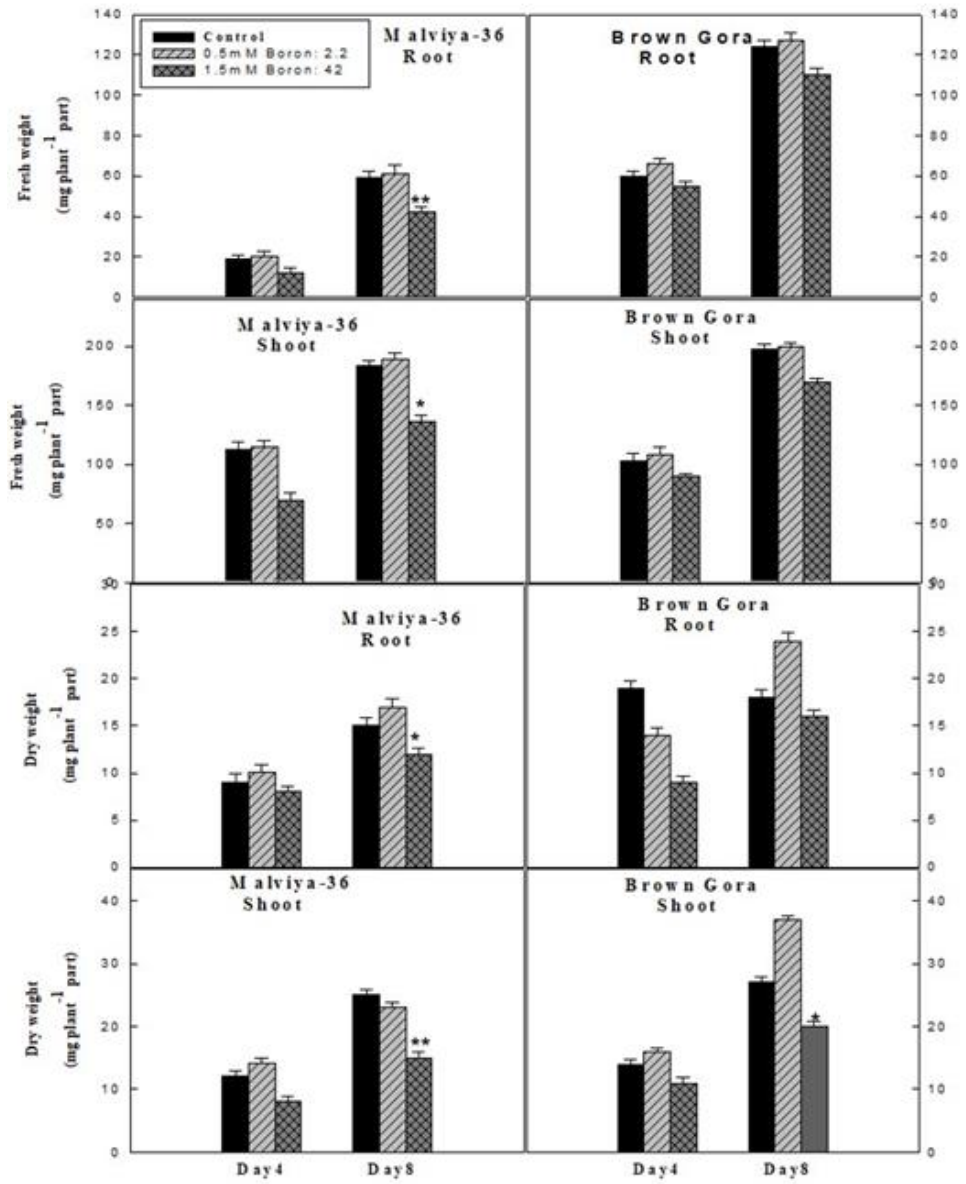



**Fig. 2. Effect of boron on fresh and dry weights of different parts (root/shoot) of the seedlings of rice *cvs.* Malviya-36 and Brown Gora**

*Seedlings were grown for 8 days in hydroponics in Yoshida nutrient solution containing 0.05 mM boron (control), 0.5 mM boron and 1.5 mM boron. Values represent means of three independent experiments and bars indicate standard deviations. Values differing significantly from controls have been represented as asterisks (*) and (**) at p<0.05 and p<0.01 respectively following Tukey's multiple range test*

Evan's blue dye uptake has been regarded as an indicator of alteration in membrane permeability. When root tips were stained with Evan's blue, increased uptake of the dye was observed in the root tips boron treated seedlings as compared to root tips from untreated control grown seedlings (Fig. 4E). This suggests that the integrity of root plasma membrane gets altered, leading to membrane destabilization due to boron treatment. More uptake of the dye was noticed in the root tips of sensitive cv. Malviya – 36 than the tolerant cv. Brown Gora.

## 3.5 Boron Treatment and ROS Production in the Tissues

Increased levels of metals and metalloids in plant tissues invariably cause increased generation of ROS [24]. Our experiments showed that boron exposure to the seedlings of rice *cv*. Malviya-36 caused increase in the levels of $O_2^{\cdot -}$ and $H_2O_2$

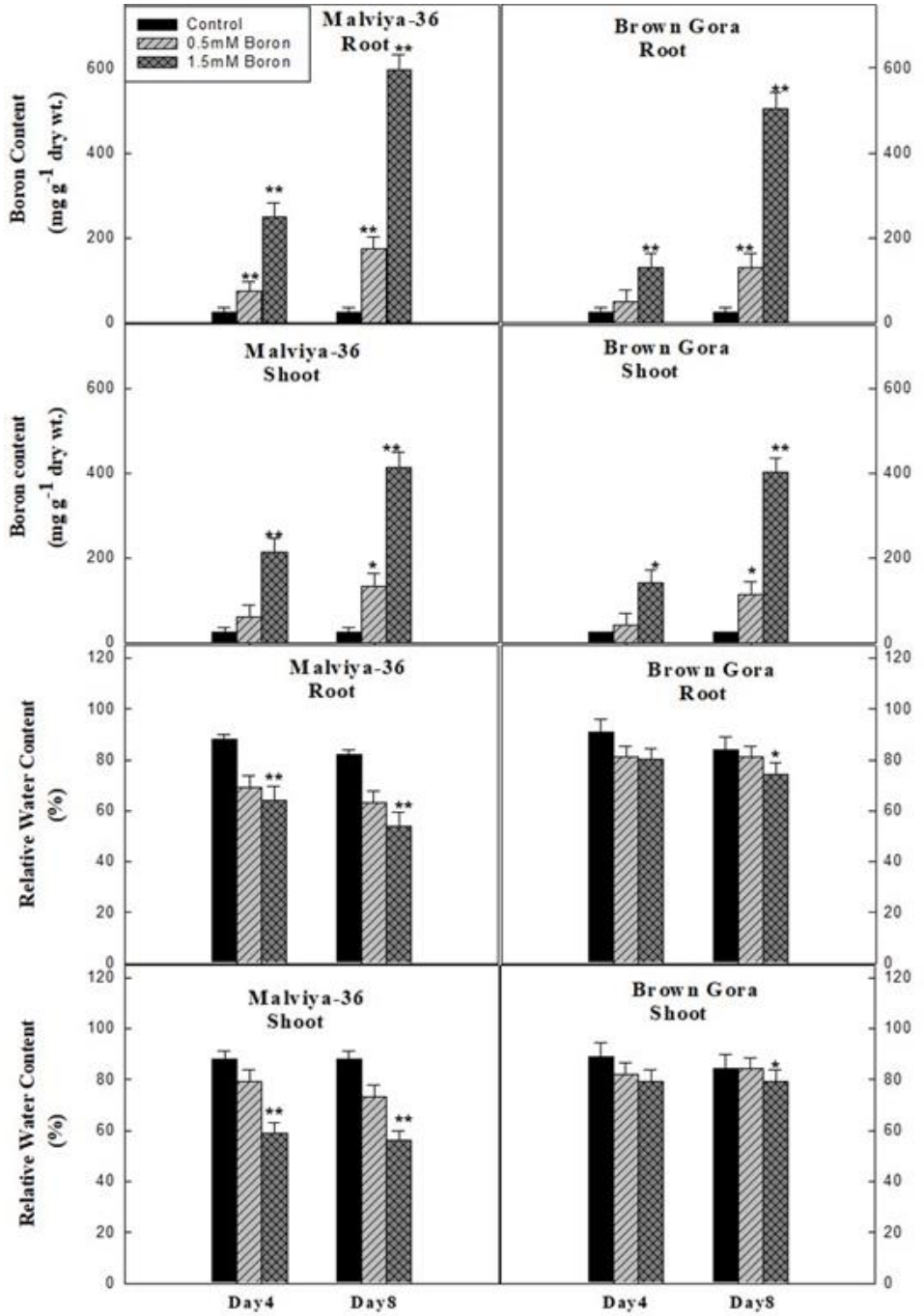
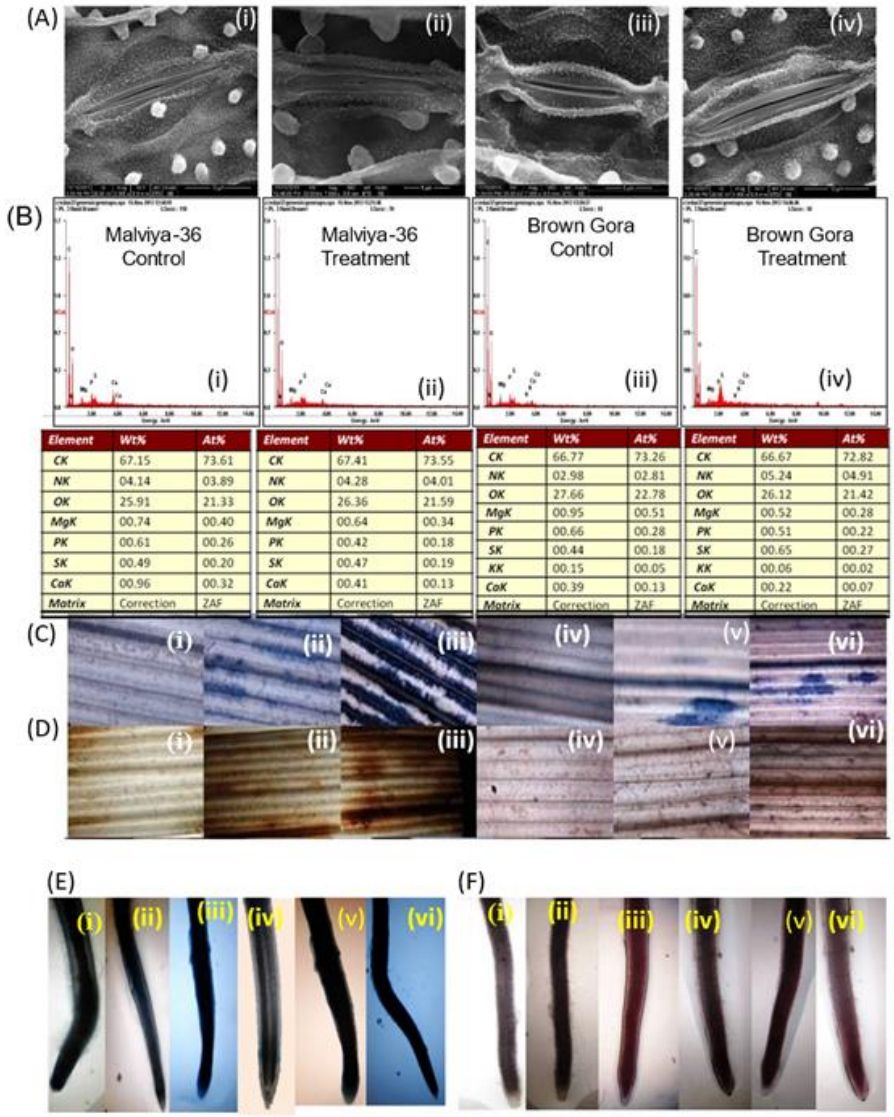



**Fig. 3. Effect of boron on its uptake and relative water content in the roots and shoots of seedlings of rice *cvs.* Malviya-36 and Brown Gora**

*Seedlings were grown for 8 days in hydroponics in Yoshida nutrient solution containing 0.05 mM boron (control), 0.5 mM boron and 1.5 mM boron. Values represent means of three independent experiments and bars indicate standard deviations. Values differing significantly from controls have been represented as asterisks (*) and (**) at p<0.05 and p<0.01 respectively following Tukey's multiple range test*

and increased lipid peroxidation in both roots as well as shoots (Fig 5). Whereas, in *cv.* Brown Gora with boron treatment $H_2O_2$ and lipid peroxides increased in roots as well as shoots, but $O_2^{\bullet -}$ level declined in roots. With 1.5 mM boron treatment for 8 days in *cv.* Malviya – 36 $O_2^{\bullet -}$ level increased by 120 % ($p$<0.01) in roots and 102 % ($p<0.01$) in shoots, whereas in *cv.* Brown Gora 106 % ($p$<0.01) increase in $O_2^{\bullet -}$ level was noted in shoots and in roots $O_2^{\bullet -}$ level declined by 68 % ($p$<0.01) under similar level and duration of treatment (Fig. 5A, B).




| Element | Wt% | At% |
|---|---|---|
| CK | 67.15 | 73.61 |
| NK | 04.14 | 03.89 |
| OK | 25.91 | 21.33 |
| MgK | 00.74 | 00.40 |
| PK | 00.61 | 00.26 |
| SK | 00.49 | 00.20 |
| CoK | 00.96 | 00.32 |
| Matrix | Correction | ZAF |

| Element | Wt% | At% |
|---|---|---|
| CK | 67.41 | 73.55 |
| NK | 04.28 | 04.01 |
| OK | 26.36 | 21.59 |
| MgK | 00.64 | 00.34 |
| PK | 00.42 | 00.18 |
| SK | 00.47 | 00.19 |
| CoK | 00.41 | 00.13 |
| Matrix | Correction | ZAF |

| Element | Wt% | At% |
|---|---|---|
| CK | 66.77 | 73.26 |
| NK | 02.98 | 02.81 |
| OK | 27.66 | 22.78 |
| MgK | 00.95 | 00.51 |
| PK | 00.66 | 00.28 |
| SK | 00.44 | 00.18 |
| KK | 00.15 | 00.05 |
| CoK | 00.39 | 00.13 |
| Matrix | Correction | ZAF |

| Element | Wt% | At% |
|---|---|---|
| CK | 66.67 | 72.82 |
| NK | 05.24 | 04.91 |
| OK | 26.12 | 21.42 |
| MgK | 00.52 | 00.28 |
| PK | 00.51 | 00.22 |
| SK | 00.65 | 00.27 |
| KK | 00.06 | 00.02 |
| CoK | 00.22 | 00.07 |
| Matrix | Correction | ZAF |

**Fig. 4. (A) Scanning Electron Microscope (SEM) imaging showing ultrastructure of rice leaf stomata**

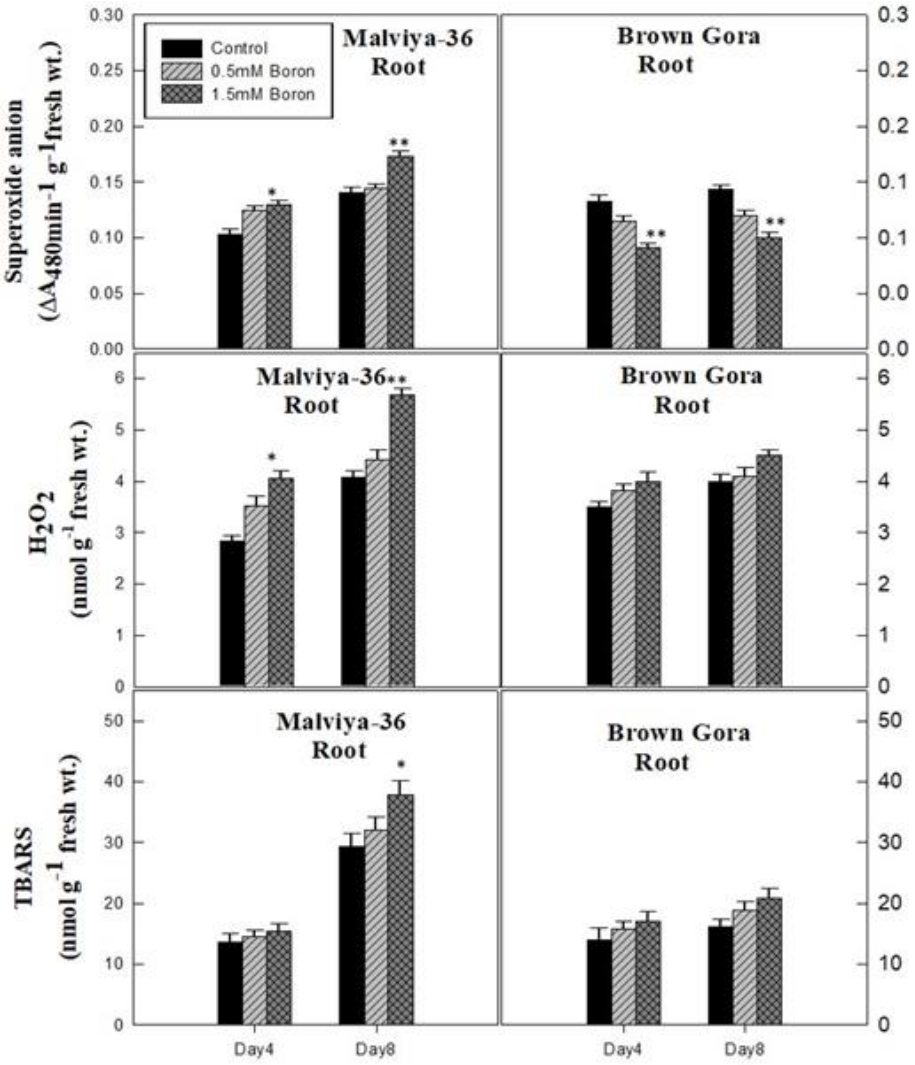
*(i) control (0.05 mM boron) and (ii) 1.5 mM boron treated seedlings of rice cv. Malviya-36 as well as (iii) control (0.05 mM boron) and (iv) 1.5 mM boron treated seedlings of rice cv. Brown Gora. Distortion in the shape of guard cells is evident in boron treated seedlings. (B) Energy Dispersive X-ray Analysis showing changes in the content of elements on leaf surfaces of (i) control (0.05mM boron) and (ii) 1.5 mM boron treated seedlings of rice cv. Malviya-36 as well as (iii) control (0.05mM boron) and (iv) 1.5 mM boron treated seedlings of rice cv. Brown Gora. (C) NBT staining showing superoxide anion ($O_2^{\cdot-}$) localization in rice leaves from (i) control (0.05 mM boron) (ii) 0.5 mM boron and (iii) 1.5 mM boron treated seedlings of rice cv. Malviya-36 as well as similarly grown seedlings of rice cv. Brown Gora under (iv) control (0.05 mM boron), (v) 0.5 mM boron and (vi) 1.5 mM boron treatments. Dark stained patches indicate $O_2^{\cdot-}$ produced. (D) $H_2O_2$ detection in rice leaves in situ using DAB. Rice seedlings raised for 8 days under (i) control (0.05 mM boron), (ii) 0.5 mM boron and (iii) 1.5 mM boron of cv. Malviya-36 as well as similarly grown seedlings of cv. Brown Gora under (iv) control (0.05mM Boron), (v) 0.5 mM boron and (vi) 1.5 mM boron treatments were used. Dark spots represent presence of $H_2O_2$. (E) Uptake of the dye Evan's blue by root tips showing loss of plasma membrane integrity. Greater intensity of blue colour retained by the roots represents more loss of plasma membrane integrity. Roots of the seedlings grown for 8 days under (i) control (0.05 mM boron), (ii) 0.5 mM boron and (iii) 1.5 mM boron of rice cv. Malviya-36 as well as from similarly grown seedlings of rice cv. Brown Gora under (iv) control (0.05 mM boron), (v) 0.5 mM boron and (vi) 1.5 mM boron treatments were used. (F) Histochemical detection of lipid peroxides in roots using Schiff's reagent. Roots of the seedlings grown for 8 days under (i) control (0.05 mM boron), (ii) 0.5 mM boron and (iii) 1.5 mM boron of rice cv. Malviya-36 as well as of cv. Brown Gora raised under (iv) control (0.05 mM boron), (v) 0.5 mM boron and (vi) 1.5 mM boron treatments were used. Intensity of pink colour represents extent of lipid peroxides produced within the roots of the seedlings*

An increase in H2O2 level was observed in the seedlings with boron treatment, with more increase in cv. Malviya than cv. Brown Gora (Fig. 5A, B). With 1.5 mM boron treatment for 8 days to the seedlings of cv. Malviya-36, 84 % ($p<0.01$) higher H2O2 levels was noticed in roots and 53 % ($p<0.01$) higher in shoots than controls, whereas in cv. Brown Gora under similar level and duration of boron treatment, compared to controls 12.5 % increased H2O2 level could be noticed in roots and 11 % increased level in shoots.

In our studies a marked enhancement in lipid peroxidation was noted in the seedlings, marked by increased level of TBARS, with boron treatment, with greater increase in cv. Malviya than Brown Gora. In Malviya-36 seedlings with 1.5 mM boron treatment for 8 days the level of TBARS was 29 % ($p<0.05$) higher in roots and 31 % ($p<0.05$) higher in shoots, whereas under similar boron treatment level in cv. Brown Gora TBARS level increased by 12 % in both roots and shoots as compared to the level in untreated control seedlings (Fig. 5 A, B).

### 3.6 Effect of Boron on Antioxidant Enzyme Activities

The activities of SOD and catalase were determined in boron treated rice seedlings. As a result of boron treatment activities of both SOD and catalase increased in the seedlings of both rice cultivars Malviya-36 and Brown Gora (Fig. 6). With 8 day of 1.5 mM boron treatment in *cv.* Malviya-36 SOD activity increased by 53 % ($p<0.01$) in roots and 140 % ($p<0.01$) in shoots, whereas under similar boron treatment level in *cv.* Brown Gora SOD activity increased by 38 % ($p<0.01$) in roots and by 122 % ($p<0.01$) in shoots (Fig. 6). In both sets of rice seedlings activity of SOD was higher in roots than in shoots under control as well as boron treatments.



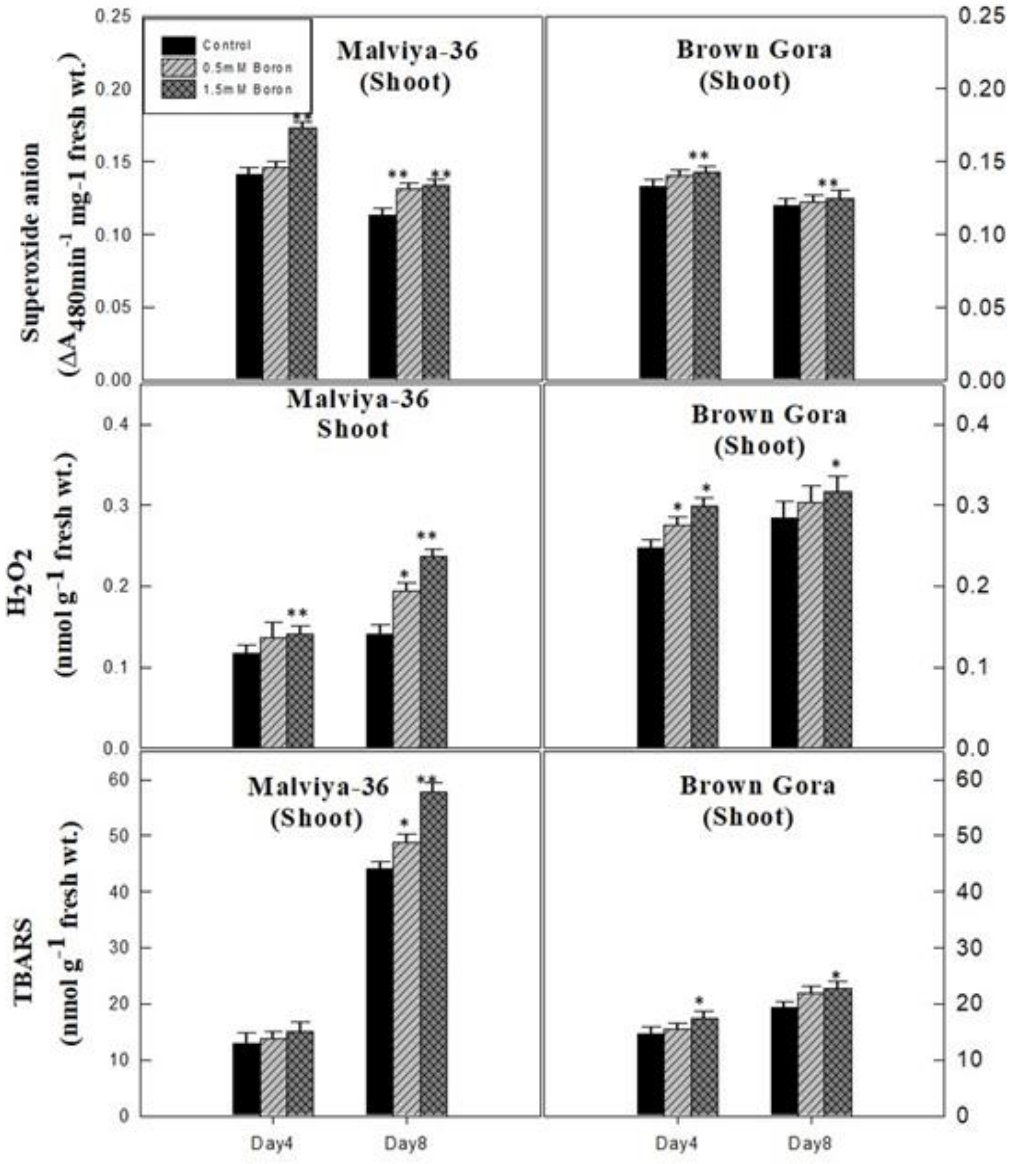



**Fig. 5. Effect of boron on the levels of $O_2^{\cdot -}$, $H_2O_2$ and lipid peroxidation products (measured in terms of thiobarbituric acid reactive substances, TBARS) in roots (5A) as well as shoots (5B) of seedlings of rice *cvs*. Malviya-36 and Brown Gora grown for 8 days in hydroponics in Yoshida nutrient solution containing 0.05 mM (control), 0.5 mM and 1.5 mM boron. Values represent means of three independent experiments and bars indicate standard deviations**

*Values differing significantly from controls have been represented as asterisks (*) and (**) at p<0.05 and p<0.01 respectively following Tukey's multiple range test*

Catalase is a key antioxidative enzyme involved in plant defense, ageing, senescence, etc. and it catalyzes $H_2O_2$ decomposition in the tissues outside the chloroplasts. In our studies increase in CAT activity was observed in the seedlings with boron treatment. Rice *cv*. Malviya-36 seedlings treated for 8 days with 1.5 mM boron had 33 % ($p$<0.01) increased CAT activity in roots and 35% ($p$<0.05) increase in activity in shoots, and under similar boron treatment level in rice *cv*. Brown Gora seedlings the activity of CAT increased by 18% in roots and 45% ($p$<0.01) in shoots (Fig. 6).

## 3.7 Gene Expression Analysis of SOD Isoforms

To further examine the possible role of SOD isoforms in boron tolerance, real time PCR study was performed. Different SOD isoforms showed

differential behavior in terms of gene expression on boron treatment. Interestingly, expression of Fe-SOD was higher in *cv.* Brown Gora seedlings, in both roots and shoots and the expression further increased with boron treatment, whereas in *cv*. Malviya-36 seedlings Fe-SOD expression was comparatively lower than in the tolerant cultivar and no apparent increase in expression could be observed in this cultivar with boron treatment (Fig. 7).

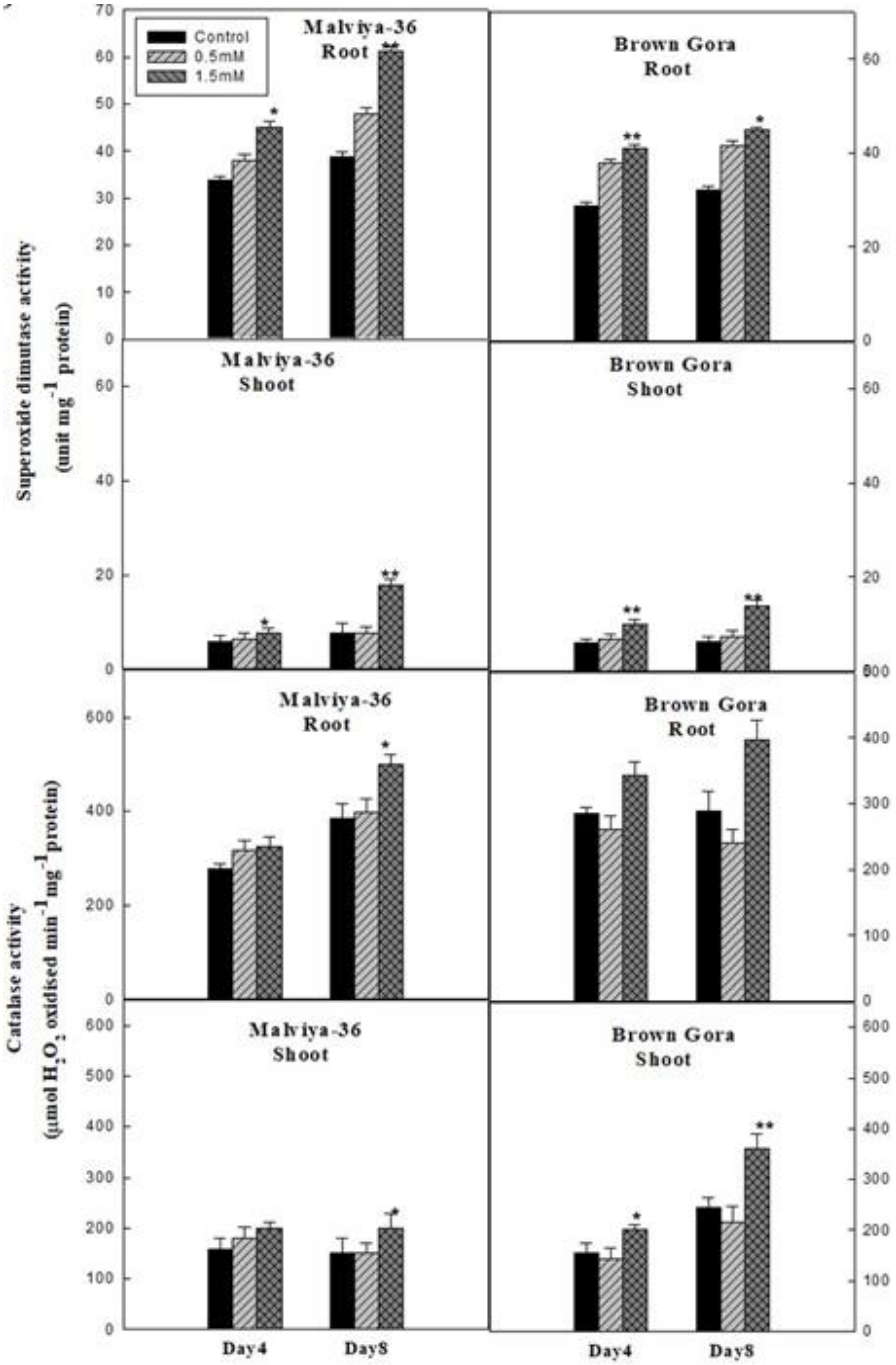



**Fig. 6. Effect of boron on superoxide dismutase and catalase activities in rice seedlings**

*Seedlings of rice cvs. Malviya-36 and Brown Gora were grown for 8 days in hydroponics in Yoshida nutrient solution containing 0.05 mM (control), 0.5 mM and 1.5 mM boron. Values represent means of three independent experiments and bars indicate standard deviations. Values differing significantly from controls have been represented as asterisks (*) and (**) at p<0.05 and p<0.01 respectively following Tukey's multiple range test*

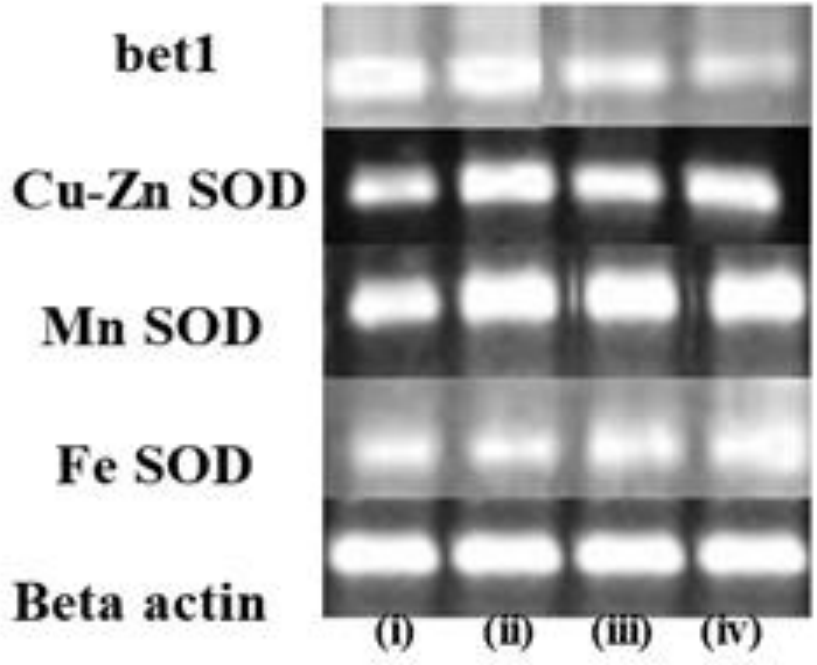



**Fig. 7. Effect of boron treatment on expression of three SOD isoforms as determined by qPCR**
*The gene beta actin was used as internal control. Shoots of the seedlings of rice cv. Malviya-36 grown under (i) control (0.05 mM boron) and (ii) 1.5 mM boron as well as shoots of seedlings of cv. Brown Gora grown under (iii) control (0.05 mM boron) and (iv) 1.5 mM boron were used*

## 4. DISCUSSION

The presence of macro and micronutrients at high concentrations in the soil leads to excessive absorption of these elements by the plants. The higher levels of these nutrients within the plant tissues cause various morphological and physiological alterations in plants leading to decrease in yield. In the present investigation, we have examined such alterations which occur in rice plants grown under high boron concentrations. We investigated morphological, histochemical, biochemical and a molecular framework of rice seedlings responding to the excess levels of boron, which is an essential plant micronutrient.

High boron level strongly inhibits seed germination [25]. A plausible reason of the inhibited germination due to boron excess may be due to a decrease in the relative water content of the germinating seeds, under boron treatment. Water is essential for germination and high boron level may restrict the availability of water for the seeds. In our experiments, boron treatment of 1.5 mM caused marked inhibition in growth of rice seedlings, as evidenced from decline in length, fresh biomass as well as dry weights of treated seedlings. A possible reason for this stunted plant growth under boron excess could be due to limiting of cell elongation leading to inhibition of root and shoot growth [26]. Decline in fresh and dry weight in wheat and rice plants has been observed with boron treatment [25]. Reduction in the fresh weight could be due to the less absorption of water via roots whereas decreased dry weight may be due to decrease in the root and shoot biomass. In our experiments with 0.5 mM boron treatment, increased fresh and dry weight of shoots was noticed in comparison to controls (Fig. 2). Such increase in growth of seedlings with low level of boron in the growth medium shows that boron promotes elongation, division and growth of cells leading to overall growth of the plants [26]. Boric acid stimulates seed germination and growth of plants when applied in low concentrations in the growth medium [27], whereas high boron concentration is inhibitory to the growth of plants [26]. In our studies, overall reduction in growth of seedlings at a high boron concentration of 1.5mM could also be partly due to accumulation of boron at the tip of roots and shoots. Excess boron when taken up by plants, primarily gets localized in the cell wall, which may restrict the further uptake of nutrients. Our data supports the findings of Choudhary et al. [26] who reported that micronutrients at higher concentration may result

in decreased growth of plants, whereas at lower concentration growth promoting effects are seen.

Reduction in the growth of rice seedlings due to boron insisted us to have the clear insight of the extent of boron uptake in the tissues. For this, we performed ICP-OES analysis to determine intracellular boron concentration. With increase in the concentration of boron in the treatment medium as well as with increase in the duration of treatment, boron uptake increased in the seedlings. A marked difference was noticed related to the level of absorbed boron among the two sets of rice seedlings. Rice *cv*. Malviya-36, a sensitive cultivar towards boron, showed much higher content of absorbed boron in both roots and shoots at $4^{th}$ and $8^{th}$ day of treatment compared to *cv.* Brown Gora, a cultivar tolerant to boron. In seedlings of both the rice cultivars, after boron uptake, its greater localization was seen in roots compared to shoots. Our results showed that *cv*. Brown Gora exposed to a high level of boron (1.5 mM) was capable of maintaining better growth as compared to *cv*. Malviya-36. It has been observed that plant species tolerant to particular element, have mechanisms to limit uptake of such elements. Boron tolerant plant genotypes show such properties [3]. However, some plant species grow well in high boron containing soils and also show high concentration of boron in aerial parts [28]. Although *cvs.* Malviya-36 and Brown Gora used by us are not boron hyper-accumulators, but it appears that some exclusion mechanism for boron exists in *cv.* Brown Gora, due to which this cultivar maintains good growth under a higher (1.5mM) boron concentration.

In our experiments inhibition of plant growth under high boron level is associated with alteration in overall physiology of rice plants such as relative water content, photosynthetic systems, antioxidative defense system etc. We observed that *cv.* Brown Gora had higher RWC than *cv*. Malviya-36, at similar level of boron treatment. This observation suggests that in *cv.* Brown Gora due to high RWC, as a result of dilution effect, toxicity of boron gets reduced. The possible reason of decreased RWC in boron treated seedlings could be due to decreased leaf water potential caused by boron toxicity. It has been reported that several metals and metalloids when taken in excess by plants cause decreased RWC in the tissues [29].

Boron toxicity causes distortion of the stomatal structure and also the structure of guard cell as evident from scanning electron microscopy (SEM). Abiotic stresses including excess boron induce a rapid distortion in stomatal structure and decline in stomatal conductance [30]. The differential boron tolerance in the two rice cultivars used in our studies might be due to the differential expression of boron transporters in two cultivars. Boron transporters control the outward and inward flow of boron in plants (Miwa and Fujiwara 2010); however, further studies are needed to get a detail and clear insight of altered structure of guard cells and expression of boron transporters under boron toxicity conditions. Our SEM results were further supported by Energy dispersive X-ray diffraction analysis (EDAX) of rice leaves that was carried out to examine the alteration in multi-elemental concentration in the leaves of boron treated seedlings. The EDAX peaks indicated a decrease in the concentration of important elements from cellular constituents such as phosphorus (DNA), calcium (plasmodesmata and cell wall) and magnesium (for cellular reactions) in boron treated seedlings (Fig. 2C). Maximum decrease in the concentration of calcium signifies the role of boron in maintenance of the cell wall structure as calcium is an essential constituent of the dimeric boron-rhamnogalacturonan complex and also signifies the interference of excess boron in the absorption of other elements [31]. Decrease in calcium concentration in the tissues under excess boron may also affect the calcium signaling cascade.

Abiotic stresses like drought, salinity, high light intensity, heat, excess of metals, etc. cause increase in production of ROS in the tissues. Superoxide anion is the first among ROS to be generated after the reduction of molecular oxygen and is considered to have strong reactivity and oxidizing ability. The main sites for its generation are the photosynthetic electron transport chain and the mitochondrial electron transport chains [32]. In our experiments we observed increased production of the ROS $O_2^{\cdot -}$ and $H_2O_2$ in boron treated rice plants. Barley plants grown under toxic concentrations of boron have been earlier shown to overproduce ROS [12]. Increased levels of $H_2O_2$ have been shown to cause damage to cell membranes [32]. As an indicator of oxidative damage in the tissues, we measured the levels of lipid peroxides and found elevated level of peroxides in boron treated tissues, marked by increased TBARS level. Greater ROS and lipid peroxides levels in boron treated cv. Malviya-36 seedlings than cv. Brown Gora suggests that more oxidative damage due to boron occurs in cv. Malviya -36 than cv. Brown Gora.

Plants defend against ROS by induction of non-enzymic antioxidants and antioxidative enzymes which scavenge ROS. Therefore, in our studies the response of antioxidative enzymes in rice seedlings against excess boron was examined. Among the antioxidative enzymes SOD plays primary role against overproduced $O_2^{\cdot-}$ and scavenges it to produce $O_2$ and $H_2O_2$ [12, 32]. The change in SOD activity has been regarded as an indicator of production of $O_2^{\cdot-}$ in the tissues [12, 32]. Increased SOD activity under excess boron treatment as observed in our experiments appears to be a protective measure adopted by the tissues against oxidative damage caused by overproduced $O_2^{\cdot-}$ [32]. Similar to SOD, the activity of $H_2O_2$ scavenging enzyme CAT increased in boron treated seedlings, with greater increase in shoots of *cv.* Brown Gora than *cv*. Malviya-36. In Brown Gora seedlings, a greater increase in shoots CAT activity than Malaviya-36, under boron treatment indicates higher efficiency of detoxification of $H_2O_2$ produced in the peroxisomes in this cultivar when exposed to excess boron. In sunflower, tomato and apple plants boron treatment has been shown to cause increase in activity of CAT [26].

SOD isoforms play important role in protection of cells against ROS because SOD directly dismutates $O_2^{\cdot-}$. They are classified on the basis of their metal cofactors such as Cu/ZnSOD, MnSOD and FeSOD. Among these isoforms, Cu/ZnSOD is present in both cytosol and chloroplasts whereas MnSOD in mitochondrial and FeSOD is chloroplastic isoform [33]. These isoforms are sensitive to $O_2$ concentration in the environment. Decrease in Fe-SOD is the first indicator of increased oxygen concentration in the environment. When Fe level decreases there is a shift in binding of oxygen from Fe to Mn and then to Cu. Cu becomes available for $O_2$ binding when Fe is completely unavailable [34].

Different SOD isoforms show differential behavior in terms of gene expression in response to environmental stresses. Interestingly, the results of our real time PCR studies revealed very low level of Fe-SOD activity in control grown seedlings of tolerant *cv*. Brown Gora. However, in this cultivar the expression of Fe-SOD increased markedly with increase in boron treatment level. Whereas in *cv.* Malviya-36 seedlings the expression of FeSOD was almost similar under control and boron treatments. This suggests a possible role of FeSOD isoform in *cv.* Brown Gora in conferring tolerance towards boron. The sequence of *Oryza sativa* Fe-SOD cDNA from rice was first reported by Kaminaka and coworkers in the year 1999 [35]. Prior to it, FeSOD was not reported from any monocot plant. Overexpression of Fe-SOD in tobacco plants has been shown to confer oxidative stress tolerance [36]. We have shown earlier that rice genotypes expressing Fe-SOD isoforms in response to Al excess, are tolerant to Al toxicity [36-49,50]. Therefore in our experiments elevated expression of Fe-SOD in the tolerant cultivar suggests that this isoform may confer oxidative stress tolerance in rice in response to toxic level of boron treatment.

## 5. CONCLUSION

The findings suggest that elevated levels of boron in rice plants impede growth, induce structural alterations in cell organelles, trigger an overproduction of reactive oxygen species (ROS) within tissues, and lead to oxidative stress. The mechanism underlying boron tolerance seems to be inherent, as tolerant genotypes accumulate lower levels of boron in their tissues compared to sensitive genotypes, irrespective of the external boron concentration. Additionally, heightened expression of Fe-SOD is observed in correlation with boron tolerance in rice.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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