Research Article

Morphological and Biochemical Responses of Wheat to Flooding Stress and Recovery

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Abstract | Wheat being flooding sensitive was analyzed for flooding response mechanism. Poorly drained fields and unexpected rains in the sowing and germination times lead to flooding stress. Wheat varieties namely Atta Habib, Siran and Ghanemat-e-IBGE were grown in glass house and studied for their flooding stress response and ability to recover after flooding stress removal at morphological and antioxidant enzymes (catalase; CAT, superoxide dismutase; SOD, and peroxidase; POD) levels. Wheat varieties were grown for 15 days, flooded for 6 days and then recovered for 8 days after removal of flooding stress. The shoot pigmentations were suppressed as they turned pale under flooding stress but recovered to green when observed at the end of recovery period. The morphological parameters of shoot & root lengths and weights were suppressed in flooded seedlings, but recovered during the recovery. CAT activity in Atta Habib was raised from 1.63 under flooding stress to 2.32 unit/mg protein at the end recovery period. SOD activity was increased from 2.27 to 3.15 unit/mg protein and POD from 1.71 to 2.39 unit/mg protein. The enzyme activities in Siran revealed the same trend as observed in Atta Habib. Recovery capacity was least in Ghanemat-e-IBGE. The results suggest that the tested three wheat varieties suffered oxidative stress due to flooding but showed a recovery trend due to increased activities of antioxidant enzymes that scavenged the oxidative radicles, thus helping the wheat to recover. Atta Habib reflected higher recovering ability than Siran and Ghanimat-e-IBGE. The findings of the current study will surely help in contributing efforts to develop flood-tolerant cultivars.

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Introduction

Wheat (*Triticum aestivum* L.) is a staple food in many parts of the world (Bhutta *et al*., 2006). Wheat is rich source of starch (58.2%) and contains sufficient quantity of sugar and fat. It contains 11.2% protein, 6.8% pentosans and 1.7% ash (Liu *et al*., 1999). Wheat has been proved as a source of fuel (Curtis and Halford, 2014). In Pakistan, the total wheat production is forecasted to 25,600 thousand metric tons in 2018-19 (USDA, 2019). Flooding adversely restricts the plant growth and lowers the productivity (Kong *et al*., 2010). Drought and flooding have increased both in frequency and severity (Mittler and Blumwald, 2010). Floods in the history of Pakistan (1973, 1976, 1988, 1992, 2010, 2011, 2012, 2013 and 2014) have caused disasters for social, economic and health sectors. Pakistan suffered total losses of 19 billion dollars to the floods in 2010, 2011, 2012, 2013 and 2014 (Rehman *et al*., 2016). Melting

of Himalaya glaciers and heavy monsoon rain has increased the frequency and hazards of floods in the country (Bukhari and Rizvi, 2016). The devastating 2010 floods had hit 2 million hectares of cotton, sugarcane, rice, maize crops (Pakistan Economic Survey, 2011). In Khyber Pakhtunkhwa, crops destroyed were 443,116 area/hectares in 2010 floods alone (Bukhari and Rizvi, 2017). These reports suggest billion dollar losses to the country during the recent floods.

Poorly-drained heavily accumulated water around the plants in the field due to flooding, pose a serious threat owing to deprived oxygen, carbon dioxide and light for photosynthesis (Jackson and Colmer, 2005). Excess of water in the soil causes reduction in the supply of oxygen as gaseous diffusion in water is ten-thousand times slower than in air (Armstrong, 1980). As a consequence of oxygen deficiency, plant's respiration is restricted to anaerobic mode only; hence, energy being produced is very much less. The ATP production in fermentative metabolism is reduced by 37%. Under these oxygen deficient conditions, cell membrane integrity is damaged (Gibbs and Greenway, 2003) and oxidation-reduction potential of soil is decreased (Pezeshki, 2001). Toxic chemicals such as formate, ethanol, acetaldehyde and lactate accumulate in the surroundings. The leakage of solutes like sugars, potassium and amino acids from tissues of hypoxia stressed plants occur to external solutions (Greenway *et al*., 1992). Flooding brings oxygen deficiency and toxics accumulation as major problems for the flooding-stressed plants (Fiedler *et al*., 2007).

Reactive oxygen species (ROS) are produced in multiple organelles such as mitochondria (Huang *et al*., 2016), chloroplasts (Dietz *et al*., 2016) and peroxisomes (Sandalio and Romero-Puertas, 2015). Flooding stress leads to oxidative damage due to rise in levels of ROS like singlet oxygen ($^1O_2$), hydroxyl radicals (OH^-), superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Hasanuzzaman *et al*., 2012). Among the ROS, H_2O_2 is toxically reactive in higher plants (Petrov and Breusegem, 2012). In response to flooding-induced oxidative damage, plants stimulate enzyme and non-enzyme-based defense mechanisms to detoxify ROS (Caverzan *et al*., 2016). Enzyme-based mechanisms include involvement of antioxidant enzymes such as peroxidases (POD) like ascorbate peroxidase, monodehydro-ascorbate reductase, glutathione reductase, catalase (CAT) and superoxide dismutase (SOD). Major cellular redox buffers like ascorbate and glutathione as well as carotenoids, tocopherol, and phenolic compounds found in cellular compartments act as non-enzymatic defense system (Gill and Tuteja 2010, Caverzan *et al*., 2016). CAT and SOD gene expression and activities affect the accumulation of ROS in wheat (Cheng *et al*., 2016). CAT and POD eliminate H_2O_2 and toxic free radicals (Lee *et al*., 2011). CAT and SOD are efficient antioxidants that reduce the cellular damage by converting toxic superoxide anion and hydrogen peroxide to water and molecular oxygen (Jaleel *et al*., 2009). The copper-zinc SOD removes superoxide radicals as well as crucial component of ascorbate-glutathione cycle of detoxification (Chew *et al*., 2003). The current study was designed to unravel the flooding response mechanism in wheat by analyzing morphological and anti-oxidative enzymes activity changes. Post-flooding recovery potential of the wheat varieties was also assessed.

Materials and Methods

Plant material and growth conditions

Three wheat varieties; Atta Habib, Siran and Ghanemat-e-IBGE (G-IBGE) were grown in glass house with completely randomized design arrangement at Institute of Biotechnology & Genetic Engineering (IBGE), University of Agriculture Peshawar, Pakistan. Average temperature was 16.6˚C in November and 11.7˚C in December. Seeds were washed for two minutes each in 70% ethanol and 15% sodium hypochlorite, followed by two minutes wash with distilled water. Seeds of all the three wheat varieties were sown in small pots (6.5 cm height and 9 cm diameter) and grown for 15 days. Seeds were sown in common field soil mixed with humus, on November 26, 2018. The seeds were provided by IBGE. All the experiments were performed as triplicates.

Flooding stress treatment and post-flooding recovery

Fifteen-days-old wheat seedlings were flooded for 6 days and then recovered for 8 days following water drainage (Figure 1). For flooding treatment, seedling pots containing the plants were fully submerged (flooded) in bucket-like pots (33 cm height and 26 cm diameter). Plants were flooded at Day 15 (December 11, 2018), flooded seedlings were sampled at Day 21 (December 17, 2018) and recovered after 8 days on Day 28 (December 24). Post-flooding recovery was achieved after removing of flooding stress and allowing plants to recover. Age-matched untreated control plants were collected on sampling points (Days 21 & 28).

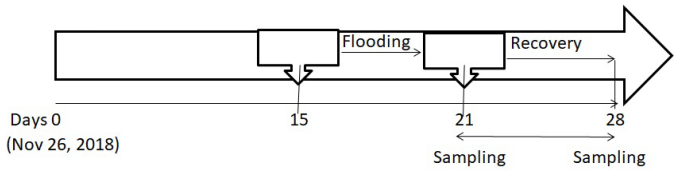

Figure 1: *Experimental design of the study. Seeds of the 3 varieties of wheat; Atta Habib, Siran and G-IBGE were sown and grown for 15 days, flooded until Day 21, and followed by removal of flooding stress and allowed to recover till day 28. Root and shoot samples were collected at Days 21 and 28 from treated and untreated (control) plants.*

Observations of shoot pigments changes

The changes in color intensities (pigmentation) were observed in control, flooded seedlings and recovering seedlings and photos were captured at each stage.

Morphological measurements

Morphological parameters such as root & shoot lengths and their weights were measured and recorded from control, flooded and recovering plants from 3 independent replications.

Enzyme assays

Catalase assay: CAT activity in the plants was measured through measuring decomposition of hydrogen peroxide at 240 nm (Havir and McHale, 1989). A 100 mg leave sample was ground with the help of mortar & pestle under liquid nitrogen and homogenized in 1mL of extraction buffer (50 mM phosphate buffer (pH 7), 2% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 10,000 rpm and 4°C for 20 minutes. The supernatant was used as enzyme extract. Bradford assay was applied for protein quantification using spectrophotometer (Bradford, 1976). Bovine serum albumin (BSA) was used as standard for standard curve derivation. For catalase reaction, 900 µL of reaction mixture (50 mM Phosphate buffer (pH 7), 10 mM hydrogen peroxide) was reacted to 100 µL of enzyme extract, vortexed and kept at room temperature for 5 minutes. The absorbance was measured at 240 nm through NanoDrop (NanoDrop-200Oc, Thermo Scientific) spectrophotometer.

Superoxide dismutase assay: SOD activity was measured by following methodology of Nagi *et al.* (1995). Principal of the method is based on SOD ability to inhibit the photo reduction of nitrobluetetrazolium (NBT). A 100 mg leave sample was homogenized in 1mL of extraction buffer (50 mM phosphate buffer (pH 7.8), 1 mM EDTA, 1% PVP). Homogenate was centrifuged at 12,000 rpm and 4°C for 20 minutes. Supernatant was re-centrifuged. The filtrate was used for enzyme assay and protein concentration determination by Bradford assay (Bradford, 1976). For analyzing activity, 900 µL enzyme reaction mixture (50 mM phosphate buffer (pH 7.8), 0.66 mM EDTA, 10 mM L-methionine, 33 µM NBT, 3.3 mM riboflavin) was reacted to 100 µL enzyme extract. The mix was vortexed and kept for 5 minutes. It was centrifuged at 12,000 rpm for 5 minutes and absorption of supernatant was recorded at 550 nm through NanoDrop (NanoDrop-200Oc, Thermo Scientific) spectrophotometer.

Peroxidase assay: POD activity was measured by slightly modifying Tewari *et al.* (2002) method. A 100 mg leave sample was ground under liquid nitrogen and homogenized in 1mL of extraction buffer (25 mM phosphate buffer (pH 7.8), 0.4 mM EDTA, 1 mM ascorbic acid, 2% PVP). The homogenate was centrifuged at 12,000 rpm and 4°C for 20 minutes. The supernatant was centrifuged again and the filtrate was used for enzyme assay. Protein concentrations were determined by Bradford assay (Bradford, 1976). For enzyme activity, 900 µL of reaction mixture (100 mM phosphate buffer (pH 7.8), 4% p-phenylenediamine, 2% H_2O_2, 5N H_2SO_4) was reacted to 100 µL of enzyme extract, vortexed and kept for 5 minutes. The mixture was finally centrifuged at 12,000 rpm for 5 minutes and absorption of supernatant was recorded at 485 nm through NanoDrop (NanoDrop-200Oc, Thermo Scientific) spectrophotometer.

Statistical analysis

Statistical significance of morphological measurements and enzyme activities were assessed by One-way ANOVA and LSD tests by using free accessed R-software (https://www.r-project.org/).

Results and Discussion

Effect of flooding stress on the shoot pigmentation of wheat varieties and post-flooding recovery

Flooding stress applied to the all three tested varieties suppressed shoot pigmentation. The results showed color changes at stressed and recovery time points as depicted in Figure 2 (2.1 Atta Habib; 2.2 Siran; 2.3 G-IBGE). The shoot color of seedlings turned pale/yellowish; showing reduction in pigmentation under flooding stress. The color changed to light green as the plants tried to recover in the post-flooding period. The trend in color change was same in all three varieties.

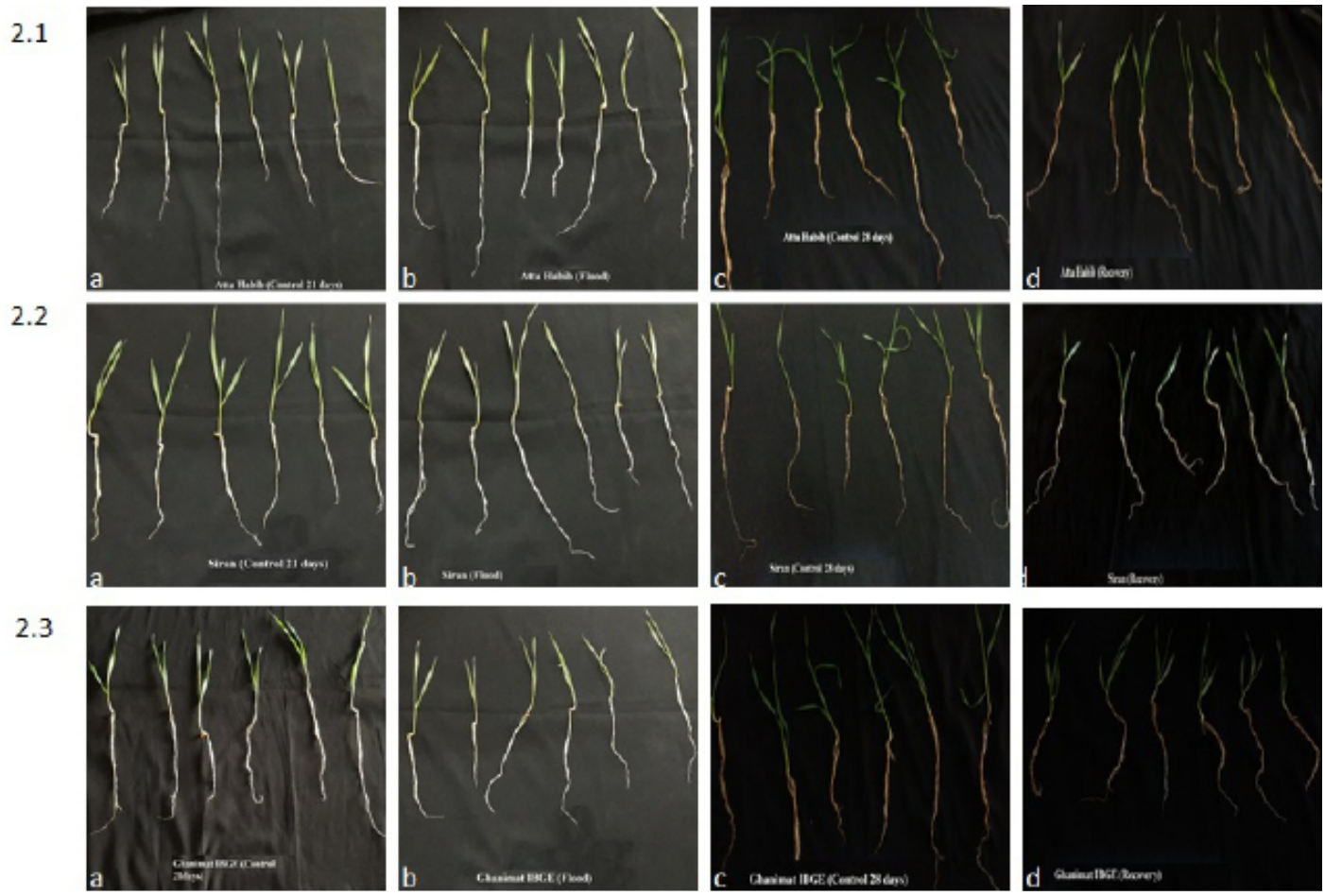

Figure 2: *Effect of flooding stress and recovery on phenotypes of Atta Habib (2.1), Siran (2.2) and G-IBGE (2.3); where a: 21 days control plants, b: flooded plants c: 28 days control plants, d: recovering plants.*

Morphological growth suppression and recovery

All three wheat varieties were grown for 15 days, flooded for 6 days and recovered for 8 days after water drainage. Flooding stress retarded shoot length in Atta Habib (28.99 cm), Siran (26.43 cm) and G-IBGE (24.57 cm); whereas; these were 33.23, 29.98 and 28.03 cm in control (untreated) plants of the respective varieties. Shoot length recorded at the end of recovery period shown increase in Atta Habib, Siran and G-IBGE (34.69, 28.98, 26.77 cm, respectively) (Figure 3A). The Shoot weight in Atta Habib, Siran and G-IBGE were decreased under flooding stress (348.25, 335.75, 305.42 mg), while it increased during recovery period to 360.73, 348.58, 316.91 mg, respectively (Figure 3B). Similarly, root length and weight were suppressed under flooding stress but recovered appreciably during recovery period. The root lengths decreased in Atta Habib (7.31cm), Siran (6.64 cm), and G-IBGE (4.92 cm); whereas, they increased during the recovery period to 7.85, 6.89, and 5.16 cm, respectively (Figure 3C). The root weight was decreased under flooding stress (35.42, 31.92, and 29.83 mg), but significantly increased during recovery period to 49, 43.25, and 41.83 mg in Atta Habib, Siran and G-IBGE, respectively (Figure 3D). The variations in root & shoot lengths and weights were statistically significant among the cultivars as well as treatments when analyzed by one-way ANOVA.

Antioxidant enzymes activity changes under flooding stress and during recovery

Antioxidant enzymes CAT, SOD, and POD were analyzed under flooding stress, post-flooding recovering as well as age-matched control plants. CAT activity in Atta Habib was only slightly decreased under 6-days of flooding stress (1.63 unit/mg protein), and increased significantly when analyzed at the end of 8-days recovery period (2.32 unit/mg protein). CAT activity in Siran only slightly decreased under flooding stress (1.58) and increased significantly while recovering (2.15 unit/mg protein). CAT activity in G-IBGE was slightly increased to 1.62 under flooding stress and raised further to 2.09 unit/mg protein at the end of 8-days post-flooding recovery (Figure 4A). CAT activity changes among the age-matched control and recovering plants as well as flooded and recovering plants were statistically significant. Activity of SOD was slightly suppressed under flooding

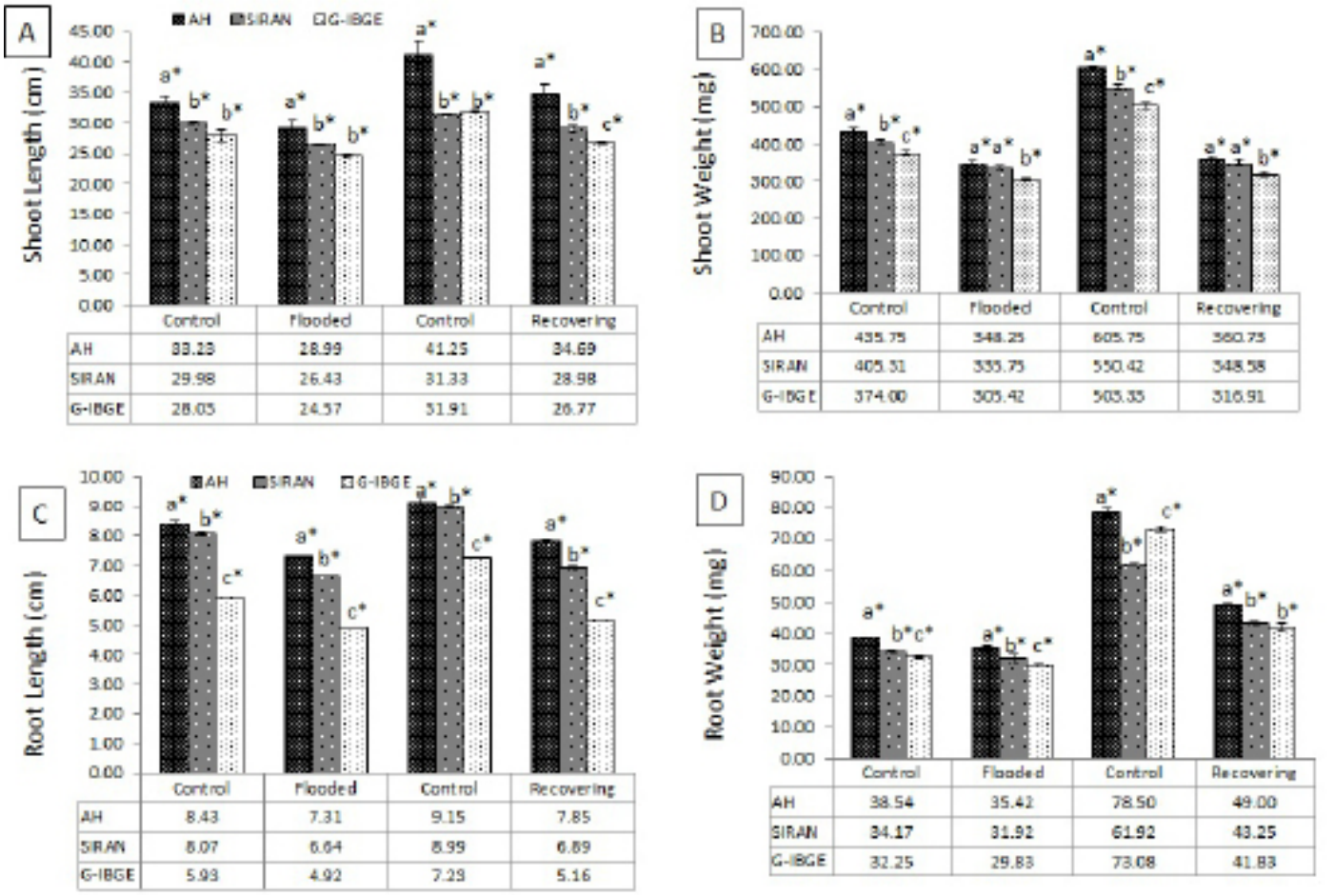

Figure 3: *Effect of flooding stress and post-flooding recovery on the shoot length, shoot weight, root length and root weight of wheat. The data represents mean of three replicates. Asterisks (*) represents significance at the level of p<0.05 when compared cultivar over time and cultivar over treatment through One-Way-ANOVA. Different alphabets depict LSD among the cultivars at a specific time-point.*

stress (2.27, 2.38, 2.10 unit/mg protein), but elevated significantly during the recovery period (3.15, 3.04, 2.72 unit/mg protein, respectively) (Figure 4B). SOD activity changes among the age-matched control and recovering plants as well as flooded and recovering plants were statistically significant. POD activity under flooding stress was almost same to the control plants i.e., 1.71 in Atta Habib, 1.64 in Siran and 1.63 unit/mg protein in G-IBGE. The activity of POD was increased to 2.39, 2.09, and 1.93 unit/mg protein, respectively (Figure 4C). POD activity changes among the age-matched control and recovering plants as well as flooded and recovering plants were statistically significant.

Flooding exerts devastating effects on plant growth, development and productivity (Kong *et al.*, 2010). In present study, green color of leaves was turned to pale yellow under flooding stress. These findings are in agreement with Yetisir *et al.* (2006) explored in watermelon, and with Khan *et al.* (2014 and 2015) in soybean, in which color of flooded plants was turned pale as compared to control plants. Flooded citrus plants shown the similar changes in color intensities (Hossain *et al.*, 2009).

Six days of flooding stress retarded the growth of the wheat cultivars in-terms of root & shoot lengths and weights (Figure 3). Growth parameters of shoot and root were recovered to significant extent after 8 days of recovery. The results were in compliance with Malik *et al.* (2002) who reported that flooding stress stopped the growth of seminal root, decreased leaf nitrogen system and stopped growth of adventitious roots. The roots resumed elongation during recovery period. Root length, weight, plant stature and shoot pigmentation were reduced in flooded soybean (Kong *et al.*, 2010). Haque *et al.* (2011) revealed that growth of aerenchymatous seminal roots is suppressed in wheat facing flooding stress. Excessive water intake due to flooding physically disrupts plant tissues leading to growth suppression (Komatsu *et al.*, 2012). In soybeans exposed to flooding stress, growth of root and hypocotyl was suppressed with increasing duration of flooding but recovered gradually during the recovery period (Salavati *et al.*, 2012; Khan *et al.*, 2014; 2015).

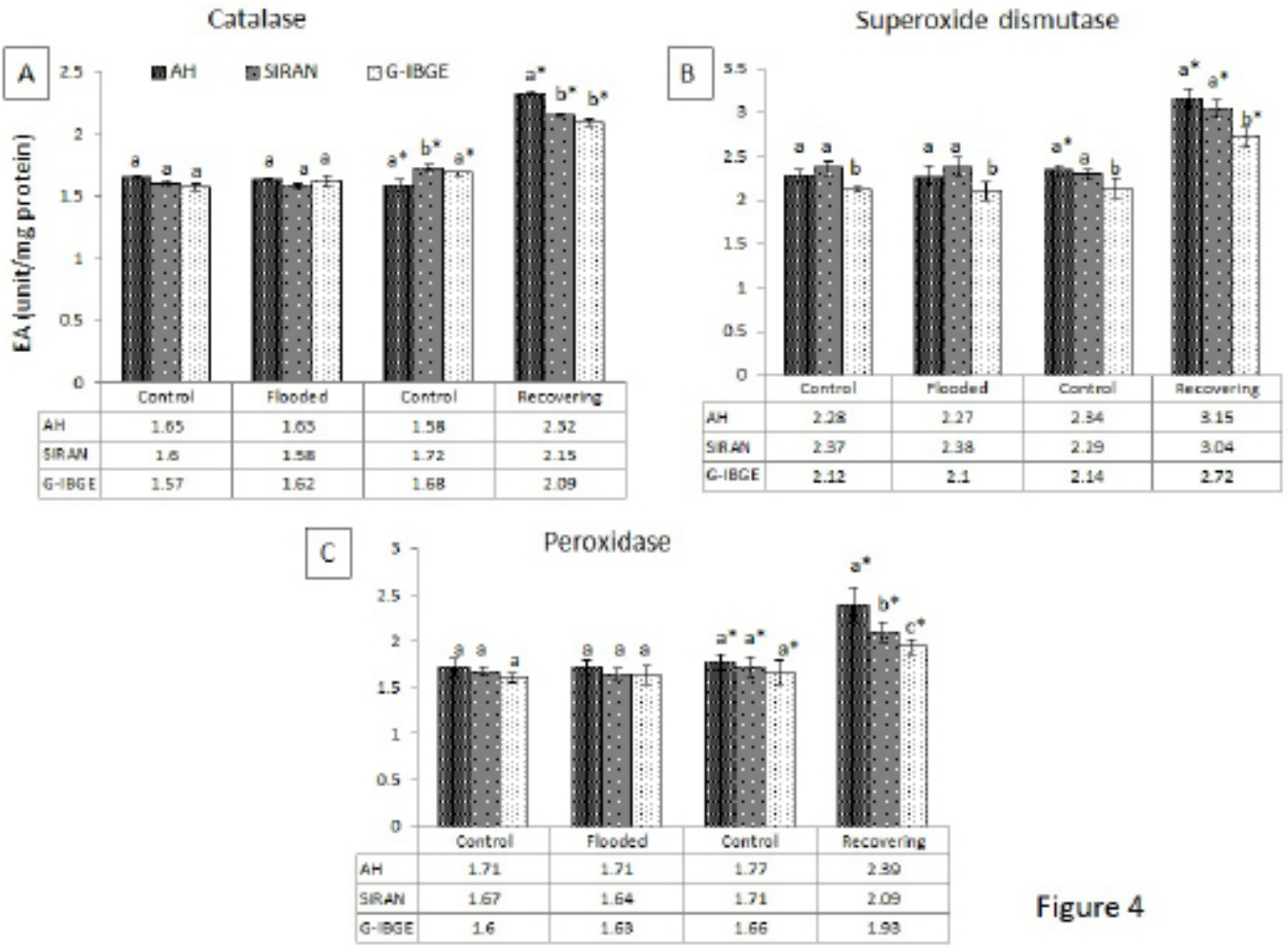

Figure 4: *CAT, SOD and POD activities changes in in wheat varieties under flooding stress and during recovery. The data represents mean of three replicates. EA is abbreviation for enzyme activity. Asterisks (*) represents significance at the level of p<0.05 when compared cultivar over time and cultivar over treatment through One-Way-ANOVA. Different alphabets depict LSD among the cultivars at a specific time-point.*

These reports in agreement with the results of current study suggest that all growth parameters of plant are suppressed under flooding stress and try to recover in the post-flooding period. Furthermore, Atta Habib showed maximum capability to recover its growth parameters, followed by Siran and then G-IBGE.

Enzymes activities of CAT, SOD and POD were analyzed in flooded wheat and during recovery after flooding removal (Figure 4). The activities of these enzymes were either suppressed or remained unchanged under flooding but raised significantly during recovery with more prominent results in Atta Habib. Similarly, CAT, SOD and POD activities were increased in citrus (Arbona *et al*., 2008). In present study, SOD activity was slightly reduced in flooded seedlings and raised significantly during recovery period. These results were in similarity with the findings in yellow iris (Monk *et al*., 1987), wheat (Biemelt *et al*., 1998), narrow leafed lupin, tobacco plants (Yu and Rengel, 1999) and barley (Yordanova *et al*., 2004). Furthermore, in the present study, POD activity was slightly decreased under flooding stress as also been reported in rice (Ushimaru *et al*., 1997), wheat (Biemelt *et al*., 1998) and pea (Kumutha *et al*., 2009). Similar trend in antioxidant enzyme activities were reported by Caverzan *et al*. (2016). From this discussion, it is concluded that antioxidant enzyme activities increase during recovery to lessen the effects of flooding-induced oxidative stress. This indicates a protective mechanism aimed at reducing oxidative damage by scavenging reactive oxygen species, thus helping the wheat to recover during post-flooding period.

Conclusions and Recommendations

Flooding stress retarded the growth of wheat roots & shoots, and reduced the pigmentation. In the post-flooding period, growth parameters of root and shoot tended to recover. Activities of CAT, SOD &

POD were suppressed under flooding stress, pointing towards accumulating ROS and increased oxidative damage. The increase in activities of these antioxidants during post-flooding recovery period describes plant use of antioxidants to scavenge accumulated ROS, and thus help the plant to move towards recovery following the flooding-removal. Moreover, Atta Habib showed best post-flooding recovery responses as compared to Siran and Ghanimat-e-IBGE, so it is recommended to be grown in flood-prone areas.

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Novelty Statement

The locally developed three wheat varieties viz. Atta Habib, Siran and Ghanimat-e-IBGE were analyzed first time for the flooding stress responses and post-stress recovery. Results revealed suppression in growth of all three varieties under flooding stress but Atta Habib revealed better recovery potential in the post-flooding recovery period. The findings are novel and will surely help in contributing efforts to develop flood-tolerant cultivars.

Author Contributions

Faryal Malik: Performed all experiments.
Mudassar Nawaz Khan: Designed and supervised the experiment as well as wrote the manuscript.
Israr-Ud-Din: Critically reviewed and edited the manuscript.

Conflict of Interest
The authors declare that they have no conflict of interest.

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