

## Drought Stress-Mediated Consequences on Enzymatic Antioxidants of Fagopyrum esculentum Moench

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ABSTRACT: *Fagopyrum esculentum* Moench is one of the vital unattended crops and is grown as a minor grain crop in the Indian Himalayas, especially in the high-altitude areas. The plant was studied to know the level of tolerance to drought stress. Seeds of *F. esculentum* were sown in nursery beds and with the appearance of first leaf; seed-lings were transferred to ceramic pots filled with mixture of soil and sand. Drought stress was imposed by watering while weighing method was used to control water potential to meet the experimental requirements. Different water potentials (-0.01, -0.02, -0.03, -0.04, -0.05, -0.06 and -0.07 MPa) were achieved after 15 days of transplanting the seedlings to pots. Response of enzymatic antioxidants to drought stress was studied in leaves and roots of *F. esculentum* at an interval of 30, 45, 60 and 75 days of plant growth. *F. esculentum* has a protection mechanism against oxidative damage by maintaining higher activities of enzymatic antioxidants (superoxide dismutase, catalase and peroxidase).

Keywords: Antioxidants; Drought; Enzymatic; Oxidative and Stress.

**INTRODUCTION:** *Fagopyrum esculentum* Moench, commonly known as common buckwheat, belongs to family Polygonaceae, is one of the vital unattended crops cultivated in the high altitude temperate zones of the North-West Himalayan region. Due to the less economic output and cultivation constraints the crop is at the verge of extinction, though it is of high medicinal and nutritive value.<sup>1</sup>

Buckwheat (*F. esculentum*) is grown as a minor grain crop in the Indian Himalayas, especially in the highaltitude areas (1600-4000 m above mean sea level). It is cultivated extensively in the high mountains of Jammu and Kashmir, Leh, Himachal Pradesh, Garwhal, Kumaon, Darjeeling, Sikkim, Assam, Arunachal Pradesh, Nagaland, Manipur, Nilgiris and Palani hills.<sup>2</sup>

Buckwheat is mainly used for the treatment of celiac disease as it is gluten free and eaten in place of wheat.<sup>3</sup> The dominant polyphenol in buckwheat is rutin and buckwheat can be described as an excellent dietary source of rutin,<sup>4, 5 & 6</sup> because no rutin was found in cereals and pseudocereals except *Fagopyrum esculentum*.<sup>7</sup> Buckwheat is also rich in vitamins, especially those of the B group and is source of macroelements (e.g., K, Na, Ca, Mg) as well as microelements (e.g., Zn, Mn, Cu, Se).<sup>8</sup>

Among the different environmental stresses, drought stress is one of the most adverse factors to plant growth and productivity. In drought situations, water potential and turgor are reduced enough to interfere with normal functions of the plant.<sup>9</sup> Plant resists drought stress by reflecting morphological, physiological and metabolic changes in their organs.<sup>10</sup> Plant response to drought depends on the length and severity of drought, and also on the species or genotype, as well as on the age and stage of its development. Drought stress often causes oxidative stress due to production of reactive oxygen species (ROS), such as singlet oxygen ( $^{1}O_{2}$ ), superoxide radical ( $O_{2}^{-}$ ), hydroxyl radical (OH<sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Plants are equipped with a complex and highly efficient antioxidative defense system which can respond and adapt to drought stress. This system is composed of protective enzymatic and non-enzymatic mechanisms.<sup>11</sup> Antioxidants are maintained in the plants in their reduced functional state that efficiently scavenge ROS and prevent damaging effects of free radicals.<sup>12&13</sup> Damage caused by oxidative stress is minimized by the cellular antioxidative defense system, which keeps active oxygen species (AOS) under control and functions as a reductant for many free radicals.13

Many underutilized species have the potential to contribute to food security at local and regional levels. Researchers, farmers, consumers and policymakers needs to recognize the significance of underutilized crops. Research on underutilized crops will help to understand the potential of these crops. The objective of the present study was to evaluate the enzymatic antioxidant defense system in plant.

## **MATERIALS AND METHODS:**

**Materials:** Seeds of *Fagopyrum esculentum* Moench were obtained from Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Research Sub-Station, Sangla, Kinnaur, Himachal Pradesh. Experiments were conducted in the laboratory and an open nursery area of School of Biological and Environmental Sciences, Shoolini University of Biotechnology and Management Sciences, Solan (latitude 30°51'N, longitude 77°07'E and altitude 1195 m), where average annual rainfall is 1315.6 mm.<sup>14</sup>

Experimental Set-Up: Homogenous seeds of F. esculentum were surface-sterilized and soaked in distilled water (control) for 12 h at 20±2 °C. Seeds of F. esculentum were sown in nursery beds of School of Biological and Environmental Sciences, Shoolini University of Biotechnology and Management Sciences. With the appearance of first leaf, seedlings were transferred to ceramic pots (23.2 cm diameter and 32.3 cm height) filled with 7 kg mixture of airdried local soil (clay-loam texture) and sand in the proportion of 2:1. Thirty replicates of three seedlings per pot were used for each stress and control treatment. Seedlings were kept in the open nursery area with mean monthly minimum and maximum temperature of 23.4-32.7 °C, respectively. Drought stress was imposed by watering while weighing method was used to control water potential to meet the experimental requirements. The gravimetric determination of water content by weighing soil samples before and after oven drying to constant weight at 80 °C was used to calibrate all measurement of the water potential of soil in pots. A preliminary experiment to select different stress levels showed that seedling failed to survive at water potential beyond -0.07 MPa. Therefore, different stress levels from water potentials -0.01 to -0.07 MPa were selected for the experiment. After determining the stress levels as -0.01, -0.02, -0.03, -0.04, -0.05, -0.06 and -0.07 MPa, soil water potential was maintained by manual watering and weighing individual pot. Different water potentials were achieved after 15 days of transplanting the seedlings to pots. Soil water potential was restored every day by watering plants daily in the morning for two months. Before the onset of the stress treatments, soil moisture was kept high by daily watering of all pots. Seedlings were fertilized by adding Hoagland nutrient solution to the tap water used for watering to each pot after every three days.

Pots were arranged in a completely randomized design and the position of the pots was changed weekly to avoid a position effect. For restoring soil water potential to the pots, water was added to the surface of the pots as well as to the bottom of the pots through previously inserted polytubes in growing medium. For destructive and non-destructive sampling pots were marked randomly within a treatment and samples were taken in triplicate at an interval of 30, 45, 60 and 75 days of plant growth. Plant parts (leaves and roots) were sampled for analysis of various parameters.

Superoxide Dismutase Assay: Plant sample (leaf and root; 0.5 g each) was homogenized with 3.0 ml of potassium phosphate buffer, centrifuged at 2,000 g for 10 min and the supernatant was used as enzyme source for the assay. The assay mixture contained 1.2 ml of 0.025 M sodium pyrophosphate buffer (pH 8.3), 0.1 ml of Phenazine methosulphate (PMS; 186 µM), 0.3 ml of Nitroblue tetrazolium (NBT; 300 µM), 0.2 ml of the enzyme preparation and water in a total volume of 2.8 ml. The reaction was initiated by the addition of 0.2 ml of nicotinamide adenine dinucleotide (780  $\mu$ M). The mixture was incubated at 30 °C for 90 sec and arrested by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was then shaken with 4.0 ml of n-butanol, allowed to stand for 10 min and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560 nm in a spectrophotometer (Systronics 2206). One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in 1 min.<sup>15</sup>

Catalase Assay: Plant sample (leaf and root; 0.5 g each) was homogenized with 10 ml of 50 mM phosphate buffer (pH 7.0). The homogenate was centrifuged at 15,000 rpm for 10 min at 4 °C and supernatant was used as enzyme source for the assay. The assay mixture in total volume of 3 ml contained 0.5 ml of 50 mM phosphate buffer (pH 7.0), 0.3 ml of 15 mM hydrogen peroxide  $(H_2O_2)$  and 0.1 ml of enzyme. The final volume was made up to 3 ml by adding distilled water. The reaction was started by adding enzyme and change in optical density was measured at 240 nm at 0 min and 3 min using a spectrophotometer (Systronics 2206). The enzyme solution containing H<sub>2</sub>O<sub>2</sub>-free phosphate buffer served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units per min. The molar extinction coefficient of  $H_2O_2$  at 240 nm was taken as 36  $\mu$ mol<sup>-1</sup> cm<sup>-1</sup>.<sup>16</sup>

**Peroxidase Assay:** Plant sample (leaf and root; 0.5 g each) was macerated with 25 ml of 0.1M phosphate buffer (pH 6.5). The homogenate was centrifuged at

5,000 rpm for 15 min and supernatant was used as enzyme source for the assay. To 3.0 ml of pyrogallol solution (0.05 M in 0.1 M phosphate buffer, pH 6.5) 0.1 ml of the enzyme extract and 0.5 ml of 1% hydrogen peroxide was added and mixed well. All procedures, stated above were carried out at 0-5 °C. Initial absorbance was read at 430 nm and then increase in the absorbance was noted at the interval of 30 sec up to 3 min using a spectrophotometer (Systronics 2206). One unit of peroxidase is defined as the change in absorbance/minute at 430 nm. <sup>17</sup>

**Statistical Analysis:** All data were analyzed by twoway analysis of variance (ANOVA) using Graph Pad Prism® 5.2. Means of all values were compared by Bonferroni posttests to detect significant difference between treatments. Least significant difference (LSD) was calculated and 'F' test was applied to assess the significance of data at 5% level of probability (p<0.05). Standard error was plotted in all the graphics.

## **RESULTS AND DISCUSSION:**

Superoxide Dismutase (SOD): The results revealed a gradual increase in SOD activity of both leaves and roots with an increase in drought stress level and period (Fig 1). The maximum increase in the SOD activity of leaf (136.74%) was observed at the 75<sup>th</sup> day of plant growth with water potential -0.07 MPa and minimum increase (3.56%) at the 45<sup>th</sup> day of plant growth with -0.01 MPa (p<0.05), which was 2.39 and 1.04 fold higher, respectively, compared with control plant. Severe drought stress levels induced a significant increase in SOD activity at all growth intervals. The minimum increase in SOD activity of root (6.85%) was observed at the 30<sup>th</sup> day of plant growth with lower stress level (-0.01 MPa) (p>0.05). However, with increase in the drought stress levels and periods, SOD activity enhanced and reached maximum (122.17%) at the 75<sup>th</sup> day of plant growth with imposition of severe drought stress (-0.07 MPa), it was 2.22 times higher with respect to control (p<0.05). No significant difference was observed between growth interval of  $60^{\text{th}}$  and  $75^{\text{th}}$  day at all stress levels (p>0.05).

In our results, it was observed that the activity of SOD enhanced both in the leaves and roots by increasing the level and duration of stress. The enhanced activity may indicate the increased quantity of  $O_2^-$  production and also reflect the possible role of SOD's dismutation effects on  $O_2^-$  and defense of the photosynthetic apparatus. An increased SOD activity by drought stress was considered important for drought tolerance as it antagonize harmful actions of superoxide radicals.<sup>18</sup> Increased activity of SOD under drought stress was reported in *Phaseolus mungo*<sup>19</sup> and *Brassica* napus.<sup>20</sup> Our results are also in agreement with Pan *et al*;<sup>21</sup> Yong *et al*<sup>22</sup> and Wang and Li,<sup>23</sup> who reported that over expression of SOD resulted in efficient stress protection against drought in *Glycyrrhiza uralensis; Radix astragali* and *Trifolium repens*, respectively.

Catalase (CAT): The CAT activity in leaves and roots was remarkably increased as a result of drought stress (Fig 2). The minimum increase in CAT activity in leaves (11.31%) was observed at the 30<sup>th</sup> day of plant growth with water potential -0.01 MPa, it was 1.11 fold higher compared to control (p>0.05). The results revealed maximum increase in CAT activity (219.69%) at the 75<sup>th</sup> day of plant growth with water potential -0.07 MPa. The significant difference (p<0.05) was observed between  $60^{th}$  and  $75^{th}$  day of growth period at all stress levels (except at -0.01 MPa). The minimum CAT activity in roots (10.17%) was recorded at the 45<sup>th</sup> day of plant growth with -0.01 MPa (p>0.05) and increased with an increase in the drought stress level and growth period of plant, the CAT activity reached maximum (174.30%) with water potential -0.07 MPa at the 75<sup>th</sup> day of plant growth in comparison to control (p<0.05). The minimum and maximum increase in CAT activity of root was 1.10 and 2.72 fold, respectively, compared to control plant. At moderate drought stress level (-0.03 MPa) a significant difference (p<0.05) was observed between all growth periods.

During drought stress, CAT activity increased in F. esculentum in order to defend cell damage by eliminating  $H_2O_2$ . Tohidi-Moghaddam *et al.*<sup>24</sup> reported that CAT activity increased under drought stress in Brassica napus. Mannivannan et al.25 reported that under drought stress Helianthus annuus showed a significant increase in CAT activity. These results are in agreement with our findings. The higher CAT activity indicated that under drought stress F. esculentum had better ability to scavenge H<sub>2</sub>O<sub>2</sub>. Similar results were reported by Ti-da et al.<sup>26</sup> in Zea mays. However, there are contradictory reports about defensive role of CAT in plants. A decline in CAT activity was reported in Glycyrrhiza uralensis<sup>21</sup>; Zea mays<sup>27</sup> and Cajanus  $cajan^{28}$  under drought stress. The reduction of CAT activity was due to the inhibition of enzyme synthesis, change in the assembly of enzyme subunits or protein degradation under drought stress.<sup>29</sup>

**Peroxidase (POD):** The leaf POD activity was higher to that in the root under drought stress treatments (Fig 3). The POD activity reached maximum (100.32%) at the  $45^{\text{th}}$  day of plant growth in leaves with water potential -0.07 MPa, it was 2.00 times higher than that in the control with a significant difference (p<0.05), and then decreased in the following growth stages ( $60^{\text{th}}$ 

and 75<sup>th</sup> day), however the POD activity was still higher than that in the control. The minimum POD activity (15.44%) was observed in leaves at the 75<sup>th</sup> day of plant growth with water potential -0.01 MPa, it was 1.76 times higher than that in the control (p<0.05). No significant difference (p>0.05) was observed between late stages of growth (60<sup>th</sup> and 75<sup>th</sup> day) at all stress levels (except at -0.01 MPa). This study showed that leaf POD activity of F. esculentum had different change patterns during different growth periods under drought stress. The POD activity in roots was observed significantly (p<0.05) lowest (13.15%) at the 30<sup>th</sup> day of plant growth under mild drought stress (-0.01 MPa), it was 1.17 fold higher than that in the control. As the level and duration of drought stress increased, the activity of POD in roots was enhanced (1.87 fold) and optimized (89.39%) at the 75<sup>th</sup> day of growth, when the plant was exposed to severe drought stress (-0.07 MPa) compared to control (p<0.05). A significant difference (p<0.05) was observed only between 30<sup>th</sup> and 75<sup>th</sup> day of plant growth period under moderate (-0.04 MPa) to severe (-0.07 MPa) drought stress.



Figure 1: Effect of different levels of drought stress on superoxide dismutase (SOD) content of leaf (A) and root (B) of *F. esculentum* over period of time (± S.E; n=9).



Figure 2: Effect of different levels of drought stress on catalase (CAT) content of leaf (A) and root (B) of *F. esculentum* over period of time (± S.E; n=9).



Figure 3: Effect of different levels of drought stress on peroxidase (POD) content of leaf (A) and root (B) of *F. esculentum* over period of time (± S.E; n=9).

The present study indicated that the activity of POD increased with the imposition of severe (-0.07 MPa) drought stress compared to control, to reduce cell membrane damage by scavenging of H<sub>2</sub>O<sub>2</sub> content produced by peroxidation of membrane lipids. PODs are engaged not only in scavenging H<sub>2</sub>O<sub>2</sub>, but also in development, plant growth, suberization, lignifications and cross-linking of cell wall compounds.<sup>30</sup>This could contribute to better drought survival of F. esculentum. Drought stress induced increase in POD activities was pronounced in drought tolerant Triticum aestivum cultivars.<sup>31</sup> The POD activity also increased in Juniperus oxycedrus;<sup>32</sup> Cajanus cajan<sup>28</sup> and Brassica napus<sup>20</sup> under drought stress. These findings demonstrated that POD activity rapidly increased in response to drought stress which is in agreement with our results of the present study.

**CONCLUSION:** Analysis of enzymatic antioxidants revealed a significant increase in the activity of superoxide dismutase, catalase and peroxidase under the influence of drought stress over period of time. An increase in enzymatic activity was observed, which indicate that *F. esculentum* has the ability to scavenge the reactive oxygen species (ROS) and can be grown successfully under drought stress conditions.

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