International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
www.chemijournal.com
IJCS 2021; 9(2): 29-52
© 2021 IJCS
Received: 10-01-2021
Accepted: 14-02-2021

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Assessment of biomolecules and enzymes during drought stress in *Echinochloa frumentacea* grown in Himalaya

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Abstract
The effect of drought stress in barnyard millets (*Echinochloa frumentacea)* varieties viz VL29, VL207, VL172 and VL181 was studied under *in vitro* environment in presence of PEG 6000 in liquid MS medium with different osmotic potential 5% w/v (-0.5MPa), 10% w/v (-0.15MPa), 15% w/v (-0.30MPa) and 20% w/v (-0.49MPa). Biochemical and enzymatic parameters were assessed in 12 days old seedling imposed to various concentration of PEG and evaluated every 24 hrs up to 7 days. Parameters of oxidative stress malondialdehyde (MDA), and hydrogen peroxide (H_2O_2) concentration, activities of stress responsive enzymes ascorbate peroxidase (APX), guaiacol peroxidase (GPX), catalase (CAT) and superoxide dismutase (SOD) and antioxidant molecules ascorbic acid (ASC) and proline were determined. Maximum response to drought stress was alleviated at 20% w/v (-0.49MPa) PEG as compared to that of control. However variety VL172 responded better among the other three varieties. The relative drought tolerant ability associated with variety VL172 due to high antioxidant enzyme activity, high proline and ascorbic acid content with low MDA content and H_2O_2 content. While variety VL29 was found to susceptible to drought stress. Due to high content of MDA and H_2O_2 and lower antioxidant activities was found in variety VL29. Data of this study clearly suggest the mechanism to limit the oxidative damaged which is important for drought tolerance for variety VL172 among the four varieties of barnyard millet.

Keywords: Barnyard millet, biochemical parameters, drought, enzymatic parameters, PEG

Introduction
Drought is an extended period where water availability falls below the statistical requirements for a region (Gupta *et al*. 2011) [35]. Drought stress is one of the major factors among the environmental stress which directly hampered plant normal growth and productivity. It has been reported that about 68% of the area in India is prone to drought and most of the areas are susceptible under persistent drought (Parida and Oinam 2015) [65]. Barnyard millet is mainly grown in India, China, Japan, and Korea for human consumption as well as fodder (Upadhyaya *et al*. 2014) [82]. The cultivation area of barnyard millet is decreasing day by day compare to major crops like rice, wheat, and maize. About 72% reduction in barnyard millet area was reported from 11 villages in Garhwal Himalayas (Maikhuri *et al*. 2001) [54].
Millet grains are rich in many nutrients, phytochemicals, and nonnutritive plant protective functional constituents (Rao *et al*. 2011; Saleh *et al*. 2013) [68, 72]. It has also reported thatbarnyard millet to be tolerant to drought and waterlogging (Zegada-Lizarazu and Iijima 2005) [91]. Drought stress alteredthe balance between antioxidant defenses and the amount of Reactive Oxygen Species (ROS) resulting in oxidative stress (Fathi and Tari 2016) [26]. Excessive ROS generated in plant cells tends to interact with different macromolecules resulting in oxidation of proteins, membrane lipids and nucleic acids and causes cellular damage, eventually affecting the growth and productivity of plants (Wang *et al*. 2003) [87]. Plants having some mechanism to cope out with drought condition to continued their normal growth conditions like accumulation of compatible solutes like amino acids, carbohydrates in plant cells that are known to play a role as security to protect cellular components (Ourcut and Nilsen 2000) [64].
Among osmolyte, proline is well known in plants which increasing in moderate or severe stress conditions, proline as a nitrogen storage reservoir or soluble which help in cytoplasmicosmotic potential decrease in acts of plant stresstolerance assists (Ghodsi *et al*.

1998) [31]. Among the plant antioxidants, L-ascorbate is a major antioxidant playing a vital role in the lessening of excessive ROS activity through enzymatic as well as nonenzymatic detoxification (Mittler 2002) [57]. Plants also try to combat from these ROS by enzymatically such as superoxide dismutase, SOD; catalase, CAT; guaiacol peroxidase, GPX; ascorbate peroxidase, APX which efficiently scavenge ROS and maintain their levels at non-damaging levels (Mittler 2002; Gill and Tuteja 2010; Anjum *et al*. 2012) [57, 33, 5]. The APX: SOD ratio plays a critical role in determining the level of oxidative stress tolerance in plants (Bhatt *et al*. 2011) [11]. A disturbance in the ROS/antioxidant homeostasis in any cell compartment leads to a situation called oxidative stress (Gill and Tuteja 2010) [33]. The peroxidation of membrane (phospho) lipids and the degradation/oxidation of proteins are among the most investigated consequences of ROS action on membrane structure and function (Blokhina *et al*. 2003; Davies 2005; Rinalducci *et al*. 2008; Foyer and Noctor 2009; Foyer and Shigeoka 2011) [12, 20, 69, 28, 29]. The overproduction of H_2O_2 has been observed in plants exposed to a number of stress conditions and is considered as one of the factors causing oxidative stress (Snyrychova *et al*. 2009) [79]. However the mechanism of drought resistance has not been studied in barnyard millet. In the present study physiological and biochemical basis of mechanism of drought tolerant in barnyard millet was evaluated in four different varieties of barnyard millet grown in Kumaun region of Himalaya viz. VL29, VL207, VL172 and VL181.

Materials and Methods

Plant culture and PEG treatment

The seeds of four varieties of barnyard millet **(***Echinochloa frumentacea) viz*. VL207, VL172, VL29 and VL181 were procured from ICAR Vivkananda Parvatiya Kirishi Anusandhan Sansthan (VPKAS), Almora, India. Seeds of Barnyard millet varieties (VL207, VL29, VL172 and VL181) were washed with distilled water followed by treatment of Tween-20 detergent for 20 min and thoroughly washed under running tap water for 20 min to remove all traces of detergent. The seeds were treated with 0.1% w/v bavastin for 1 min, 70% v/v ethanol for 2 min and 0.1% w/v $HgCl_2$ for 1 min. After each step seeds were washed with autoclave distilled water. These seeds were blot dried in filter paper and ten seeds per bottle were placed on liquid MS medium aseptically with the help of sterilized and kept in tissue culture chamber for 12 days at temp of 25±1°. Twelve days old seedlings were treated with different concentration of PEG 6000 i.e. 5% w/v (-0.5MPa), 10% w/v (-0.15MPa), 15% w/v (-0.30MPa) and 20% w/v (-0.49MPa) in liquid MS medium. Biochemical and enzymatic parameters wereassessed in 12 days old seedling was imposed to various concentration of PEG and evaluated every 24 h up to 7 days.

Proline

Free proline was determined by the method of Bates *et al*. (1973) [9]. Leaf, 0.2 g, was homogenized in sulfosalicylic acid (3% w/v in distilled water) in the ratio of 1:10 and the homogenate was centrifuged at 10,000 g for 20 min. at room temperature. Supernatant 2 mL was mixed with 2 mL of glacial acetic acid and 2 mL of acid ninhydrin reagent. The content was incubated for one hour at 100 °C in a water bath. The reaction was stopped by keeping the tubes in ice bath. 4 mL of toluene was added to each tube and the chromophore was extracted by vigorous stirring on a vortex mixer. The absorbance of chromophore containing toluene layer was measured at 520 nm. Concentration of proline in the sample was calculated in µg of free proline/ g fresh weight.

Hydrogen peroxide content

Hydrogen peroxide was measured spectrophotometrically by the method of Alexieva *et al*. (2001) [3]. 0.5 g of leaf was crushed in 4 mL of 0.1% (w/v) trichloroacetic acid (TCA) (1:10 ratio) and centrifuged at 10,000 g for 30 min. at 4°C. In a reaction mixture 0.5mL of supernatant, 0.5 mL of 0.1M potassium phosphate buffer (pH 7.4) and 2 mL of 1M KI reagent was taken. The blank consisted of 0.1% (w/v) TCA in place of leaf extract. The tubes were placed for 1 h in dark and absorbance was measured at 390 nm. The amount of H_2O_2 was calculated using standard curve prepared with a working standard of 100µM of H_2O_2.

Estimation of total, reduced and oxidised ascorbate content

Ascorbate content was assessed in acidic solution by the method of Law *et al*. (1983) [51]. 0.2 g of fresh leaf tissue was homogenized in 2 mL of 5% metaphosphoric acid (1:10) and centrifuged at 20,000 g for 15 min. In a reaction mixture 0.2 mL of tissue extract, 0.5 mL of 150 mM sodium phosphate buffer (pH 7.4) containing 5mM EDTA and 0.1 mL of 10mM dithiothreitol was added. The mixture was kept for incubation at room temperature for 10 min and 0.1mL of 0.5% N-ethylmeleimide was added to remove DTT. A Color was developed by adding 0.4 mL 10% TCA, 0.4 mL 44% orthophosphoric acid, 0.4 mL 4% alpha dipyridyl in 70% ethanol and 0.2 mL 3% ferric chloride and kept for incubation at 40°C for 40 min. While for the estimation of reduced ascorbate, DTT and N-ethylmeleimide was not added to the reaction mixture, instead 0.2 mL of water added to the reaction mixture. The absorbance was taken at 525 nm. oxidized ascorbate was calculated by taking the difference between the total and reduced ascorbate. The amount of total ascorbate and reduced ascorbate in all the sample was computed in per gram fresh weight from the standard curve of total ascorbate and reduced ascorbate.

Malondialdehyde content

Malondialdehyde content was assessed by the standard method Heath and Packer (1968) [38]. 0.2 g of leaf material was homogenized in 0.25% (w/v) 2-thiobarbituric acid (TBA) prepared in 10% (w/v) TCA, in the ratio of 1:10. The homogenate was incubated at 95 °C for 30 min. in a water bath and centrifuged at 12,000 g for 30 min. The clear supernatant was collected and allowed to cool at room temperature. The absorbance was measured at 532 nm and 600 nm respectively. Absorbance at 600 nm was subtracted from the absorbance at 532 nm for non-specific absorbance. The concentration of MDA was calculated by using an extinction coefficient of $155 mM^{-1} cm^{-1}$.

Preparation of extracts for Enzymatic analysis

For determination of antioxidant enzyme activities, 0.5 g of leaf was homogenized in 10 mL, 50 mM Sodium phosphate buffer pH 7.0 in a pre-chilled mortar and pestle using liquid nitrogen. The homogenate was filtered through four layers of cheesecloth and centrifuged at 20,000 g for 20 min. at 4 °C. The supernatant was re-centrifuged again at 20,000 g for 20 min. at 4 °C. Concentration of protein in extract was determined by Bradford (1976) [14].

Ascorbate peroxidase activity (APX)

Ascorbate peroxidase specific activity was assayed according to Nakano and Asada (1981) [63]. The assay mixture consisted of 50 µL of the enzyme extract in 50 mM phosphate buffer (pH 6.0), 0.1 µM EDTA, 0.5 mM ascorbate and 1 mM H_2O_2 in a total volume of 1.5 mL. The decrease in absorbance was measured at 290 nm for 3 min. at an interval of 5s. The difference in absorbance (ΔA_{290}) was dividing by the ascorbate molar extinction coefficient (2.8 $mM^{-1}cm^{-1}$) and the enzyme activity expressed as µM of H_2O_2 min^{-1} mg^{-1} protein, taking into consideration that 1M of ascorbate is required for the reduction of 1M of H_2O_2 (McKersie and Ya'acov 1994) [56].

Guaiacol peroxidase activity (GPX)

Guaiacol peroxidase activity was assessed by the procedure of Urbanek *et al*. (1991) [83]. 1 g of leaf material was homogenized in 5 mL of homogenization buffer (100 mM Phosphate buffer (pH 7.0) containing 0.1 mM EDTA) and centrifuged at 12,000 g for 30 min. at 4°C. Total reaction mixture volume was 2mL which contains assay buffer 100 mM phosphate buffer (pH 7.0) and 0.1 µM EDTA and 50 µL of enzyme extract were added. Reaction was started by adding 50 µL of guaiacol (5 mM) and 50 µL of H_2O_2 (15 mM) and the increase in absorbance was recorded at 470 nm for 3 min. at an interval of 5s. The specific activity of guaiacol peroxidase was expressed in µmol/ min/mg protein using molar extinction coefficient 26.6 mM^{-1} cm^{-1}.

Catalase Activity (CAT)

Specific catalase (CAT, EC 1.11.1.6) activity was measured according to Beers and Sizer (1952) [10], with minor modifications. The assay mixture consisted of 50 µL of the enzyme, 100 mM phosphate buffer (pH 7.0), 0.1 µM EDTA, and 20 mM H_2O_2 in a total volume of 1.5 mL. The decrease of H_2O_2 was monitored by reading the absorbance at 240 nm at the moment of H_2O_2 addition and 1 min. later. The difference in absorbance (ΔA_{240}) was divided by the H_2O_2 molar extinction coefficient ($36M^{-1}cm^{-1}$) and the enzyme activity expressed as µmol of H_2O_2 min^{-1} mg^{-1} protein.

Superoxide dismutase activity (SOD)

Specific activity of superoxide dismutase was determined by measured as described by (Giannopolitis and Ries (1977) [32]. The assay mixture consisted of 50 µL of the enzyme extract, 50 mM phosphate buffer (pH 7.4), 0.1 µM EDTA, 13 mM methionine, 75 µM nitroblue tetrazolium and 2 µM riboflavin in a total volume of 1.5 mL. Riboflavin was added at the end and the tubes were shaken and placed under fluorescent lighting from two 20 W tubes. The reaction was allowed to proceed for 15 min. under dark conditions. Absorbance of the reaction mixture was measured at 560 nm and one unit of SOD activity (U) was defined as the amount of enzyme required to cause 50% inhibition of the nitroblue tetrazolium photoreduction rate. The results were expressed as $unit^{-1}$ min^{-1} mg^{-1} protein.

Stastical analysis

All analyses were performed in triplicate. Data presented are means ± SE of three independent experiments. Data subjected to analysis of variance (ANOVA), using SPSS16 (Stastical Package for the social science). Mean were separated by the Duncan when analysis of variance (ANOVA) was significant ($P< 0.05$).

Results

Proline accumulation

Proline accumulated in all the four varieties of barnyard millet from 5% to 20% PEG (Figure 1 a, b, c and d). Proline gets accumulated in linear order as the level of drought stress increased through different percent of PEG. Maximum proline content reached at 20% PEG in all the four varieties. However maximum content of proline was observed in variety VL172 (401.13%) and minimum was observed in variety VL29 (177.26%). In other variety VL207 and VL181 proline content was 295.54% and 180.43% respectively compared to control in 20% PEG.

Hydrogen peroxide content

Hydrogen peroxide content was significantly increased in all the varieties of barnyard millet as the percent of PEG concentration was increased from 5% to 20% (Figure 2a, b, c and d). In variety VL29 hydrogen peroxide content increased to 94.43% compared to control in leaves of seedling grown in MS medium containing 20% PEG containing. It was followed by variety VL181 (70.2%), VL207 (68.04%) and minimum was observed in variety VL172 (60%) hydrogen peroxide compared to control in 20% PEG.

Total, reduced and oxidized ascorbic acid content

Total ascorbic acid content was increased as the percent PEG increases from 5% to 20% in all the varieties of barnyard millet (Figure 3a, b, c and d). Maximum increased in total ascorbic acid content was observed in variety VL172 (13.7%) compared to control in presence of PEG 20% and minimum in variety VL29 (10.5%). However in variety VL207 increased ascorbic acid content was 11.5% compared to control and 10.6% in variety VL181 compared to control in presence of 20% PEG.

Reduced ascorbic acid content was also increased in linear way as the level of PEG was increased from 5% to 20% (Figure 4a, b, c and d). Maximum 21% reduced ascorbic acid was observed in variety VL172 and minimum 14.4% reduced ascorbic acid was observed in variety VL29 compared to control in presence of 20% PEG containing. However in variety VL207 reduced ascorbic acid was 20% and in variety VL181 reduced ascorbic acid was 19.8% compared to control in presence of 20% PEG.

Oxidized ascorbic acid was also increased linear way as the level of PEG was increased from 5% to 20% (Figure 5a, b, c and d). Minimum 1.8% increase oxidised ascorbic acid was observed in Variety VL172 and maximum increased 5% was observed in variety VL29 compare to control in presence of 20% PEG. In variety VL207 4.8% increased oxidized ascorbic acid was found compared to control in 20% PEG.

Ratio of reduced to oxidised ascorbic acid (ASC/DHA) was observed maximum percent increase in variety VL172 (18.82%) compared to control on the maximum day of stress (7^{th}day) at 20% PEG. However minimum percent increase was found in variety VL29 (14.4%). In others two varieties it was VL207 (14.87%) and VL181 (14.09%) compared to their respective controls (Table 1).

Malondialdehyde content

Malondialdehyde is a marker of lipid peroxidation which was increases as the level of PEG increased from 5% to 20% in all the varieties of barnyard millet (Figure. 6a, b, c and d). The levels of MDA content reached the highest values in the treatment of PEG 20%. The levels MD Awas higher in all the

stressed plants compared to control. In variety VL29 during unstressed condition maximum content of MDA was found among the others varieties. Minimum content of MDA was found in variety VL172. While as the seedlings were subjected to drought stress the maximum MDA content percent increased (45.42%) was found in variety VL29 compared to control. However minimum (29.04%) increased was found in variety VL172 compared to control. In the variety VL207 MDA content was 34.06% increased and in variety VL181 37.63% increase compared to control.

Ascorbate peroxidase activity (APX)

Ascorbate peroxidase activity (APX) was increased in all varieties of barnyard millet (Figure. 7a, b, c and d). Highest activity of APX was observed in variety VL172 (61.3%) and minimum activity of APX was observed in variety VL29 (19.3%) compared to control in 20% PEG concentration. However in variety VL207 (36.6%) increased APX activity was observed. APX content was 33.2% in variety VL181 compared to control in 20% PEG.

Guaiacol peroxidase activity (GPX)

GPX was increased in all the four varieties of barnyard millet as shown in (Figure. 8a, b, c and d). Maximum GPX activity was observed in variety VL172 (85.42%) and lowest was observed in VL29 (49.52%) compared to control in presence of 20% PEG concentration. However in variety VL207 and VL181 GPX activity was increased to 74.84% and in variety VL181 to 71.40% respectively compared to control in 20% PEG.

Catalase activity (CAT)

CAT was increased in all the four varieties of barnyard millet as shown in (Figure. 9a, b, c and d). Experimental finding indicated that highest activity of CAT was observed in variety VL172 (36.01%) and lowest CAT activity was observed in variety VL29 (13.81%) compared to control in 20% PEG. However in variety VL207 and VL181 the CAT activity was increased 31.06% and 26.29% respectively compared to control in presence of 20% PEG.

Superoxide dismutase activity (SOD)

SOD was increased in all the four varieties of barnyard millet as shown in (Figure. 10a, b, c and d). Experimental finding indicated that highest activity of SOD was observed in variety VL172 (51.98%) and lowest SOD activity was observed in variety VL29 (34. 80%) compared to control in 20% PEG. However in variety VL207 and VL181 the SOD activity was increased 41.49% and 35.32% respectively compared to control in presence of 20% PEG.

APX ratio SOD

At 20% PEG concentration on 7th day of stress the maximum APX/SOD ratio was observed in variety VL172 to 2.12. Minimum and approximately same ratio of APX/SOD was observed in both the variety VL29 and VL181 to 1.34. However in variety VL207 ratio of APX/SOD was observed to 1.64 at 20% PEG concentration on 7th day of stress.

Discussion

Proline is an osmolyte or an osmoprotectant accumulated in response to drought, salt, and extremes temperature (Csonka 1989) [19]. Proline accumulation in the cell suggested its active involvement in the scavenging of free radicals by reducing damage caused by various kinds of oxidative stress (Vendruscolo *et al*. 2007; Tatar and Gevrek 2008) [84, 80]. Accumulation of free proline is a typical response to drought stress (Kotapati *et al*. 2016) [50]. It had been reported that proline get accumulated in pearl millet on increasing NaCl concentration (Sneha *et al*. 2013) [78], Finger millet (Satish *et al*. 2016) [73] and in as anti-drought defense protein under drought condition (Hong-Bo *et al*. 2006; Islam *et al*. 2015) [41, 42]. The similar results specified that proline was found to increase in all the four varieties of barnyard millet during PEG mediated stress. However, in variety VL172, it increases 1.9 fold from 5% to 20% PEG on 7th day of observation among the others varieties (Figure 1).

H_2O_2 is generated in the cells under normal as well as wide range of stressful conditions (Sharma *et al*. 2012). H_2O_2 is moderately reactive and is relatively long-lived molecule with a half-life of 1ms (Mittler and Zilinskas 1991)**.** Increased production of H_2O_2 is a commonly observed feature of plant in stress response (Chakraborty and Pradhan 2012). Hydrogen peroxide accumulation during stress greatly depends on the balance between H_2O_2 production and H_2O_2 scavenging (Mittler *et al*. 2004). H_2O_2 can be injurious for the cells when present in excess (Halliwell and Gutteridge 1999a) [37]. Drought induced H_2O_2 accumulation correlated with decreases in soil water content in wheat (Luna *et al*. 2004). The accumulation of H_2O_2 and lipid peroxidation increased with increase in the days of stress was observed in the leaf of wheat (Chakraborty and Pradhan 2012). Accumulation of H_2O_2 was also found to increase in all the four varieties of barnyard millet as the level of drought stress increased from 5% to 20% through PEG. In our study variety VL172 found to be less accumulated with H_2O_2 content (60%) compared to control in 20% PEG containing MS medium among the other varieties (Figure 2). It indicates that VL172 had better ROS scavenging ability among the other three varieties.

MDA content, which is the final product of lipid peroxidation and it, is a well-known marker of oxidative damage (Moller *et al*. 2007). Its less content is commonly considered as one of the best physiological components of drought tolerance in plants (Xu *et al*. 2008). The MDA content was found to increase in durum wheat following a period of water shortage (Ahmadizadeh *et al*. 2011). MDA significantly increased after 30 days of drought stress in two oranamental shrubs *Eugenia* and *Photinia* (Toscano *et al*. 2016). The level of MDA content was found to high in sensitive genotype of maize compared to tolerant, the unchanged level of lipid peroxidation seem to a characteristic of tolerant plant which are better equipped with better free radical quenching system than sensitive genotypes that offered protection against oxidative stress (Chugh *et al*. 2011). Our result indicates that increased in lipid peroxidation rate on drought encounter but the variety VL172 showed better adaptability under drought stress as the rate of change in MDA content was lower among the others varieties. And this variety VL172 also had low content of H_2O_2, which is main cause of membrane damaged factors. This indicated that variety VL172 maintained better correlation against oxidative stress by enzymatic and non-enzymatic factors. Positive correlation (r = 0.872) was observed in between hydrogen peroxide (H_2O_2) content and in Malondialdehyde (MDA) (Figure 11a). Where H_2O_2 was independent variable and MDA was dependent variable. Positive correlation (r = 0.923) was observed in between (MDA) and in electrolyte leakage (Figure 11b). This showed significant correlation as r-value near to 1.0 showed a perfect positive correlation. As the high level MDA causes lipid peroxidation responsible for damaged to the membrane

resulted in electrolyte leakage. MDA was independent variable and electrolyte leakage was dependent variable.

L-Ascorbate is a major antioxidant play a vital role in the mitigation of excessive ROS activity through enzymatic as well as non-enzymatic detoxification and as a result, monodehydroascorbate (MDHA) and dehydroascorbate (DHA) accumulate in the cells (Mittler 2002) [57]. The ascorbic acid (ASA) pool size is dependent, on both the rate of synthesis and the rate of reduction of MDHA and DHA back to ascorbate. MDHA and DHA produced as a result of activities of ascorbate peroxidase (APX) and ascorbic acid oxidase (AAO), respectively, should be efficiently recycled to maintain the reduced pool of ASA (Venkatesh and Park 2014) [85]. The antioxidant activity of ASA is associated with resistance to oxidative stress and longevity in plants. In peroxisomes, H_2O_2 can be destroyed either by CAT or APX. However, APX has a high affinity for H_2O_2 but it requires a reducing substrate; ascorbate (Khan *et al*. 2011) [48]. Ascorbic acid is used as a reference compound in a large number of studies associated with stress tolerance in plants (Lopez-Munguia *et al*. 2011) [52]. A remarkable increase in ascorbic acid levels indicated the induction of an antioxidant mechanism such as glutathione ascorbate cycle, as reported for a number of plants (Koca *et al*. 2007) [49]. On encountering stress condition there is gradual decrease of ascorbic acid content could be due to its participation in reduction of H_2O_2 through the increased APX activity and there by increased synthesis of reduced form could be maintained by the monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) in tolerant plant (Amor *et al*. 2005) [4]. Maximum increased percent of reduced ascorbic acid content was observed in variety VL172 (21%) and minimum in variety VL29 (14.4%) compared to their respective control in our study (Figure 4). The change in the ASC/DHA ratio, an important indicator of the redox status of the cell, is one of the first signs of oxidative stress (Foyer *et al*. 2005). The ratio of reduced to oxidized (ASC/DHA) where maximum increased percent was found in variety VL172 (18.82%) and minimum in variety VL29 (9.41%) on the 7th day of stress at 20% PEG (Table 1). Similar kind of results was studied in cotton where the salt-tolerant cultivars of cotton also had a higher ASC/DHA ratio than the salt-sensitive lines under salt conditions (Ashraf and Harris 2004) [6].

Ascorbate peroxidase (APX, EC 1.1.11.1) is a central component of ascorbate-glutathione (AsA-GSH) cycle, and plays an essential role in the control of intracellular ROS levels. APX uses two molecules of ascorbic acid (ASA) to reduce H_2O_2 to water with a concomitant generation of two molecules of monodehydroascorbate (MDHA) (Welinder 1992) [88]. APX isoenzymes are labile in the absence of ASA. It was observed from many works on drought stress that APX is an antioxidant enzyme that plays a key role in drought stress responses (Faize *et al*. 2011; Mittler and Zilinskas 1994; Fini *et al*. 2012) [25, 60, 27]. APX activity was found to increase with the level of increasing drought stress and reached maximum under severe drought condition (Bai *et al*. 2009) [7], similar finding is observed in the present work. Similar result was observed in another experiment on genotype of *Vicia sativa* L. under stress condition the activity of APX was found to increased (Abbasi *et al*. 2014) [1]. Our results indicated maximum increased percent in APX activity in variety VL172 and variety VL29 had minimum APX activity, which also had maximum percent increased ratio of reduced ascorbic acid to oxidized ascorbic acid among the others three varieties. This indicated that variety VL172 maintained regular availability of ascorbic acid for enzymes APX which used it as substrate for the maintenance of redox state. The APX activity was found to also increase in all the four varieties with increased in percent PEG. The tolerance level in among the variety was VL172> VL207> VL181> VL29. Our results was supported by similar kinds of finding in others as it found out that lower levels of lipid peroxidation are associated with higher APX activity in drought- or salt tolerant tomato (Shalata and Tal 1998) [75], sugar beet (Bor *et al*. 2003) [13] and rice (Demiral and Turkan 2005) [22] plants. Water deficit stress led to the upregulation of APX activity in the endosperms of wheat (Devi *et al*. 2010) [23]. Highly Positive correlation (r = 1.000) was observed in between Ascorbic acid and in APX (Figure 11c). This showed significant correlation as r-value near to 1.0 showed a perfect positive correlation. Ascorbic acid is substrate for APX enzyme for the completion of dismutation reaction of H_2O_2. Ascorbic acid was independent variable and APX was dependent variable.

Guaiacol peroxidase (GPX) is a heme-containing protein (EC 1.11.1.7). Like all peroxidases, they mediate the one electron oxidation of organic compounds that is reduced glutathione (GSH) with a concomitant reduction of H_2O_2 and lipid hydroperoxides. GPXs are widely accepted as a stress ''enzyme'' (Sharma *et al*. 2012) [77]. Their role in detoxifying lipid hydroperoxides and other reactive molecules has been shown in different species and under several stress conditions (Roxas *et al*. 1997; Csiszár *et al*. 2004; Basantani and Srivastava 2007; Edwards and Dixon 2009) [71, 18, 8, 24]. In our result it was found that maximum percent increased activity was observed in variety VL172 and minimum was in variety VL29 compared to control. However GPX activity increased linearly in all the four varieties on increasing drought stress and reached maximum on the 7th day of stress, this showed the crucial role of GPX in circumventing drought stress in barnyard millet. The tolerance level in among the variety was VL172> VL207> VL181> VL29. In another study four caprifig varieties were studied for their GPX activity under drought stress and the tolerant genotype showed highest activity of same enzyme among all the varieties under stress (Rostami and Rahemi 2013) [70]. In sunflower plants water deficit condition significantly increased the activity of GPXs as compared with fully-irrigated control plants (Pourtaghi *et al*. 2011) [67]. Other research suggests that drought increases enzymatic GPXs activity in leaves of sugar beet (*Beta vulgaris* L.) genotypes (Sayfzadeh and Rashidi 2011) [74]. Increased GPX activity under drought stress (Zhang *et al*. 1995) [92] and saltstress (Gondim *et al*. 2012) [34] has also been reported in maize plant**.** In finger millet varieties during the drought stress study, the activity of GPX was found to increased, however the activity of GPX come to less than APX which showed major role of APX in decomposition of H_2O_2 in response to ROS (Bhatt *et al*. 2011) [11].

CAT plays an important role in removing of H_2O_2 which generated in peroxisomes by fatty acids oxidation and photorespiration. Catalase does not require a reducing substance for its action. CAT has one of the highest turnover rates for all but a much lower affinity for H_2O_2 than APX. CAT isozymes extensively studied in higher plants (Polidoros and Scandalios 1999) [66]. It is important to note that CAT is highly sensitive to light. This may be a result of light absorption by the heme group or perhaps of H_2O_2 inactivation. Various stress conditions that reduce the rate of protein turnover, such as salinity, drought, and heavy metals,

reduce CAT activity which could be compensated by increasing the activity of APX or SOD enzymes by the plants (Karuppanapandian *et al*. 2006a, c; Karuppanapandian and Manoharan 2008; Hojati *et al*. 2010) [45, 46, 44, 40]. In the seedling leaves of wheat, the CAT can efficiently break down high concentrations of H_2O_2 and reduce the damage of $^{\cdot}OH$ produced by H_2O_2. The level of hydrogen peroxide is controlled by CAT in plant cells (Weng *et al*. 2015) [89]. A similar finding concluded that variety VL172 had maximum percent increase in CAT activity and minimum was observed in variety VL29. The CAT played an important role in elimination of ROS in variety VL172. The CAT activity was also increases with increased percent of PEG in all the four varieties. The tolerance level in among the variety was VL172> VL207> VL181> VL29. Our result is supported by many others studies. Antioxidant enzyme activities in pearl millet under salt stress showed that remarkably enhanced CAT antioxidant enzyme activity at both vegetative and reproductive stages (Heidari and Jamshidi 2011) [39]. From the study of drought tolerant genotypes of wheat it was found that maximum activity of catalase (CAT) which was upregulated by more than 50% in the roots of water-stressed seedlings of drought tolerant genotype comparison with non-stressed seedlings (Devi *et al*. 2010) [23]. CAT activity showed maximum activity at –0.4 MPa osmotic potential in melon seedlings cultivars which exposed to drought stress levels (Kavas *et al*. 2013) [47].

The Superoxide dismutase (SOD) (EC 1.15.1.1) belong to metalloenzymes family which catalyzes the disproportionation of $O_2^{\cdot-}$ into H_2O_2 and O_2 and is present in all aerobic organisms and subcellular components like chloroplast, mitochondria, cytosol and peroxisome which are susceptible to oxidative stress (del Rio *et al*. 1996; Halliwell and Gutteridge 2000; Moussa and Abdel-Aziz 2008; Chen *et al*. 2010) [21, 36, 62, 16]. SOD acts as the first line of defense converting O_2^- to H_2O_2, followed by detoxification of H_2O_2 by APX, GPX and CAT (Mittler 2002) [57]. In durum wheat (*T. durum*) leaves after *in vivo* treatment against drought stress on the 37 genotypes, showed that the genotype which showed resistant property against drought conditions having higher activity of SOD (Ahmadizadeh *et al*. 2011) [2]. Increased activities of SOD have been correlated with drought in maize (Malan *et al*. 1990), *O. sativa* (Sharma and Dubey 2005) [76], *P. vulgaris* (Zlatev *et al*. 2006) and *Alternanthera philoxeroides* (Wang *et al*. 2008) [86]. A significant increase in superoxide dismutase specific activity was recorded under stress, in all the five finger millet varieties tested (Bhatt *et al*. 2011) [11]. SOD activity increased in stress condition there by help to reduce superoxide radicals produced under oxidative stress. But H_2O_2 is a by-product of SOD activity which can easily penetrate the biological membrane as it exists in a neutral form (pKa=11.8) at physiological pH (Joshi *et al*. 2011). Therefore SOD activity could help in relief from stress condition only there by increasing the activity of other H_2O_2 metabolizing enzymes like APX or GPX (Sayfzadeh and Rashidi 2011) [74]. A similar result was also reported in our study on barnyard millet where SOD activity was found maximum percent increased in variety VL172 (51.98%) and minimum percent increased in variety VL29 (34.80%) compared to their respective control on 7th day of stress (Figure 10). SOD activity was increased linear way in all the four varieties on increasing percent of PEG. However, the tolerance level in among the variety was VL172> VL207> VL181> VL29.

The ratio of APX: SOD activity is of critical importance in determining the stress tolerance potential than the absolute value of SOD or APX specific activities (Bhatt *et al*. 2011) [11]. In our finding at 20% PEG concentration on 7th day of stress the maximum APX/SOD ratio was observed in variety VL172 to 2.12. Minimum and approximately same ratio of APX/SOD was observed in both varieties VL29 and VL181 to 1.34. However in variety VL207 ratio of APX/SOD was observed to 1.64 at 20% PEG concentration on 7th day of stress. In three varieties of Brassica juncea, study *in vitro* effect of salt stress showed resistant variety had maximum ratio of APX/SOD (Joshi *et al*. 2011). Among the enzymes, the activity of APX was found to be maximum in barnyard millet variety VL172 and APX is the main enzymes of ascada-helliwell pathway and considered for the detoxification of H_2O_2. After APX the SOD was found to maximum activity which acts as main line of defense against ROS.

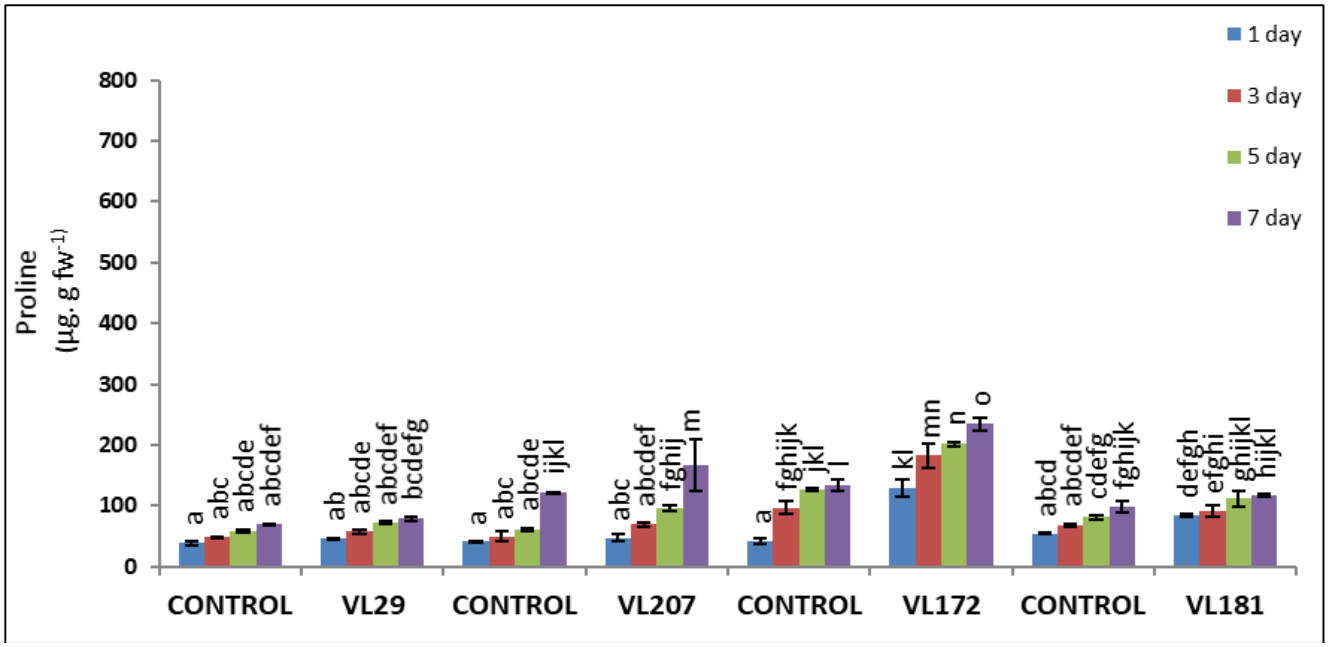

Fig. 1a

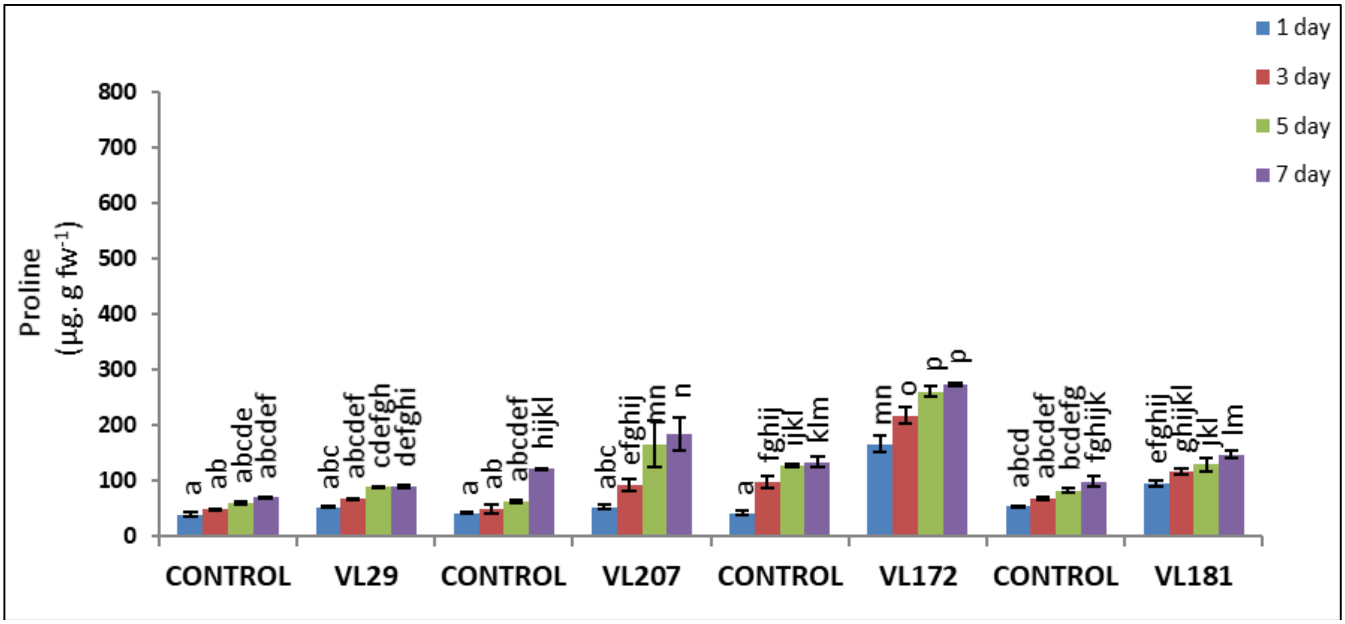

Fig. 1b

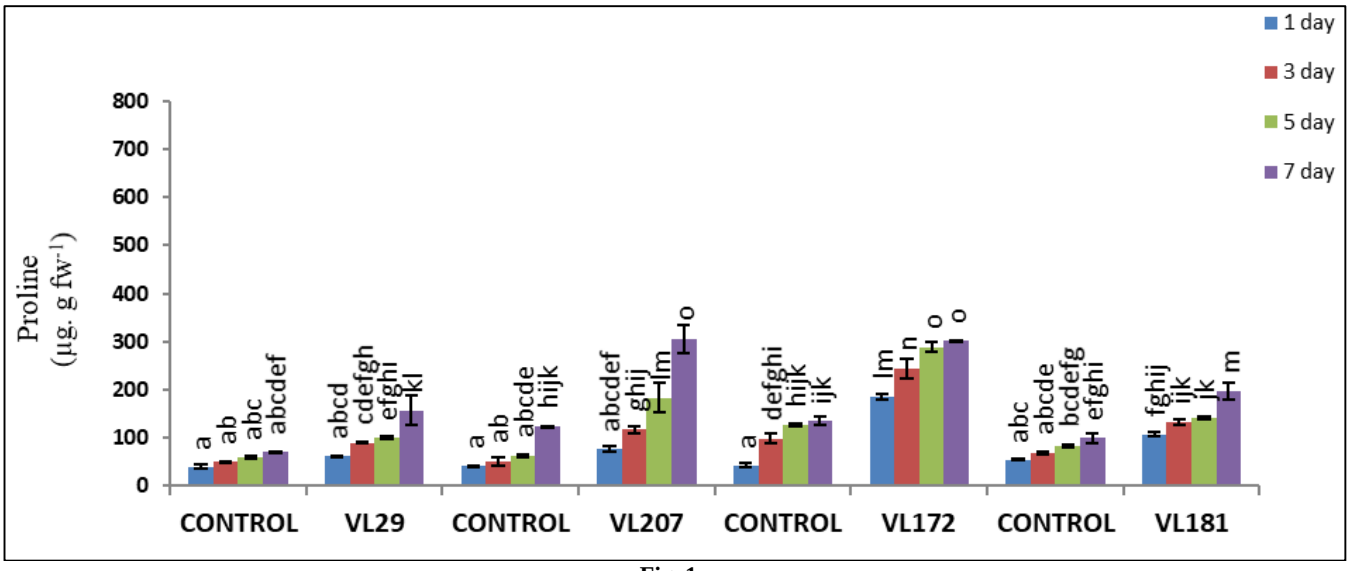

Fig. 1c

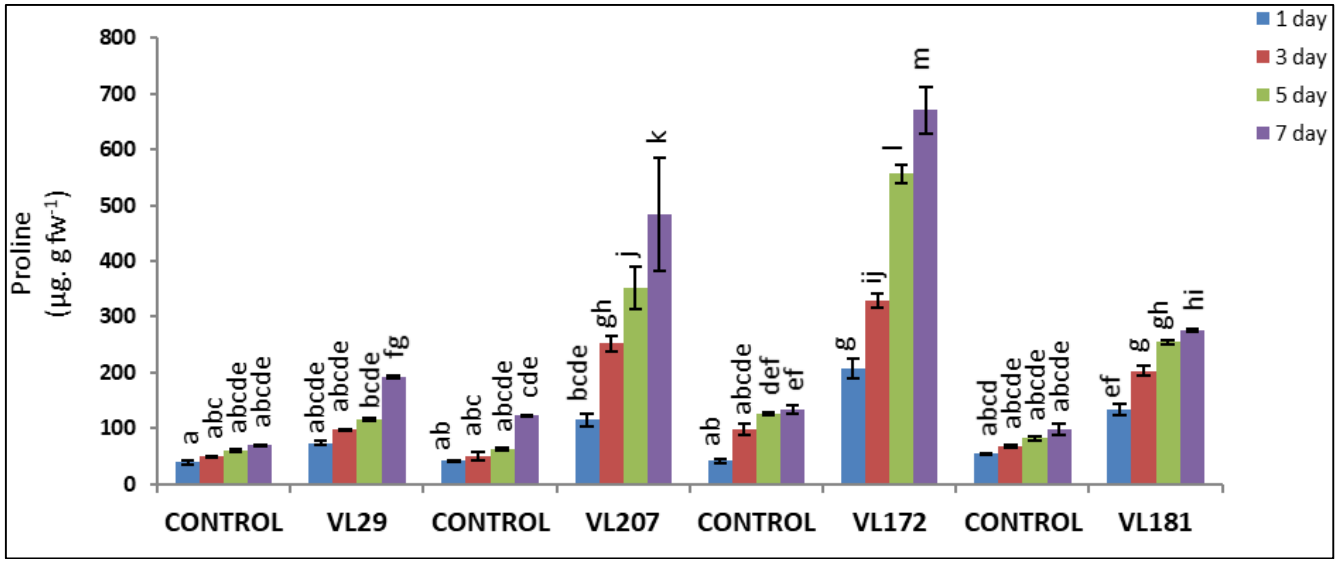

Fig. 1d

Fig 1: Effect of progressive drought stress by different concentration of PEG (%) on proline in four varieties of barnyard millet (value represent mean ± SE (n=3)). (a) 5% PEG, (b) 10% PEG, (c) 15% PEG, (d) 20% PEG. Different letters denotes significant differences ($P< 0.05$) among four varieties in control and drought stressed plants. Line above bars represents mean ±standard error.

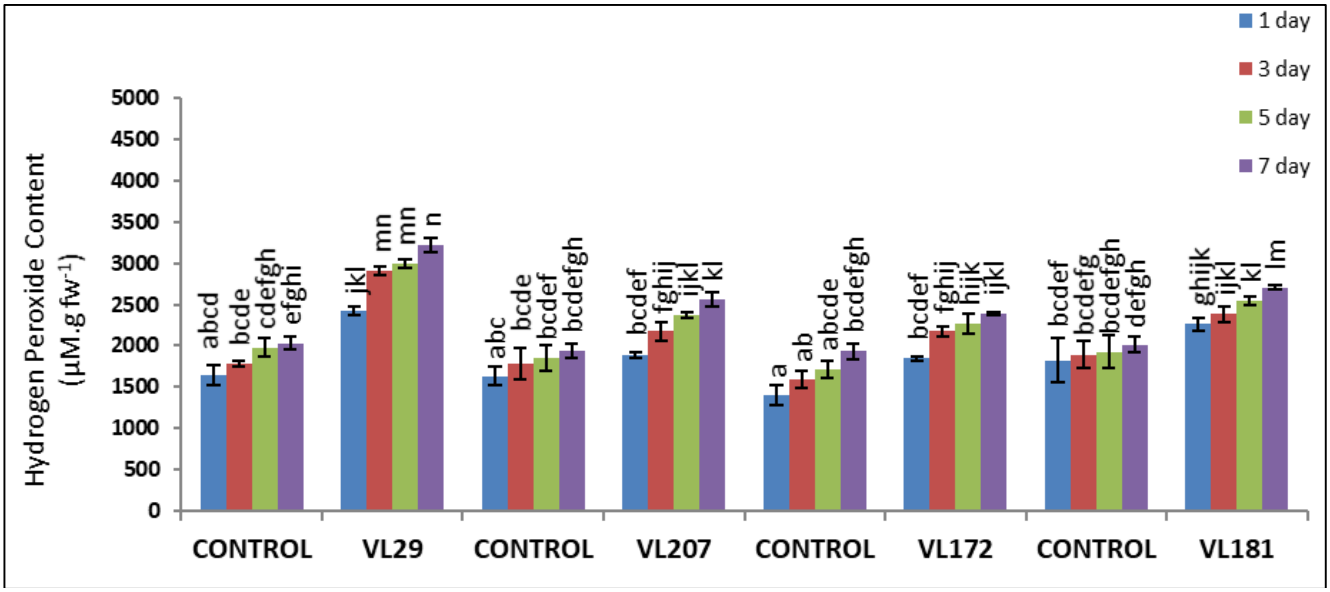

Fig. 2a

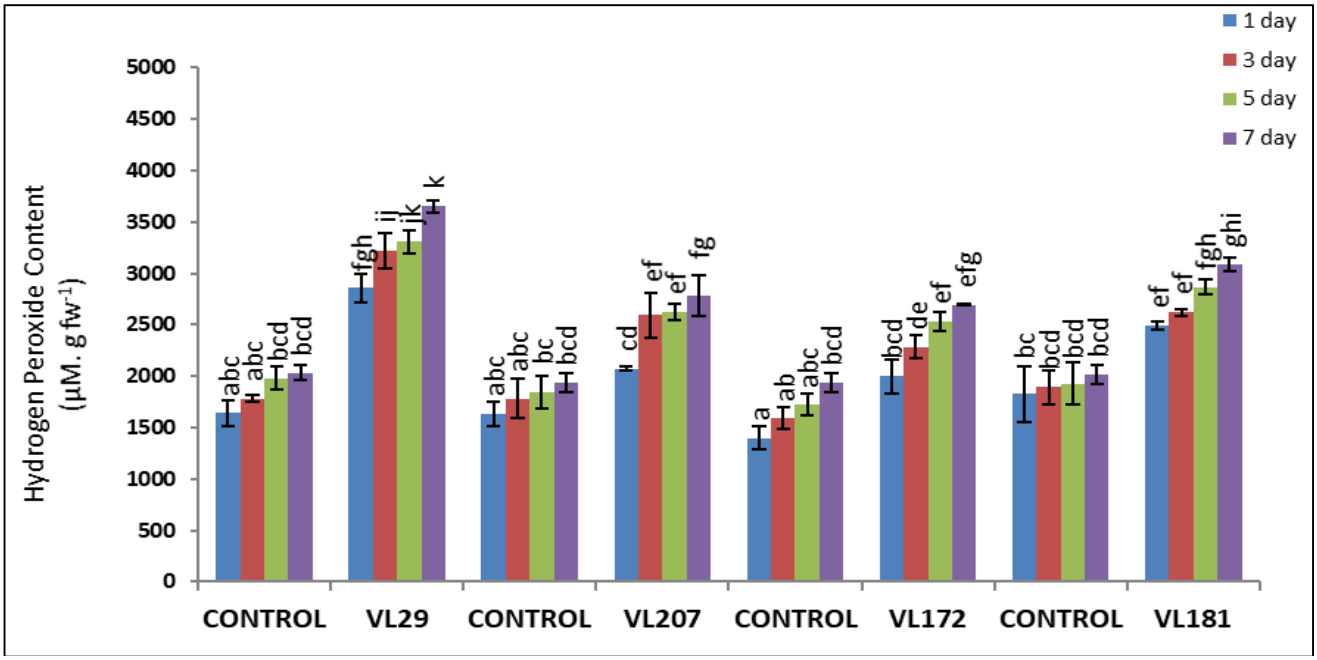

Fig. 2b

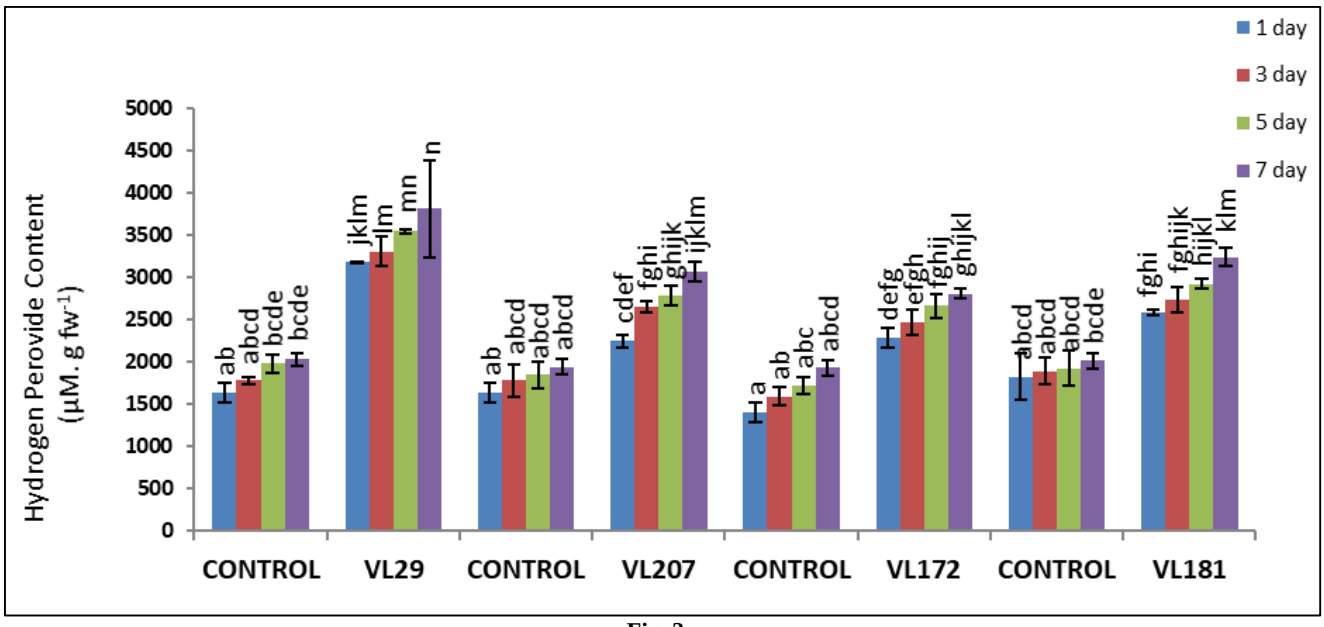

Fig. 2c

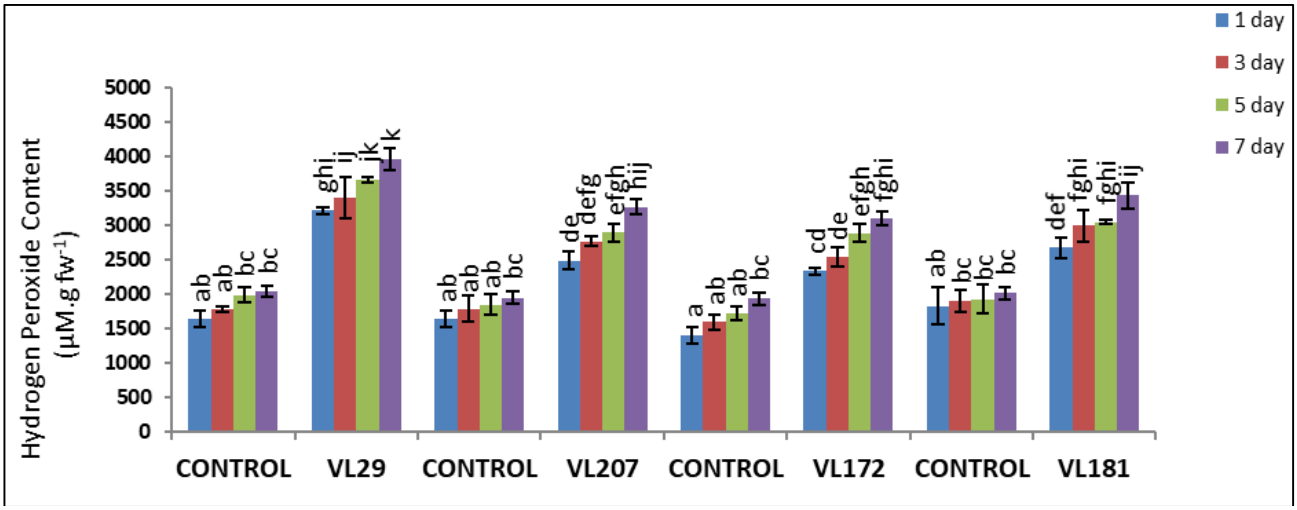

Fig. 2d

Fig 2: Effect of progressive drought stress by different concentration of PEG (%) on Hydrogen peroxide in four varieties of barnyard millet (value represent mean ± SE (n=3)). (a) 5% PEG, (b) 10% PEG, (c) 15% PEG, (d) 20% PEG. Different letters denotes significant differences ($P<$ 0.05) among four varieties in control and drought stressed plants. Line above bars represents mean ±standard error.

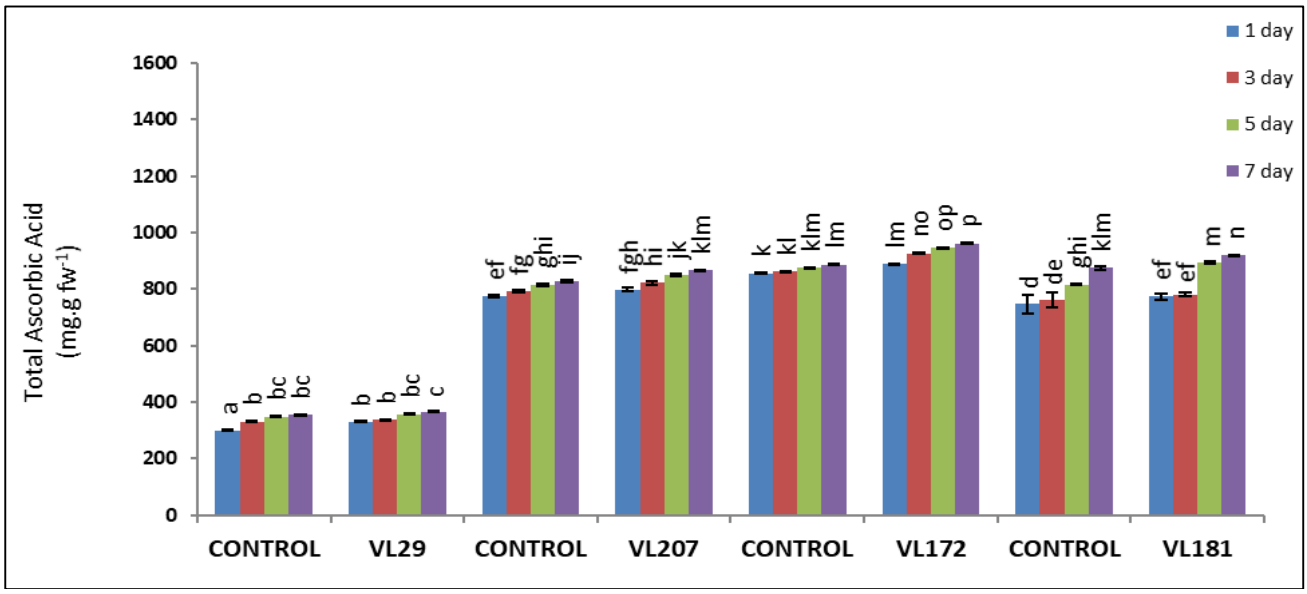

Fig. 3a

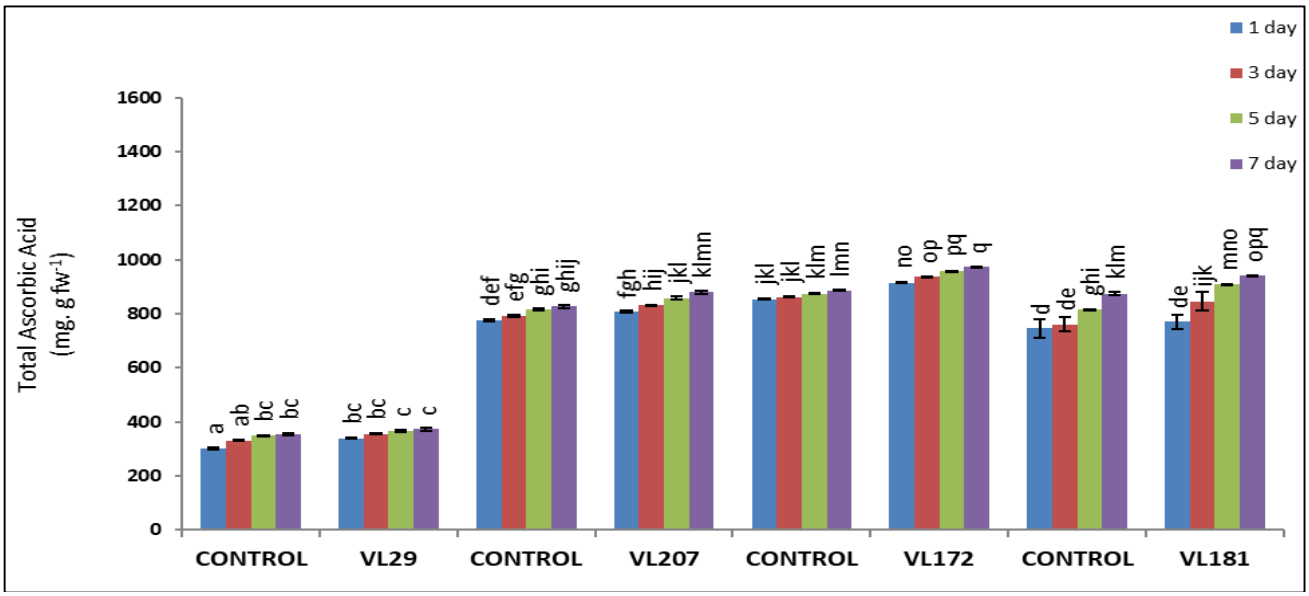

Fig. 3b

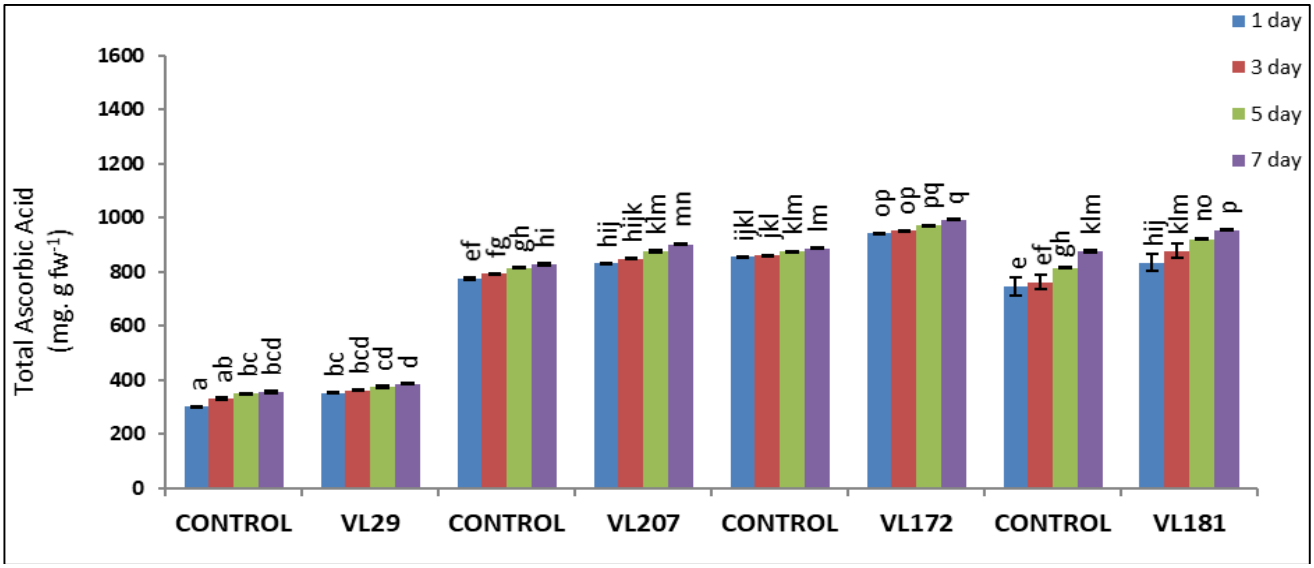

Fig. 3c

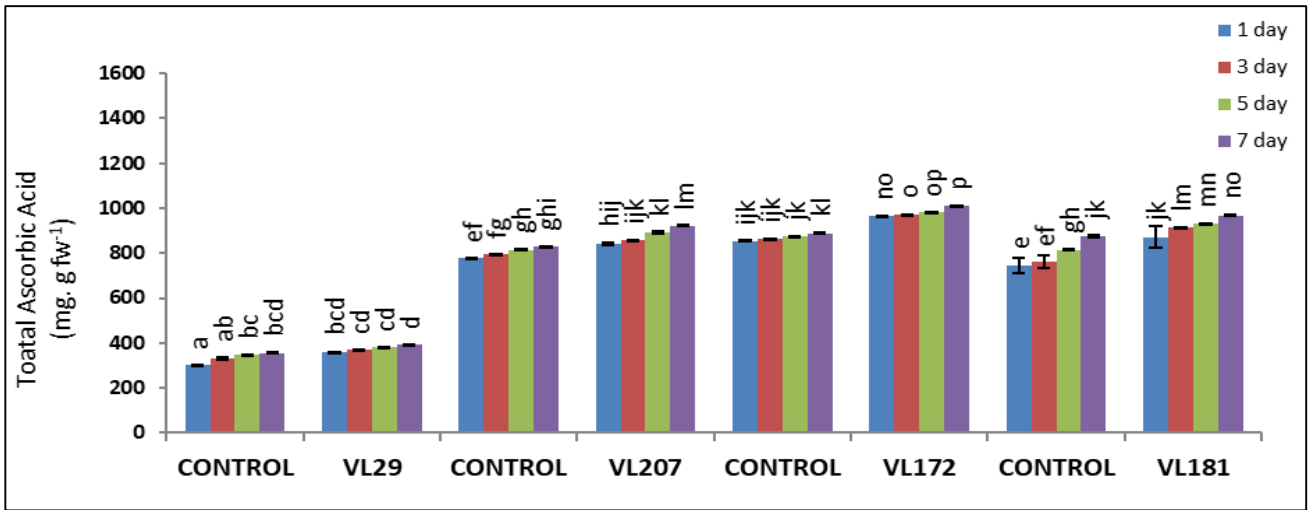

Fig. 3d

Fig 3: Effect of progressive drought stress by different concentration of PEG (%) on total ascorbic acid content in four varieties of barnyard millet (value represent mean ± SE (n=3)). (a) 5% PEG, (b) 10% PEG, (c) 15% PEG, (d) 20% PEG. Different letters denotes significant differences (P< 0.05) among four varieties in control and drought stressed plants. Line above bars represents mean ±standard error.

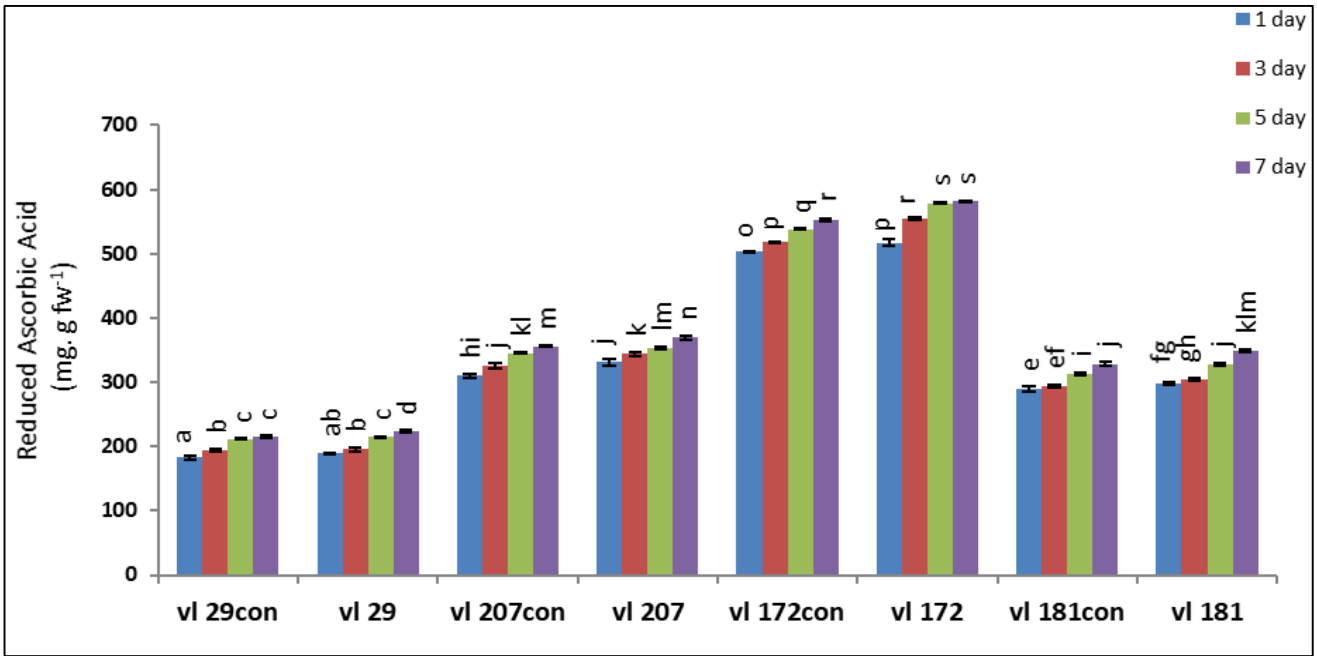

Fig. 4a

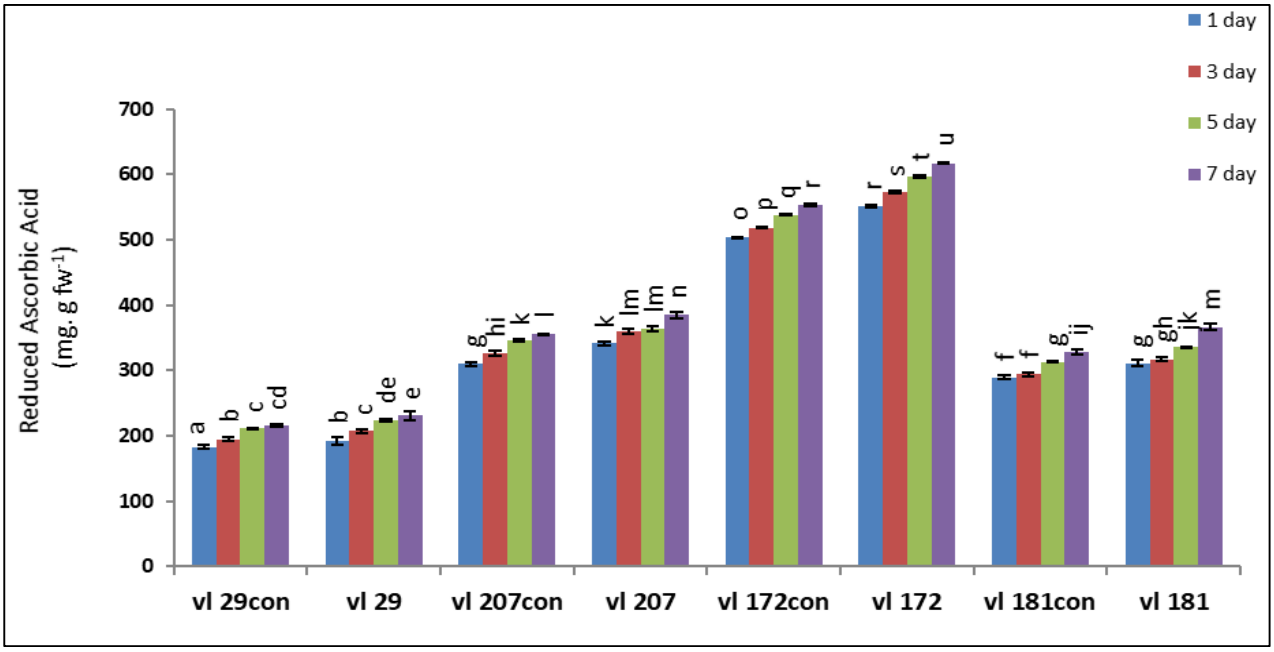

Fig. 4b

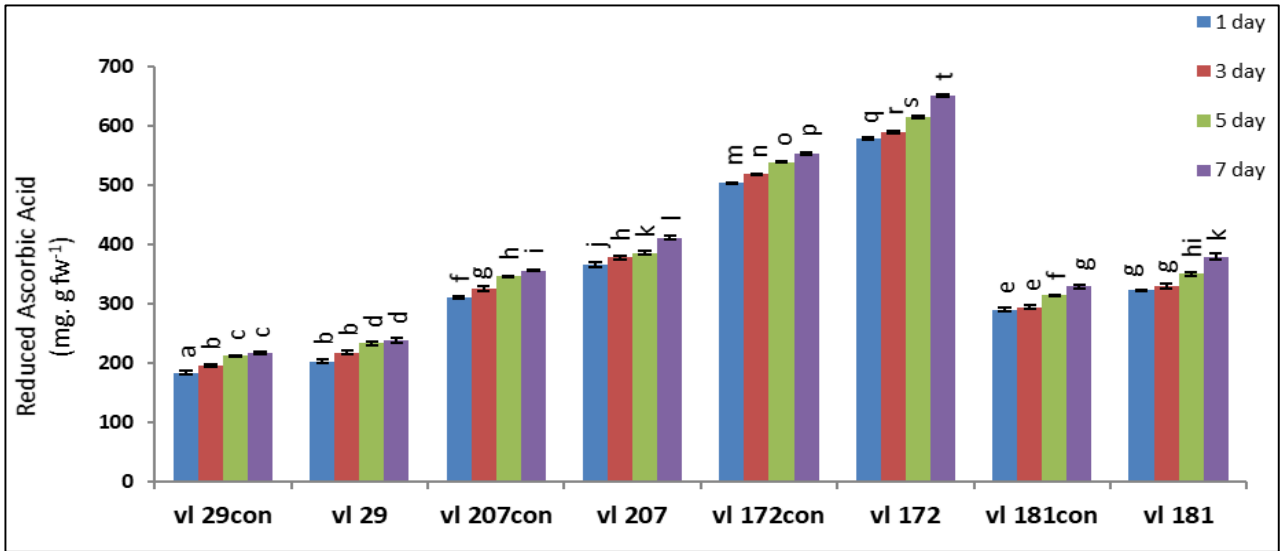

Fig. 4c

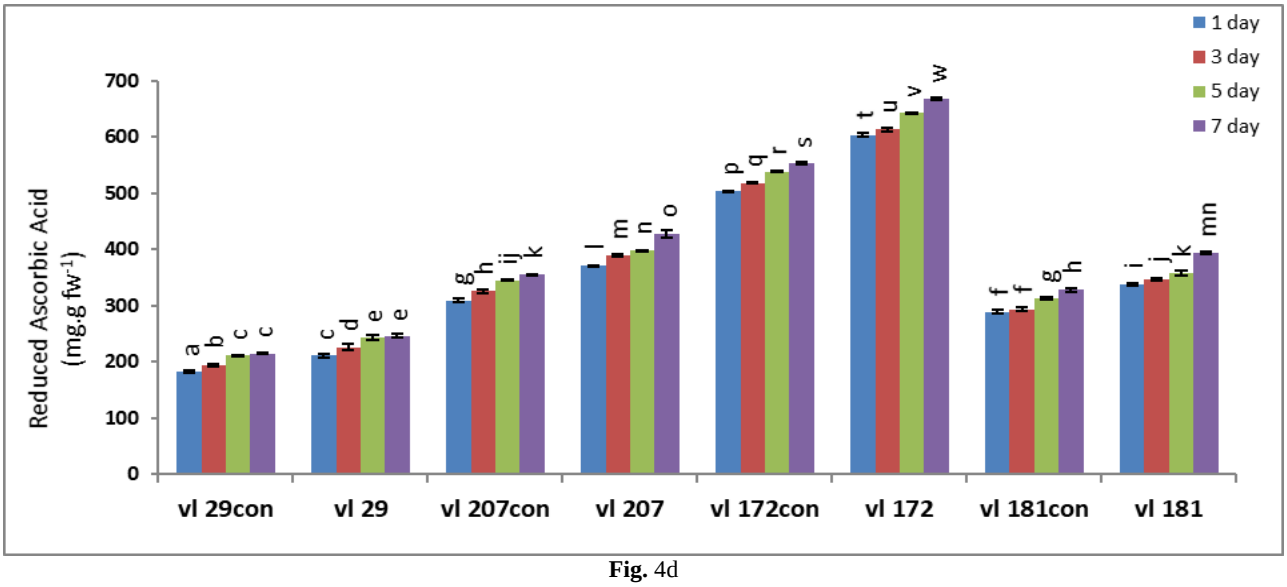

Fig. 4d

Fig 4: Effect of progressive drought stress by different concentration of PEG (%) on reduced ascorbic acid content in four varieties of barnyard millet (value represent mean ± SE (n=3)). (a) 5% PEG, (b) 10% PEG, (c) 15% PEG, (d) 20% PEG. Different letters denotes significant differences (P< 0.05) among four varieties in control and drought stressed plants. Line above bars represents mean ±standard error.

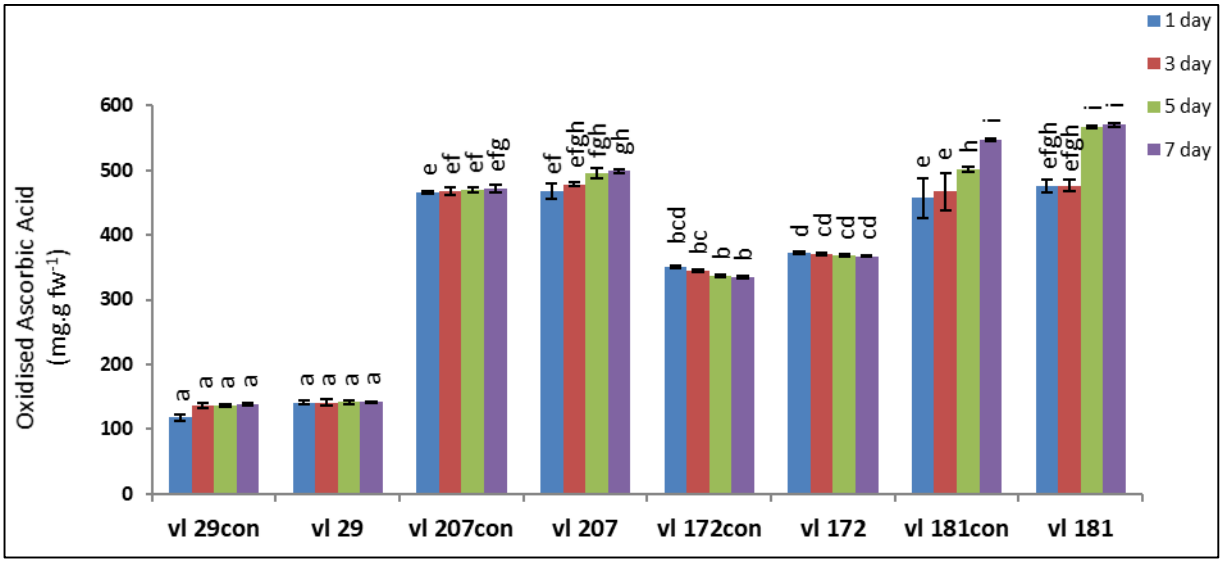

Fig. 5a

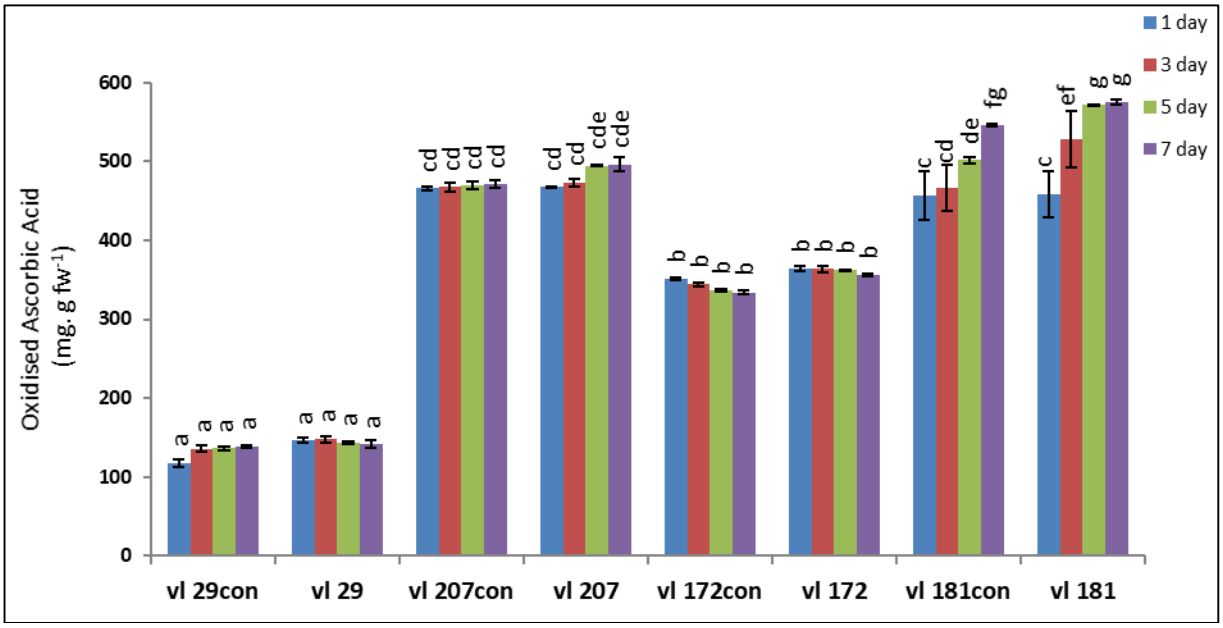

Fig. 5b

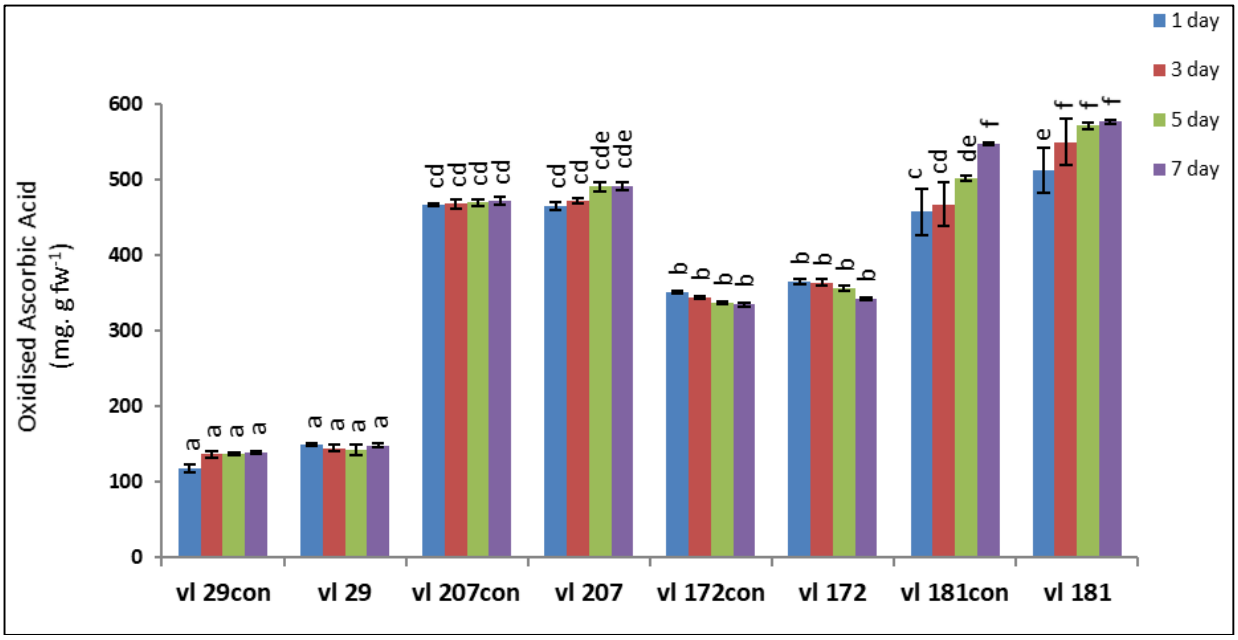

Fig. 5c

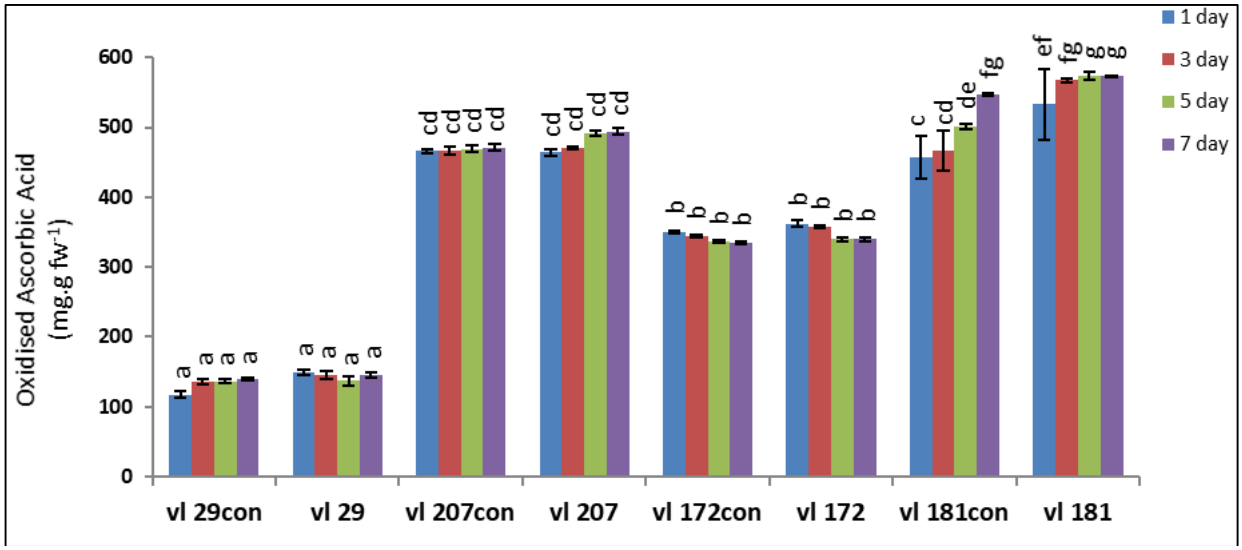

Fig. 5d

Fig 5: Effect of progressive drought stress by different concentration of PEG (%) on oxidised ascorbic acid content in four varieties of barnyard millet (value represent mean ± SE (n=3)). (a) 5% PEG, (b) 10% PEG, (c) 15% PEG, (d) 20% PEG. Different letters denotes significant differences ($P< 0.05$) among four varieties in control and drought stressed plants. Line above bars represents mean ±standard error.

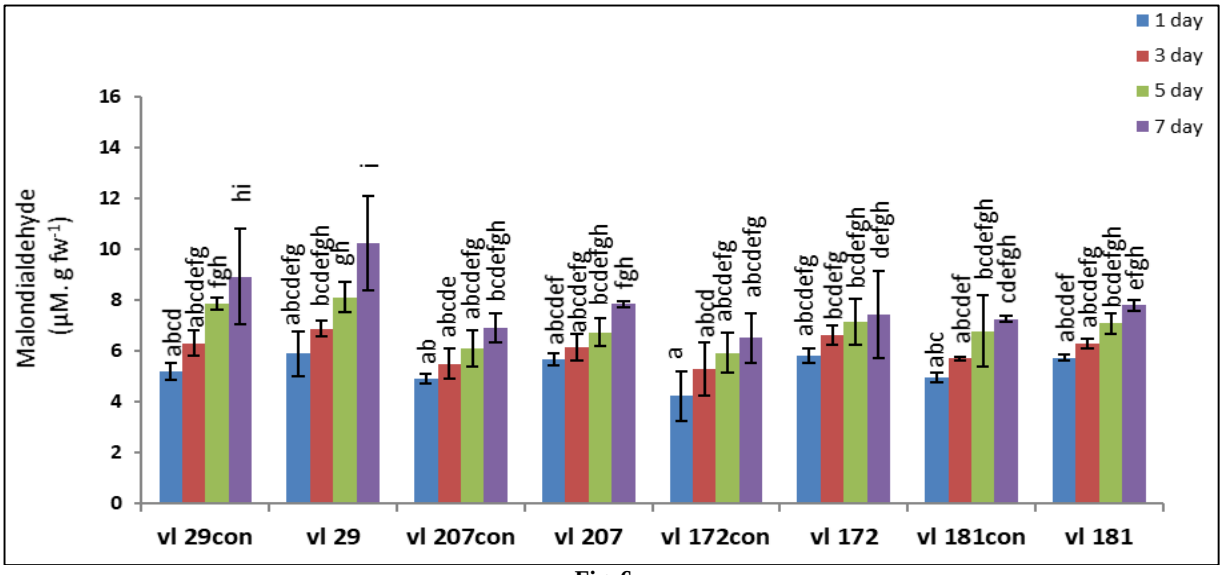

Fig. 6a

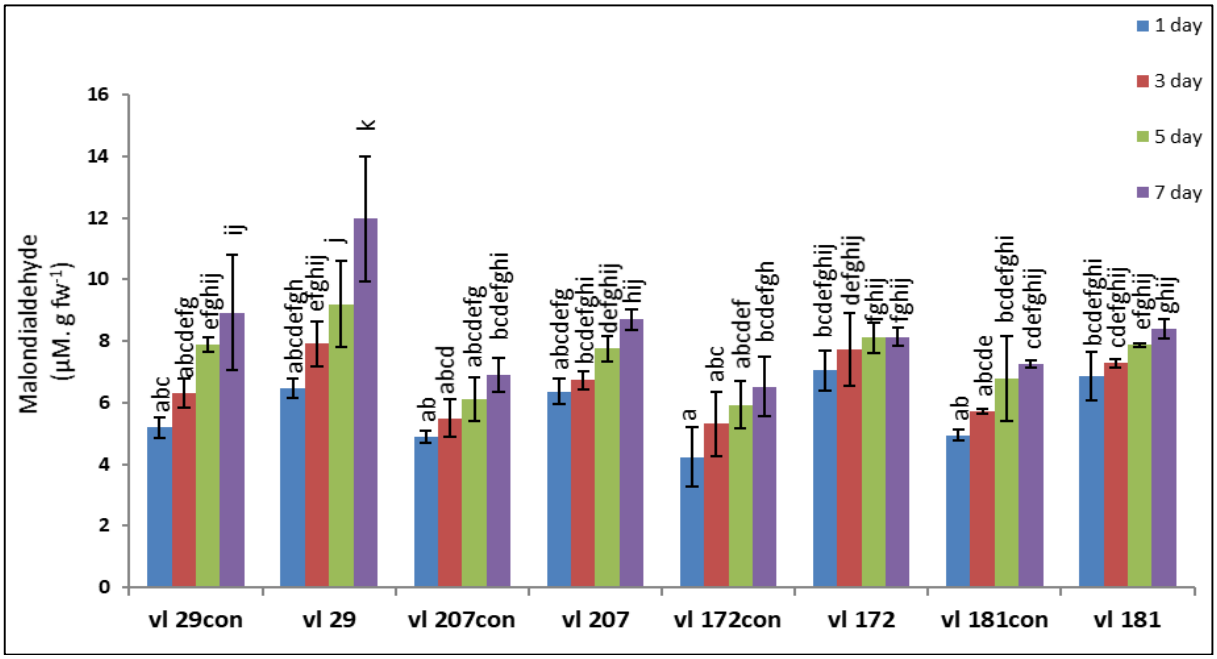

Fig. 6b

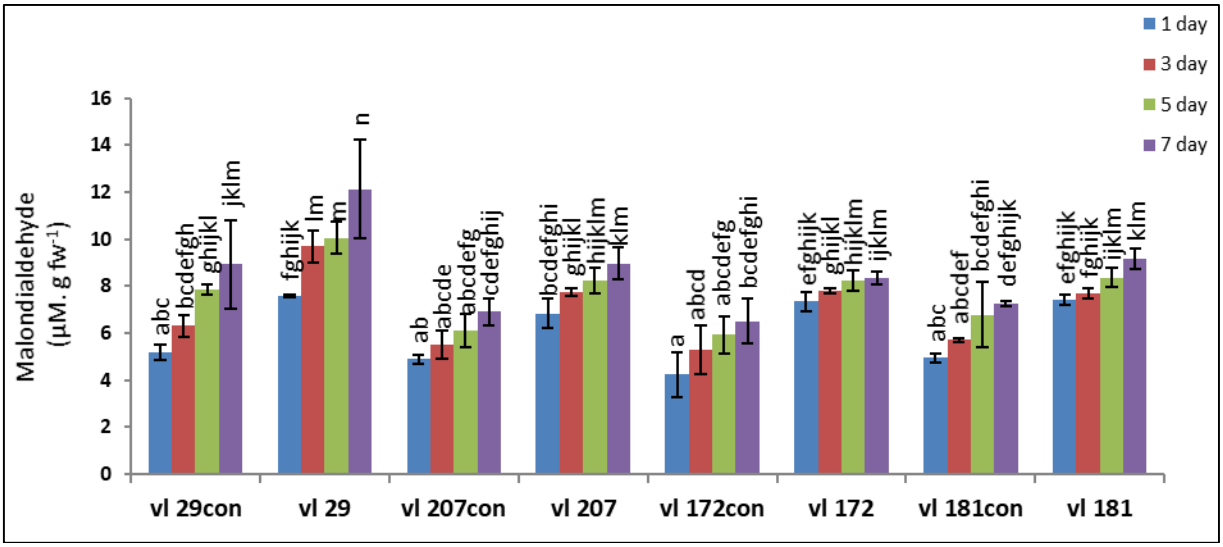

Fig. 6c

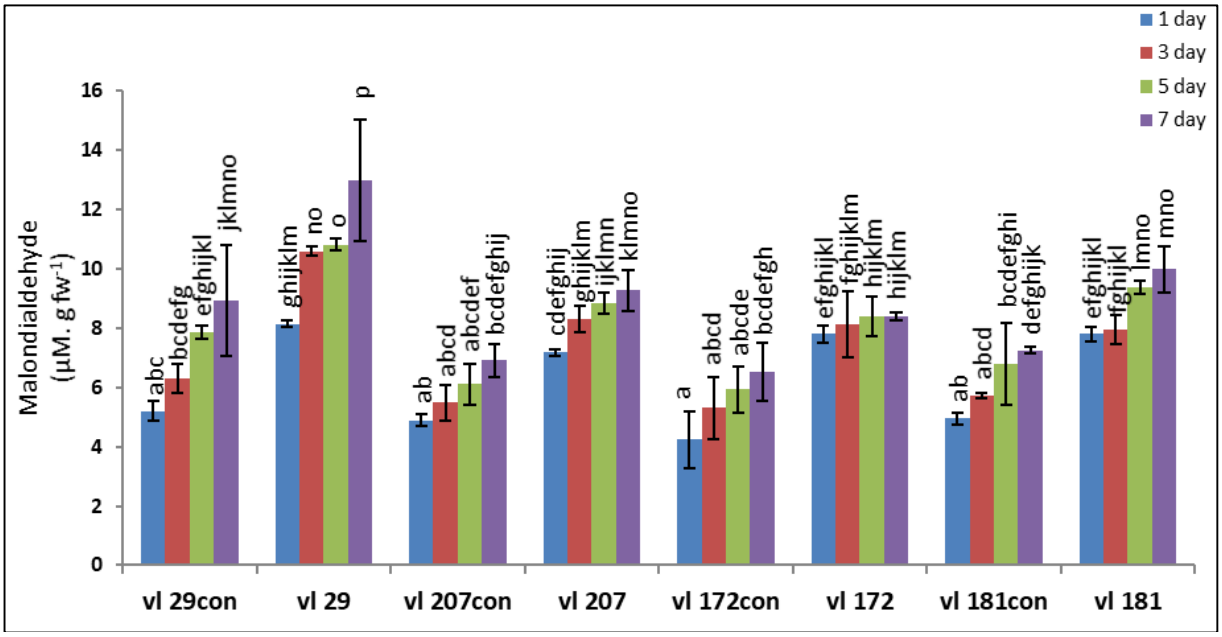

Fig. 6d

Fig 6: Effect of progressive drought stress by different concentration of PEG (%) on MDA content in four varieties of barnyard millet (value represent mean ± SE (n=3)). (a) 5% PEG, (b) 10% PEG, (c) 15% PEG, (d) 20% PEG. Different letters denotes significant differences (P< 0.05) among four varieties in control and drought stressed plants. Line above bars represents mean ±standard error.

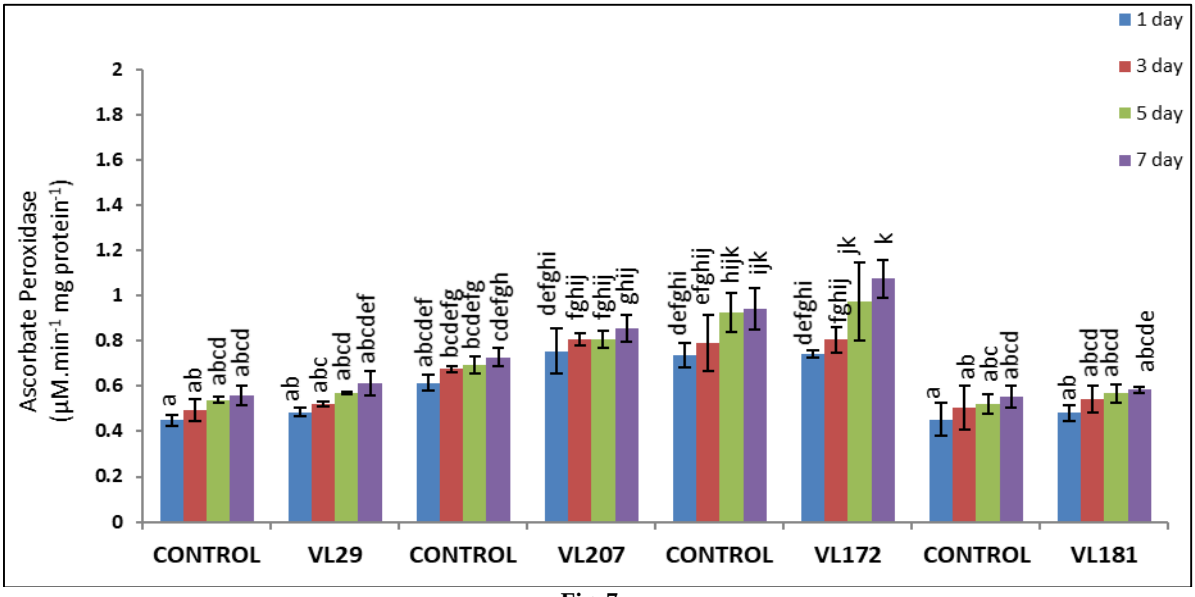

Fig. 7a

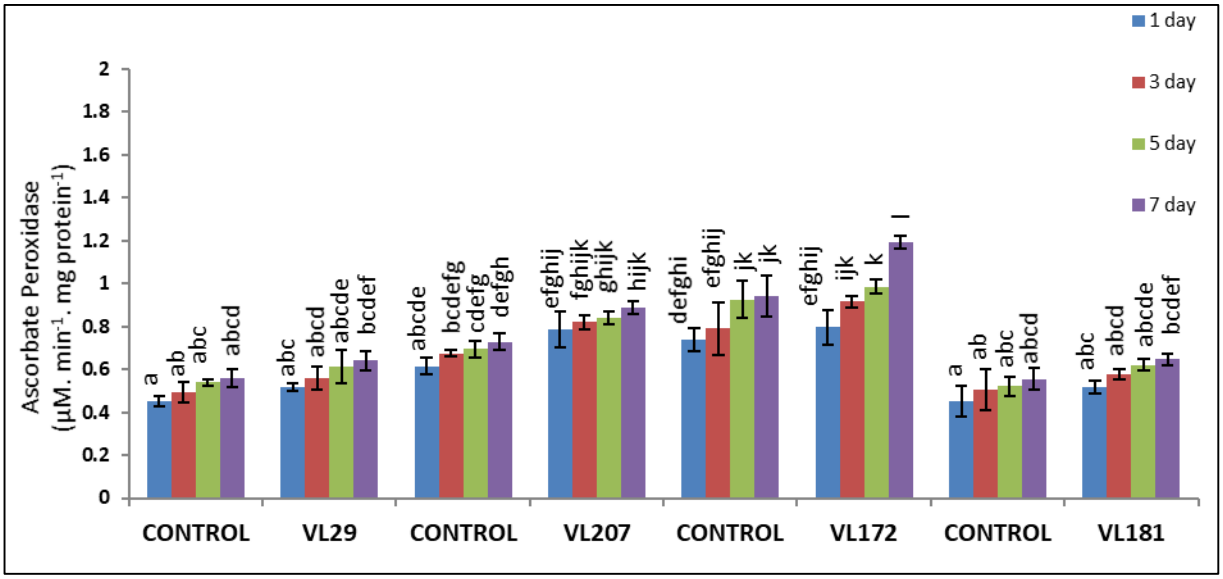

Fig. 7b

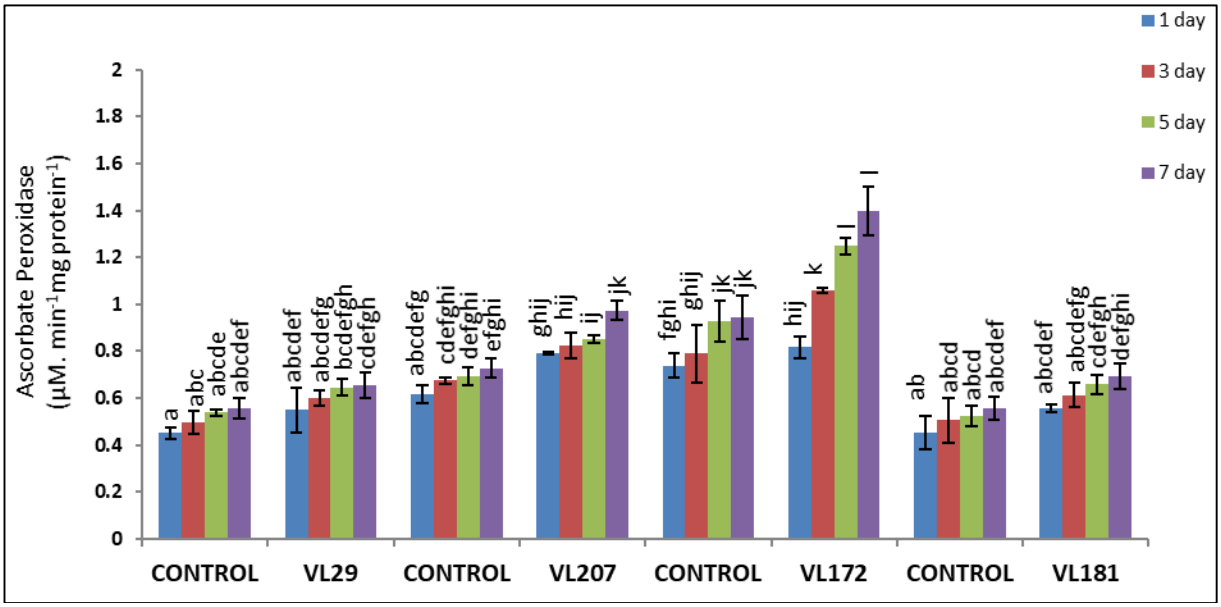

Fig. 7c

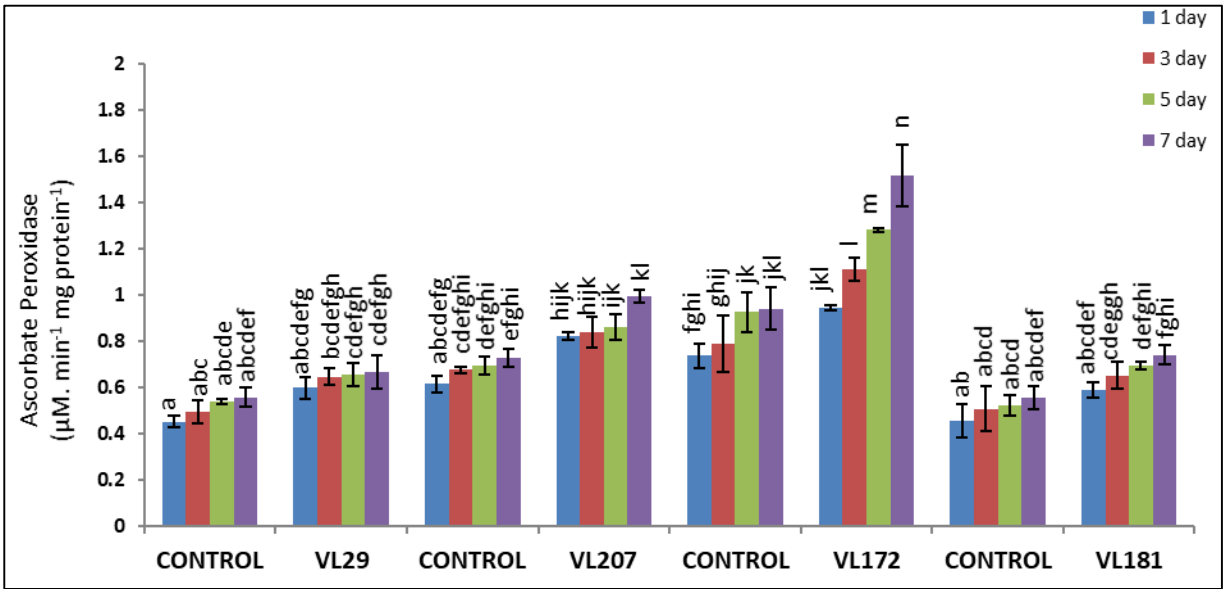

Fig. 7d

Fig 7: Effect of progressive drought stress by different concentration of PEG (%) on APX activity in four varieties of barnyard millet (value represent mean ± SE (n=3)). (**a**) 5% PEG, (**b**) 10% PEG, (**c**) 15% PEG, (**d**) 20% PEG. Different letters denotes significant differences (P<0.05) among four varieties in control and drought stressed plants. Line above bars represents mean ±standard error.

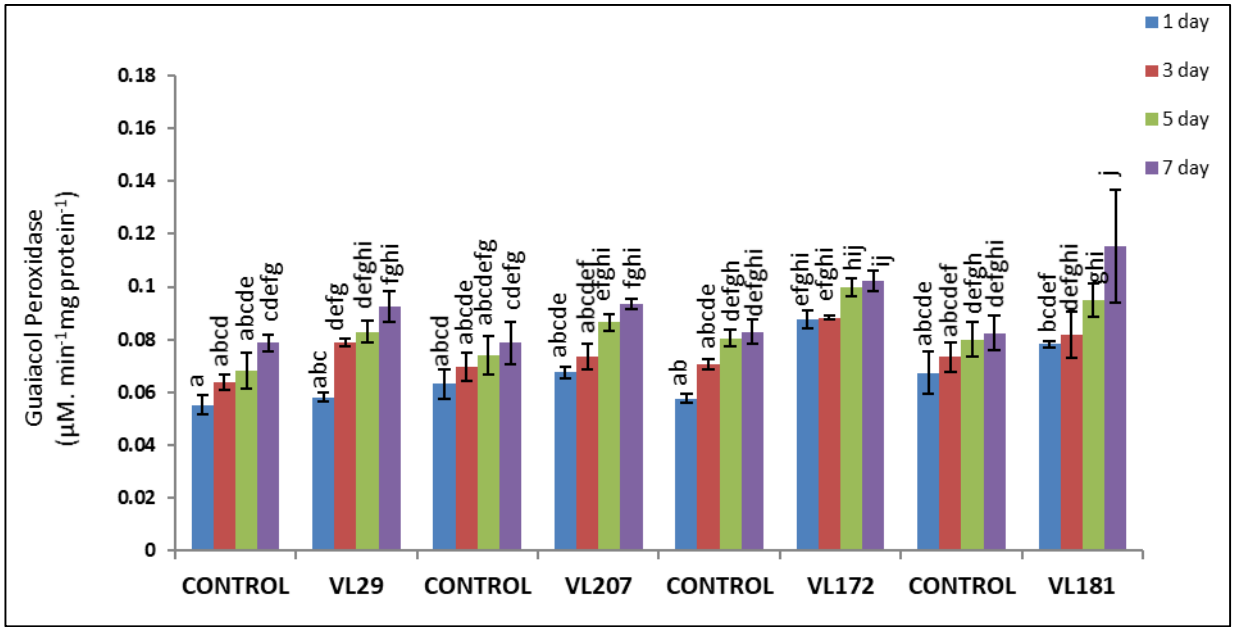

Fig. 8a

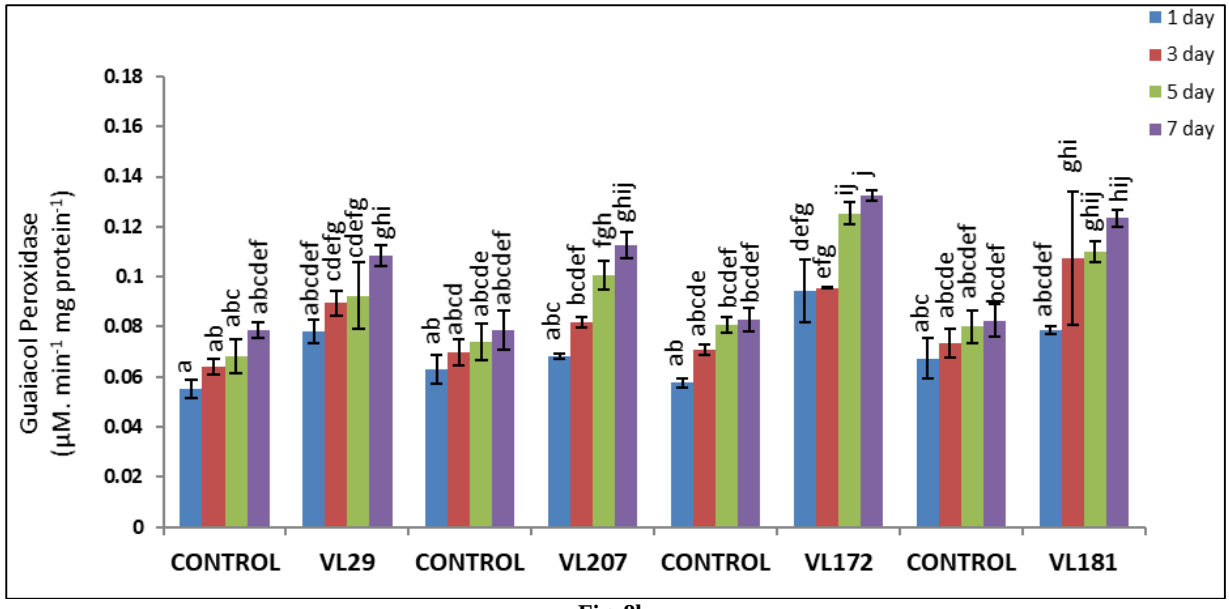

Fig. 8b

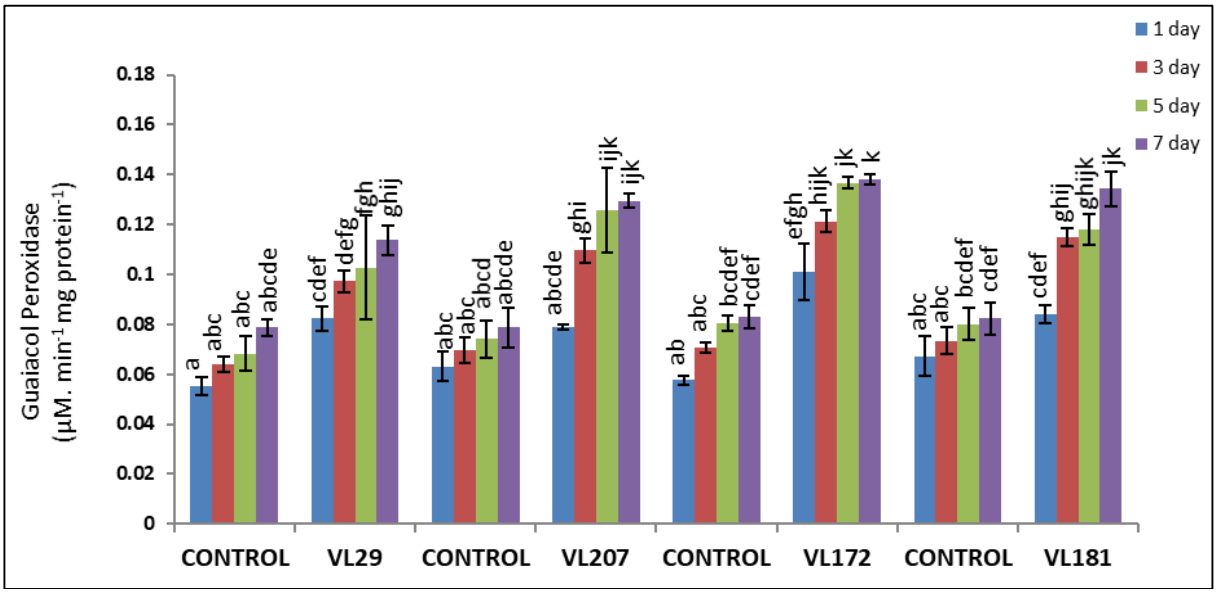

Fig. 8c

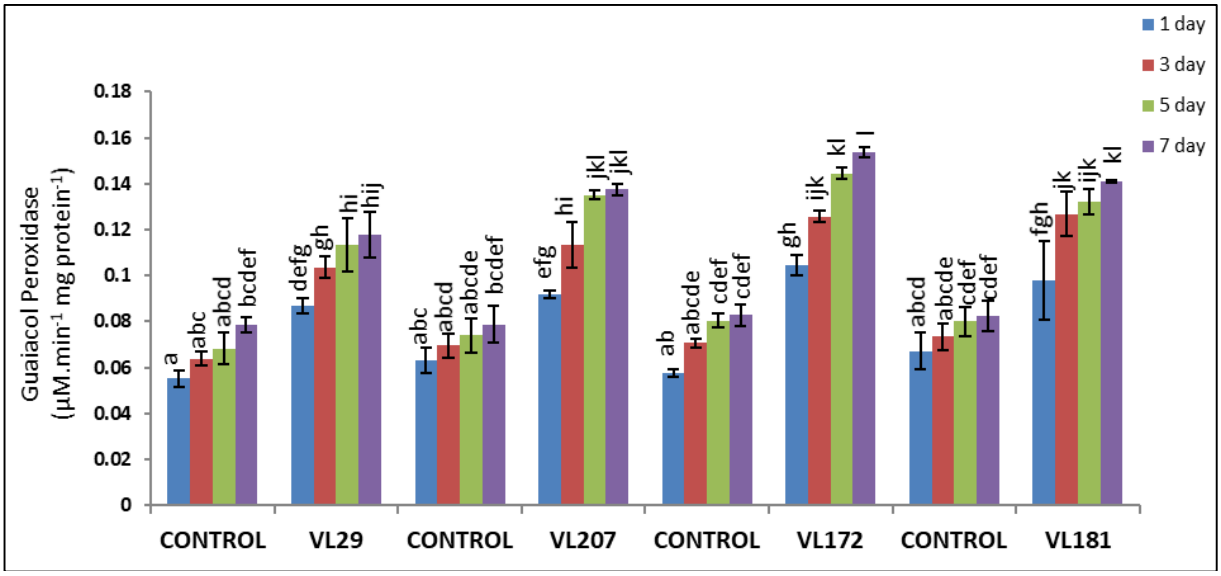

Fig. 8d

Fig 8: Effect of progressive drought stress by different concentration of PEG (%) on GPX activity in four varieties of barnyard millet (value represent mean ± SE (n=3)). (a) 5% PEG, (b) 10% PEG, (c) 15% PEG, (d) 20% PEG. Different letters denotes significant differences ($P< 0.05$) among four varieties in control and drought stressed plants. Line above bars represents mean ±standard error.

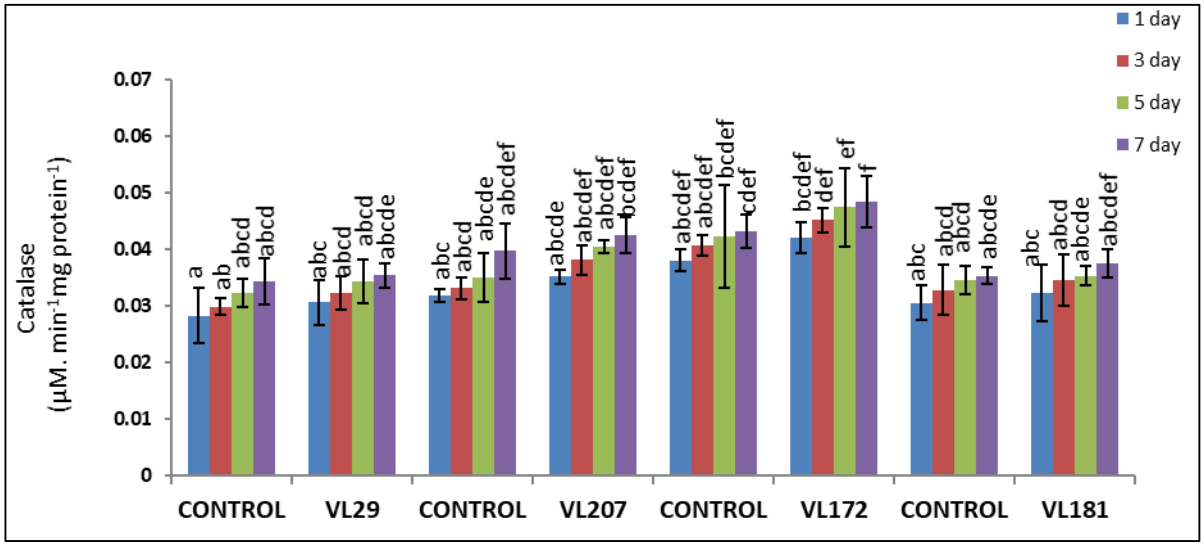

Fig. 9a

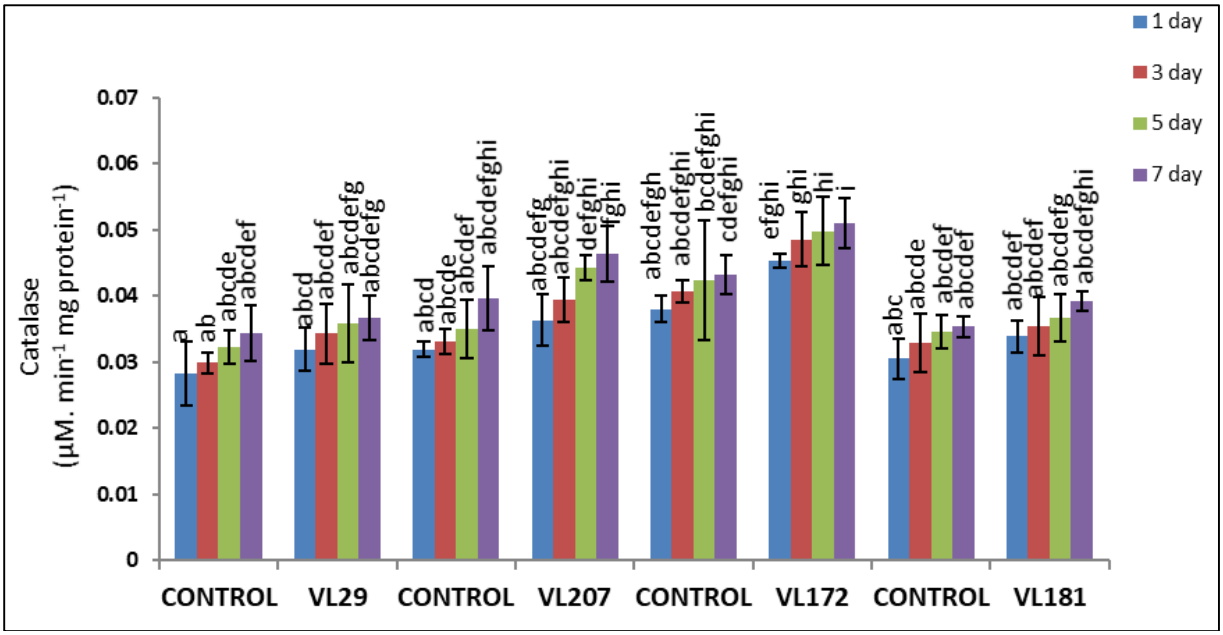

Fig. 9b

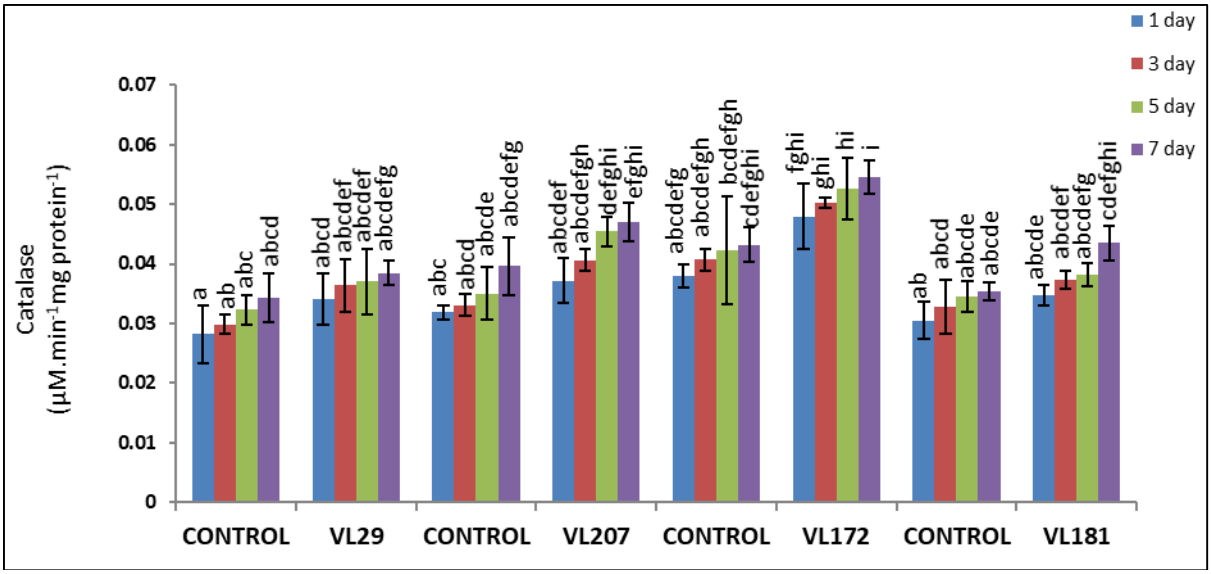

Fig. 9c

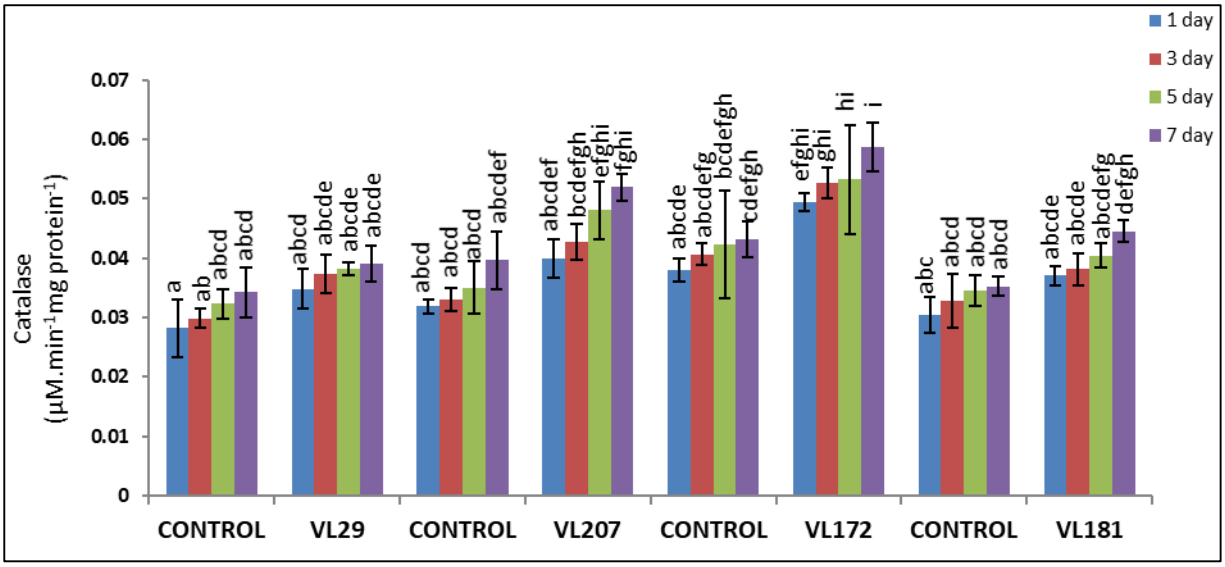

Fig. 9d

Fig 9: Effect of progressive drought stress by different concentration of PEG (%) on catalase activity in four varieties of barnyard millet (value represent mean ± SE (n=3)). (a) 5% PEG, (b) 10% PEG, (c) 15% PEG, (d) 20% PEG. Different letters denotes significant differences (P< 0.05) among four varieties in control and drought stressed plants. Line above bars represents mean ±standard error.

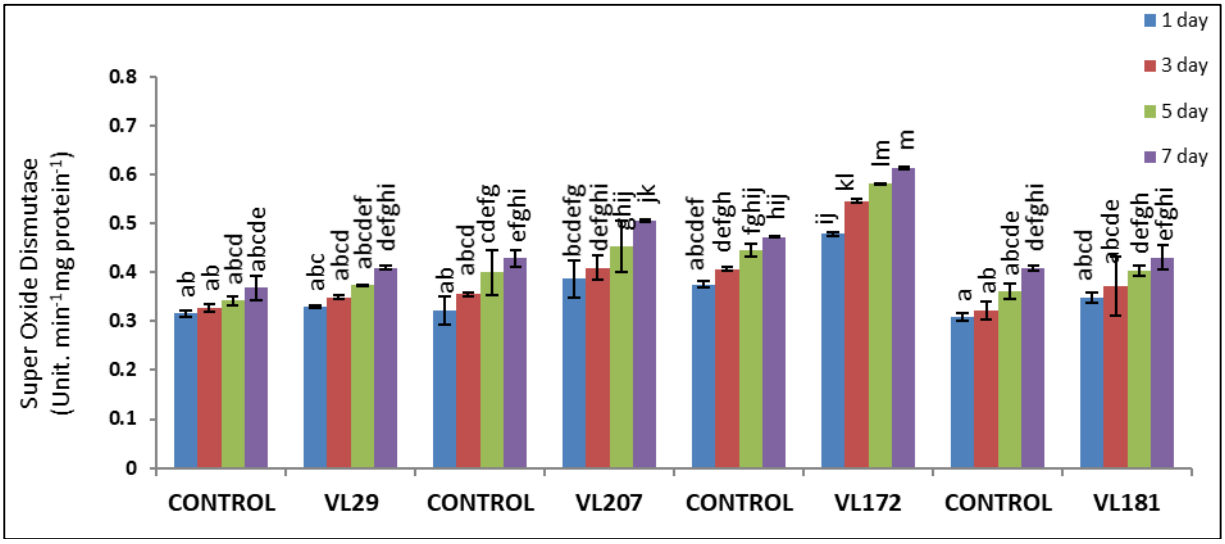

Fig. 10a

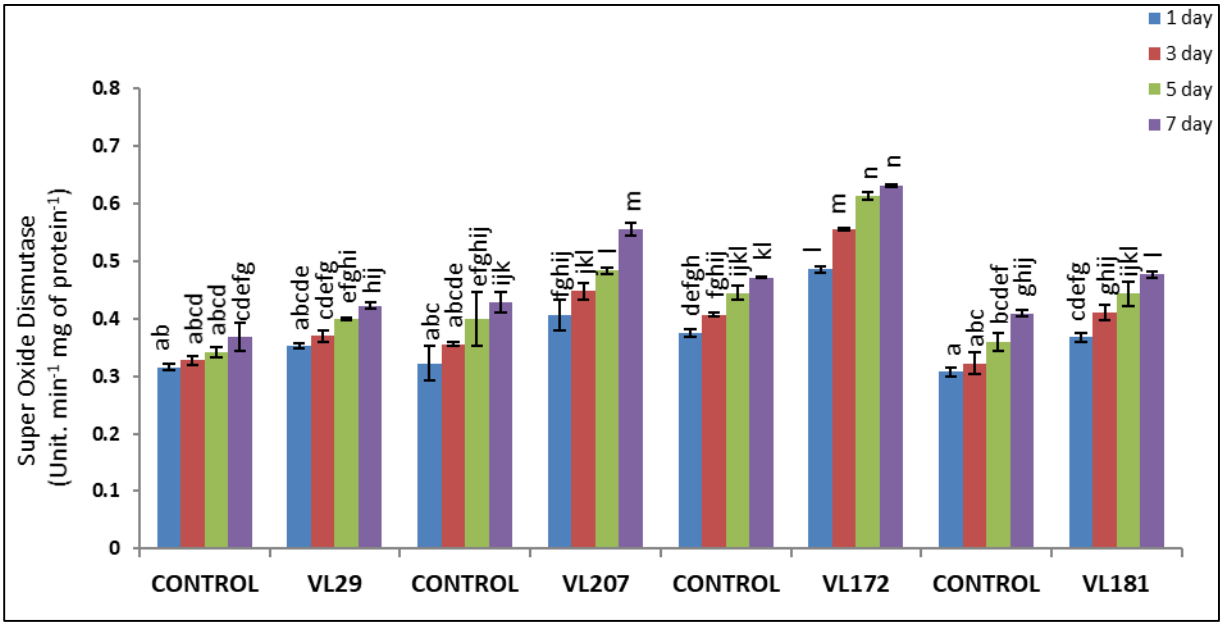

Fig. 10b

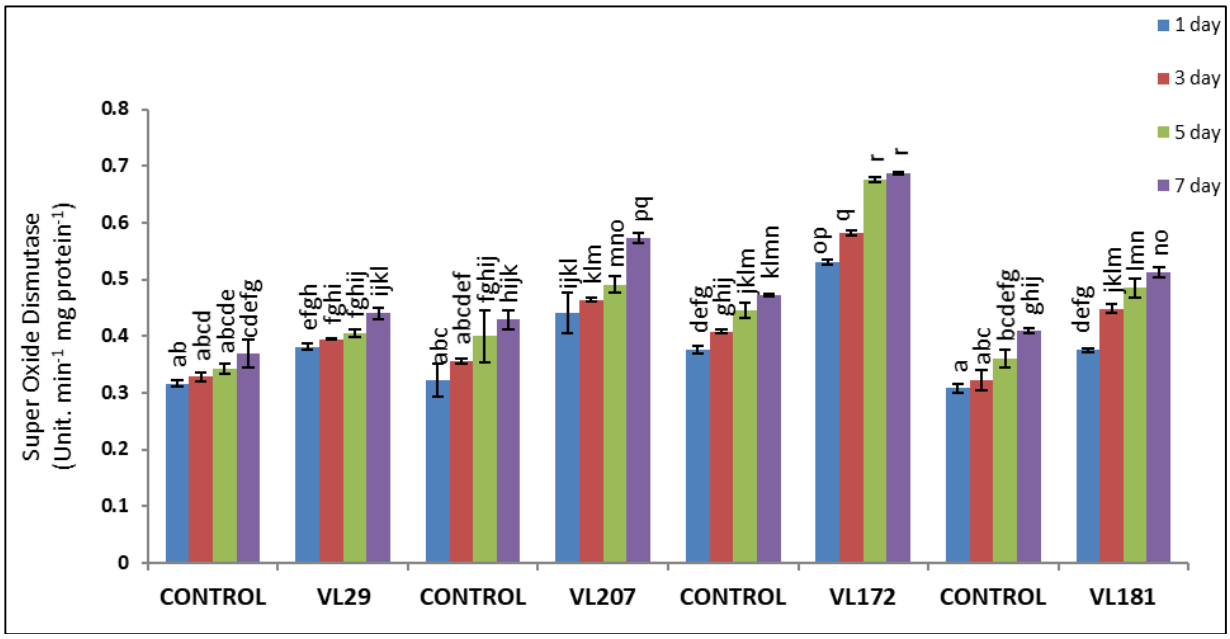

Fig. 10c

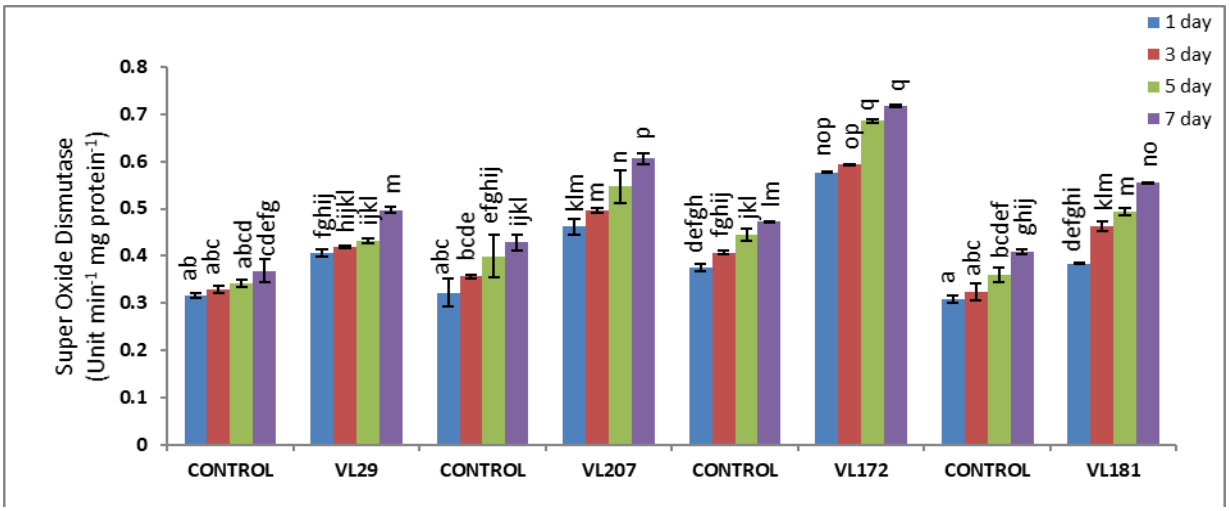

Fig. 10d

Fig 10: Effect of progressive drought stress by different concentration of PEG (%) on SOD activity in four varieties of barnyard millet (value represent mean ± SE (n=3)). (a) 5% PEG, (b) 10% PEG, (c) 15% PEG, (d) 20% PEG. Different letters denotes significant differences ($P< 0.05$) among four varieties in control and drought stressed plants. Line above bars represents mean ±standard error

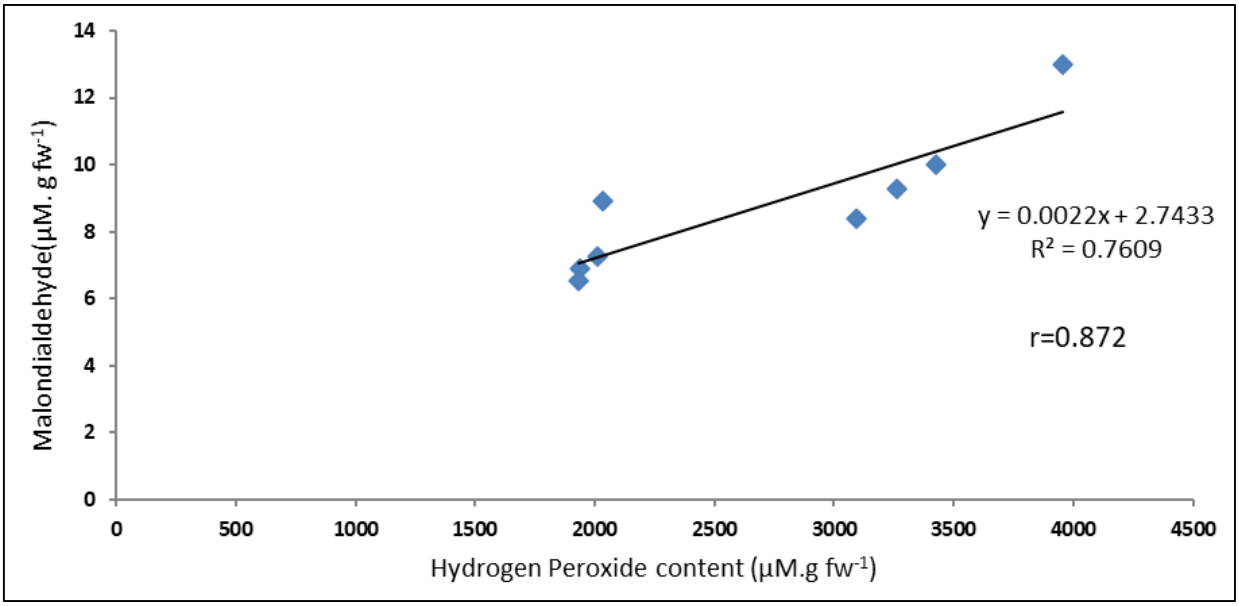

Fig. 11a

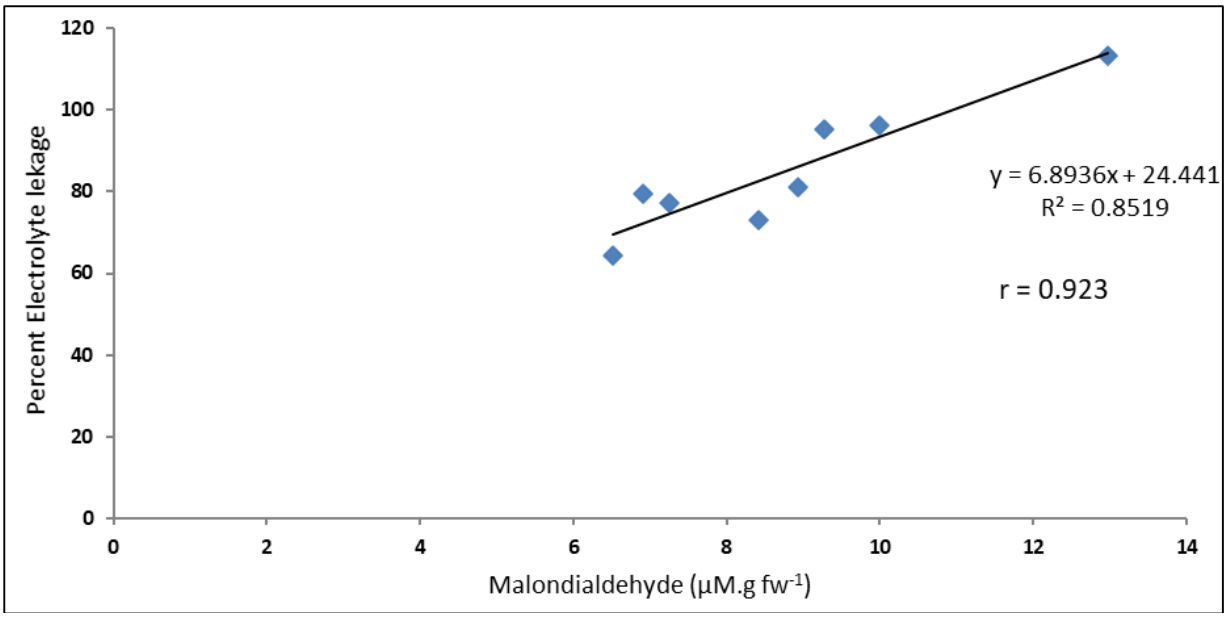

Fig. 11b

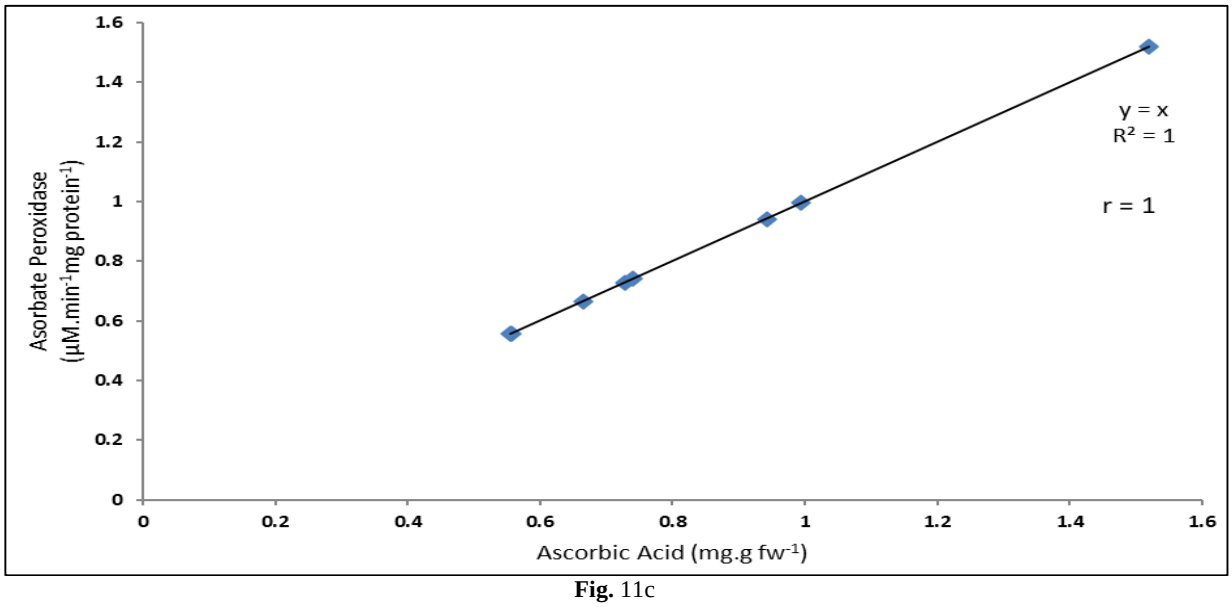

Fig. 11c

Fig 11: Correlation analysis performed between different parameters of all four varieties of barnyard millet (a) Hydrogen peroxide content and lipid peroxidation, (b) Lipid peroxidation and electrolyte leakage and (c) ascorbic acid and ascorbate peroxidase.

Table 1: Effect of increasing PEG concentration on ascorbic acid (reduced ascorbate and dehydroascorbate) contents of four varieties of Barnyard millet leaves of seedling grow in MS medium containing PEG. Means of three replicates ± SE. Rows with different letters are significantly different ($P< 0.05$).

Antioxidant Compound (mg. g fw-1)				
Varieties	**PEG (%)**	**ASC**	**DHA**	**ASA/DHA**
VL29	**0**	**215.5 ±2.06c**	**139.0±1.86b**	**1.55±0.02a**
	5	223.0±2.21cd	142.5±0.68b	1.56±0.02a
	10	231.4±6.95de	141.4±4.78b	1.64±0.10a
	15	237.7±3.79e	147.8±2.77b	1.61±0.05a
	20	246.4±3.44f	145.2±3.89b	1.70±0.07a
VL207	0	355.6±1.05b	471.2±5.55g	0.75±0.01a
	5	369.4±3.10c	498.8±2.53h	0.74±0.01a
	10	384.9±4.47d	496.1±9.16h	0.78±0.02a
	15	411.1±2.60e	490.8±4.67h	0.84±0.01a
	20	428.2±5.93f	494.0±5.16h	0.87±0.02a
VL172	0	553.2±2.10f	334.1±2.11b	1.66±0.01a
	5	581.4±1.43g	367.4±0.38e	1.58±0.00a
	10	617.5±1.73h	356.0±1.92d	1.73±0.01a
	15	650.8±2.21i	342.3±1.64c	1.90±0.01a
	20	669.1±2.06j	340.0±2.93c	1.97±0.02a
VL181	0	328.6±3.83b	546.6±1.82g	0.60±0.01a
	5	349.2±2.10c	570.0±3.46h	0.61±0.01a
	10	366.7±5.60d	575.1±3.90h	0.64±0.01a
	15	379.0±5.07e	575.9±2.84h	0.66±0.01a
	20	393.7±1.73f	573.9±0.69h	0.69±0.00a

Conclusion

Our results indicated that drought stress increased oxidative level inside the plant and it resulted in accumulation of H_2O_2 and MDA content. However, variety VL172 among the other three varieties responds better by having good correlation mechanism to minimize these effects by increasing others factors like higher accumulation of proline and ascorbic acid. Reduced ascorbic acid play an important role in maintaining redox state of the cell which was reported maximum in variety VL172. To dismutised H_2O_2, various antioxidant enzymes like APX, GPX, CAT and SOD activity were found to increase in variety VL172. However, all these parameters were minimum quantified in variety VL29. However to understand more elaborate mechanism of drought defense, further study can be made at molecular level.

Conflict of interest

Authors declare that they have no conflict of interest.

Contributions

Dipti Singh initiated and performed most of experiments under the guidance of AKV and AD. All authors proofread the manuscript.

Acknowledgment

This work is highly acknowledged to Department of Science and Technology (DST) for provided facilities under DST-FIST programme. We sincerely thank Director Research, Dean CBSH, G. B. P. U. A. and T., Pantnagar for providing necessary requirements for the research work.

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