

Full Length Research Paper

Analysis of Biochemical Responses in *Vigna Mungo* Varieties Subjected to Drought Stress and Possible Amelioration

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Abstract. A complex response (in terms of physiological, biochemical and molecular level) is shown by plants exposed to drought and depending on that, plants show differential adaptation and tolerance mechanisms. Drought stress effects on plants are generally evident in terms of reduced growth, loss of membrane stability and integrity, reduction in essential pigments like chlorophyll etc. The pulse crops black gram (*Vigna mungo* L.) is an important protein source that is grown in all over India. In addition, it also plays an important role in sustaining soil fertility by fixing atmospheric nitrogen. However, productivity is adversely affected by various biotic and abiotic stresses necessitating screening of newer varieties for better adaptation to local conditions. In the present study, three varieties of *Vigna mungo*, viz., IPU 94-1, PU 40 and PU 19, widely grown in the northern India were evaluated for their response to short term drought stress at early growth stage. Drought stress had detrimental effect in several biochemical parameters like chlorophyll content, lipid peroxidation, oxidative stress, generation of H₂O₂. Modulations of several metabolic components like phenolics, proline and antioxidant enzymatic responses of superoxide dismutase, peroxidase and catalase were observed in drought and recovery to combat the stress.

Keywords: *Vigna mungo*, drought, anti-oxidant enzymes, oxidative stress

1. INTRODUCTION

Vigna is a genus of plants that comprises a number of cultivated legumes belonging to family Fabaceae and sub-family Faboideae. It is widely cultivated because of its economic and nutritional importance being a low cost source of protein. *Vigna mungo* var. *silvestris* belongs to the subgenus *Ceratropis* in the genus *Vigna*. Commonly known as black gram it has its origin in India, cultivated since ancient times and is an important constituent in the dietary practices of the rural and urban communities. It is cultivated after rice cultivation as fallow crop in India in various agro ecological zones with diverse cropping systems and practices. However, here there has been a significant decline in its production in India in recent times. Lack of newer varieties and genotypes adapted to local environment is among the factors affecting its production necessitating evaluation of existing varieties for stress tolerant traits (Kundu et al., 2011; 2013). Study of the genetic and biochemical variations of qualitative and quantitative characteristics is essential to screen better genotypes and is routinely done in many crop plants (Graham and Vance, 2003; Emami and Eivazi, 2012)

Drought or soil water deficit, a major environmental stress factor is widespread in regions with limited water availability or unpredictable rainfall, more-so with climate change and growing water scarcity which is a severe constraint to plant productivity. It has been observed that loss in crop yield due to drought can surpass that due to all other factors is critical in long duration. Drought stress reduces leaf size, stems extension and root proliferation, disturbs plant water relations and reduces water-use efficiency and is extremely detrimental at early growth stage (Reddy et al., 2004). Plants undertake several protective mechanisms to withstand drought stress by increasing water uptake through deep and / or prolific root systems, reducing transpiration loss by various physical and biochemical mechanisms and activating an array of enzymatic responses. Several genes and transcription factors that respond to drought have been studied including aquaporins, dehydrins, HSP, dehydration-responsive element-binding (DREB), late embryogenesis abundant proteins to name a few (Bray et al., 2004; Ngugi et al., 2013). Several strategies for the management of drought tolerance through marker assisted selection and breeding, exogenous application of several compounds like osmoprotectants and

engineering plants for drought resistance have been adapted (Dita et al., 2006).

The aim of the present work is to screen varieties of *Vigna mungo*, viz., PU 40, PU 19 and IPU 94-1 which are grown in Rajasthan for biochemical response under short term drought stress at early growth stage. This study will provide a theoretical basis for improving water use efficiency in pulse cultivation by analyzing the relationship between various biochemical factors during drought and subsequent re-watering.

2. MATERIALS AND METHODS

2.1. Planting material and procedure

Uniformly mixed field soil was filled in well labelled pots with height 14 cm and diameter 12 cm. The varieties of genus *Vigna* selected for the experiments viz, PU 40, PU 19 and IPU 94-1 were obtained from Krishi Vigyan Kendra, Banasthali, Rajasthan.

Seeds of each variety were soaked in distilled water for 24 h and kept in plant growth chamber in order to allow them to germinate. After 24 hours of soaking, seeds were transferred to autoclaved petri dishes by using sterilised forceps having wet double layered filter paper. Petri dishes were kept in plant growth chamber for providing suitable conditions for germination.

The plants were grown in pots for 21 days till the appearance of the second tri-foliolate leaf (21 DAS). A set of biochemical experiments were done with the control plants. The plants were then subjected to drought stress by with-holding water for the next three days. Another set of biochemical experiments were done of the drought stressed plants three days after the stress period (25 DAS). The plants were then watered for recover and the final set of biochemical experiments performed two day after watering the plants (27 DAS)

2.2. Estimation of chlorophyll content

Leaves were directly homogenized in 80% acetone for the estimation of chlorophyll pigments (Chlorophyll a, chlorophyll b and total chlorophyll content) and determined spectrophotometrically following the method of Metzner et al., 1965. The extract was centrifuged at 4000 rpm for 10 min, diluted and the extinction coefficient of the supernatant was measured at three wavelengths of 452.5, 644 and 663 nm using a UV-vis spectrophotometer (EC- UV5704SS).

2.3. Estimation of Hydrogen peroxide content

Leaves were homogenized in 0.1% (w/v) trichloroacetic acid and then centrifuged at 15000 rpm, for 15 min at 4 °C, the supernatant was used for the estimation of hydrogen peroxide. The supernatant (0.5 mL) was added to 1mL, 1 M potassium Iodide (KI) solution and incubated for 5min. The oxidation product was observed at 390nm in a spectrophotometer. H₂O₂ level was calculated using the extinction coefficient, $E=0.28 \mu\text{mol cm}^{-1}$.

2.4. Estimation of total phenolic content

Total phenolic content was measured following Folin-Ciocalteau method with modifications (Singleton et al., 1999; Chakraborty et al., 2008) and expressed as gallic acid equivalent (GAE). Leaves were homogenized with 80% ethanol and the homogenate was used for the extraction and estimation of total phenolic content.

2.5. Estimation of proline content

The amount of proline was calculated in the leaf sample using the Ninhydrin method following extraction in freshly prepared 3% sulho-salicylic acid. The extract was centrifuged at 10000 rpm for 20 minutes at 4°C. The absorbance of chromophore was taken at 520 nm.

2.6. Determination of the amount of lipid peroxidation

Lipid peroxidation was estimated in the leaves by homogenizing 0.2 g of freshly collected leaves in 0.25%TBA in 10%TCA. The extract was heated, cooled down and then centrifuged at 10000 rpm for 10 minutes. Absorbance of the product was taken at 532 and 600 nm. Correction of nonspecific absorbance was done and the difference in absorbance at 600 nm from absorbance at 532 nm calculated. Lipid peroxidation was expressed in $\mu\text{g MDA/gm f. wt.}$ by using extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

2.7. Antioxidant enzyme assay

For the estimation of different enzymes, extraction was performed in extraction buffer prepared by dissolving 1mM EDTA, 2% PVP, 0.05% Triton-X-100 and 1mM ascorbic acid in phosphate buffer (50 mM, pH-7). Leaves were homogenized with extraction buffer in prechilled mortar-pestle and centrifuged at 10000 rpm for 20 min. The supernatant thus obtained was used for the estimation of different enzymes.

Protein was estimated by the Bradford method (1976). Absorbance was taken at 595 nm with the help of spectrophotometer and estimation of protein done by comparison with a standard curve of BSA.

Superoxide dismutase, SOD (E.C. 1.15.1.1.) activity was determined by measuring the inhibition of photoreduction of NBT (nitroblue tetrazolium) by the method given by Beauchamp et al., 1971. One unit of the enzyme activity is defined as the amount of enzyme required to inhibit the photoreduction of NBT by 50%. Catalase, CAT (E.C. 1.11.1.6) activity was determined spectrophotometrically by measuring the rate of disappearance of H₂O₂ at 240 nm; taking extinction coefficient of 39.4 mM⁻¹ cm⁻¹ (Miyagawa et al., 2000). Peroxidase (E.C. 1.11.1.7) activity was determined spectrophotometrically by measuring the

breakdown of H₂O₂ using guaiacol as a substrate following the method of Kar and Mishra (1976). The activity of peroxidase and catalase is expressed in terms of nkat mg⁻¹ protein. The activity of superoxide dismutase is expressed as U mg⁻¹ protein.

2.8. Statistical analyses

For the experimental setup, a randomized block design was used. The data are represented as mean ± standard deviation of three biological replicates wherever applicable. Analysis of Variance (ANOVA) - Duncan multiple range test (DMRT) was conducted to detect significant differences between means (p<0.05) using SPSS software (17.0.0, 2008, SPSS Inc.).

Table 1: Effect of drought stress on chlorophyll contents (mg g⁻¹ fw) of *V. mungo* leaves (IPU 94-1, PU 40 and PU 19)

Treatment	Chl a		Chl b		Total Chl	
	mg/ g f.wt.*		mg/ g f.wt.*		mg/ g f.wt.*	
<u>IPU 94-1</u>						
21 DAS	0.067	± 0.011 ^f	0.619	± 0.029 ^b	0.685	± 0.018 ^{ab}
25 DAS (D)	0.006	± 0.001 ^a	0.641	± 0.025 ^b	0.646	± 0.025 ^a
27 DAS (R)	0.020	± 0.006 ^{bcd}	0.636	± 0.027 ^b	0.656	± 0.021 ^a
<u>PU40</u>						
21 DAS	0.028	± 0.001 ^d	0.440	± 0.012 ^a	0.724	± 0.013 ^b
25 DAS (D)	0.013	± 0.002 ^{abc}	0.639	± 0.026 ^b	0.651	± 0.024 ^a
27 DAS (R)	0.019	± 0.002 ^{abcd}	0.636	± 0.024 ^b	0.655	± 0.026 ^a
<u>PU 19</u>						
21 DAS	0.049	± 0.008 ^e	0.625	± 0.028 ^b	0.674	± 0.020 ^{ab}
25 DAS (D)	0.007	± 0.006 ^{ab}	0.641	± 0.027 ^b	0.648	± 0.021 ^a
27 DAS (R)	0.022	± 0.002 ^{cd}	0.635	± 0.026 ^b	0.657	± 0.024 ^a

* Values are mean±standard deviation (SD); data followed by same alphabets are not significantly different at p≤0.05 according to ANOVA and DMRT for each column. DAS: days after sowing, D: 3 days under drought stress, R, recovery.

Table 2: Effect of drought stress on proline content (µM/g f. wt.) *V. mungo* leaves (IPU 94-1, PU40 and PU19)

Treatments	Proline content	
	µM/g f. wt.	
<u>IPU 94-1</u>		
21 DAS	44.17± 0.883 ^a	
25 DAS (D)	53.93 ± 1.918 ^b	
27 DAS (R)	47.15 ± 1.278 ^a	
<u>PU 40</u>		
21 DAS	55.74 ± 1.918 ^{bc}	
25 DAS (D)	73.83 ± 1.918 ^e	
27 DAS (R)	61.39 ± 0.959 ^d	
<u>PU 19</u>		
21 DAS	44.44 ± 1.278 ^a	
25 DAS (D)	58.23 ± 2.877 ^{cd}	
27 DAS (R)	52.35 ± 0.959 ^b	

* Values are mean±standard deviation (SD); data followed by same alphabets are not significantly different at p≤0.05 according to ANOVA and DMRT for each column. DAS: days after sowing, D: 3 days under drought stress, R, recovery.

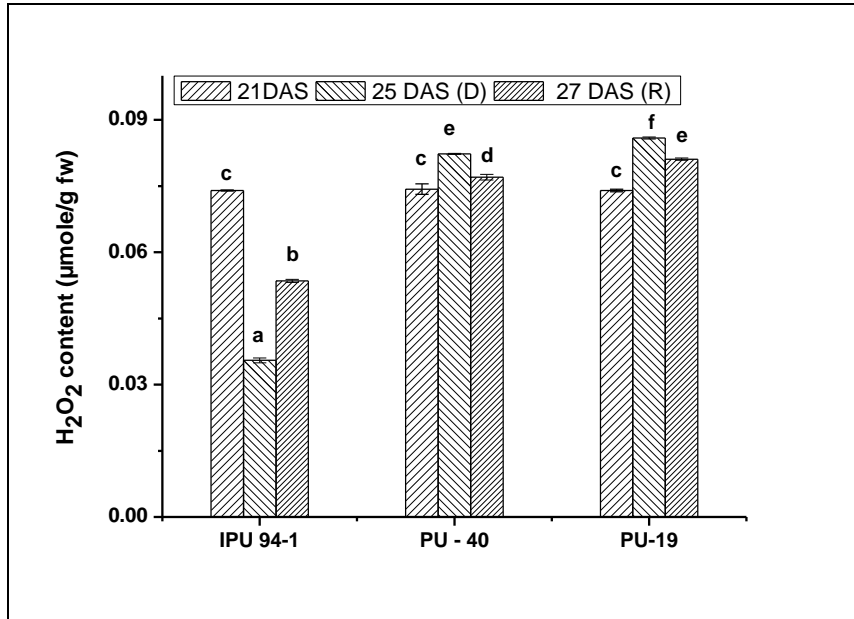


Fig.1: Effect of drought stress on H₂O₂ content (µ mole g⁻¹f.wt.) of *V. mungo* leaves. (IPU 94-1, PU 40 and PU 19) Bars are mean ± SD; bars followed by same alphabets are not significantly different at p≤0.05 according to ANOVA and DMRT. DAS: days after sowing, D: 3 days under drought stress, R, recovery.

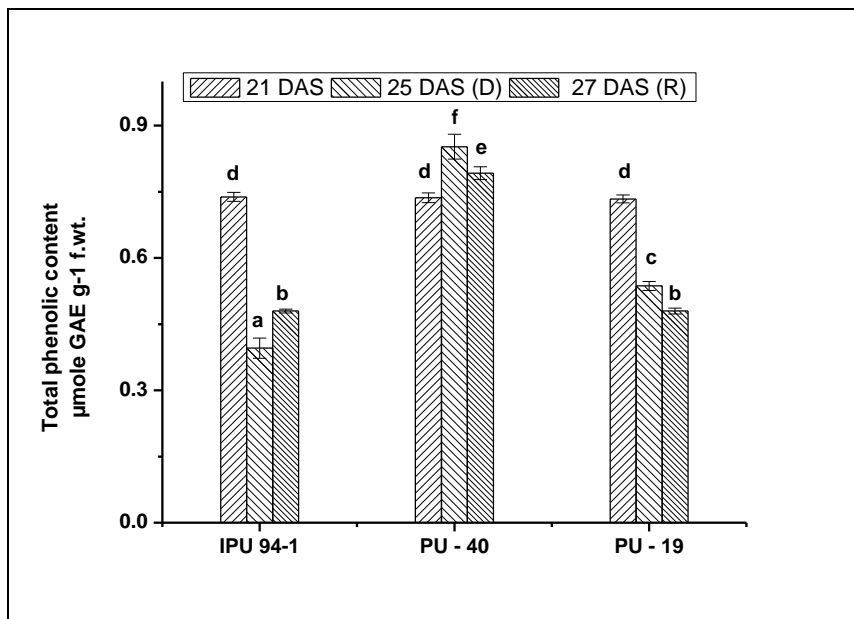


Fig. 2: Effect of drought stress on total phenolic content (µ mole GAE g⁻¹f.wt.) of *V. mungo* leaves. (IPU 94-1, PU 40 and PU 19)

Bars are mean ± SD; bars followed by same alphabets are not significantly different at p≤0.05 according to ANOVA and DMRT. DAS: days after sowing, D: 3 days under drought stress, R, recovery.

3. RESULTS AND DISCUSSIONS

Plants respond to drought stress and acclimatize through various physiological and biochemical changes. Drought stress affects the plants variably depending on the severity of stress and the developmental stage of the plants. Water stress at early stage of plant growth is highly limiting for vegetative growth and adversely affects reproductive health and yield. The reduction in plant growth in terms of height is due to the loss of cell turgor which greatly suppresses cell expansion and cell growth

thereby inhibiting the linear growth of shoot. Plant species, stage of growth and drought stress, its intensity and duration are manifested in drought response of plants (Singh et al., 2012). Additionally, increasing atmospheric pollution, depletion and contamination of ground water resources and indiscriminate application of pesticides, insecticides and fertilisers also adversely affect plant productivity (Ansari, 2014; Rakib and Bhuiyan, 2014).

All varieties of *V. mungo* subjected to drought stress in the present study show a rapid loss of chlorophyll a content (Table 1). Incidentally, the

content of chlorophyll b does not change significantly. Overall content of chlorophyll in the present study showed a decreasing trend with drought which was statistically significant for the variety PU40 indicating that these photosynthetic pigments are sensitive to water deficit condition. But at recovery stage chlorophyll content increased to the level of that of

the control plants showing better adaptability of the varieties to drought stress. The reduction in leaf chlorophyll content under drought stress might be due to excessive swelling of chloroplast membranes, distortion of the lamellae vesiculation and the appearance of lipid droplets (Massacci et al., 2008).

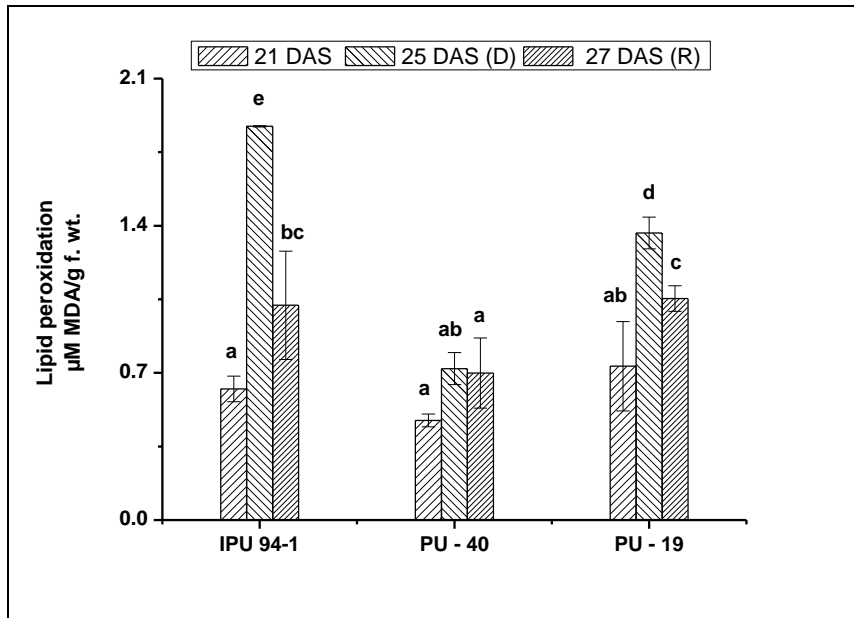


Fig. 3: Effect of drought stress on lipid content ($\mu\text{M MDA/g f. wt.}$) *V. mungo* leaves. (IPU 94-1, PU40 and PU19) Bars are mean \pm SD; bars followed by same alphabets are not significantly different at $p \leq 0.05$ according to ANOVA and DMRT. DAS: days after sowing, D: 3 days under drought stress, R, recovery.

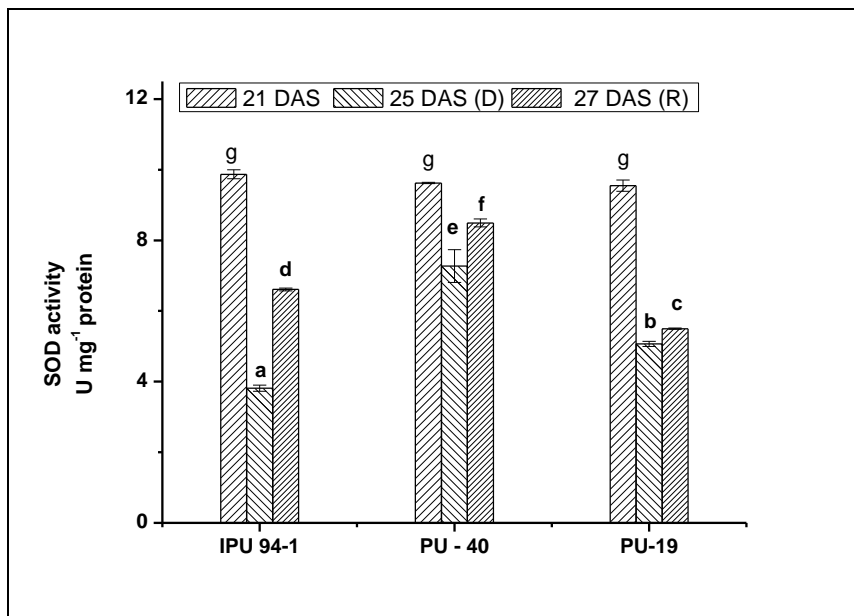


Fig. 4: Effect of drought stress on SOD activity ($\text{U mg}^{-1}\text{Protein}$) *V. mungo* leaves. (IPU 94-1, PU40 and PU19) Bars are mean \pm SD; bars followed by same alphabets are not significantly different at $p \leq 0.05$ according to ANOVA and DMRT. DAS: days after sowing, D: 3 days under drought stress, R, recovery.

The H_2O_2 content of different varieties of *V. mungo* on drought stress and its recovery are represented in Fig. 1. In varieties PU 40 and PU 19 content of H_2O_2 enhanced under stressed condition. Increase in H_2O_2 content in the leaves under drought was evident by the

rapid increase in oxidative stress in the plants. While in variety IPU 94-1, content of H_2O_2 decreases under stressed condition. H_2O_2 acts as a signalling molecule in several biotic and abiotic stresses, its prolonged presence is detrimental for the living tissues. H_2O_2 and

other ROS molecules may also act as second messengers to activate proteins for plant defence providing resistance to biotic and abiotic stresses, but at higher concentration may initiate derogative mechanisms (Quanet et al., 2008). Hydrogen peroxide

has been implicated in signalling cascade and in control of gene expression and is also linked to auto-oxidation of components associated with photosystem I (Foyer et al., 2003).

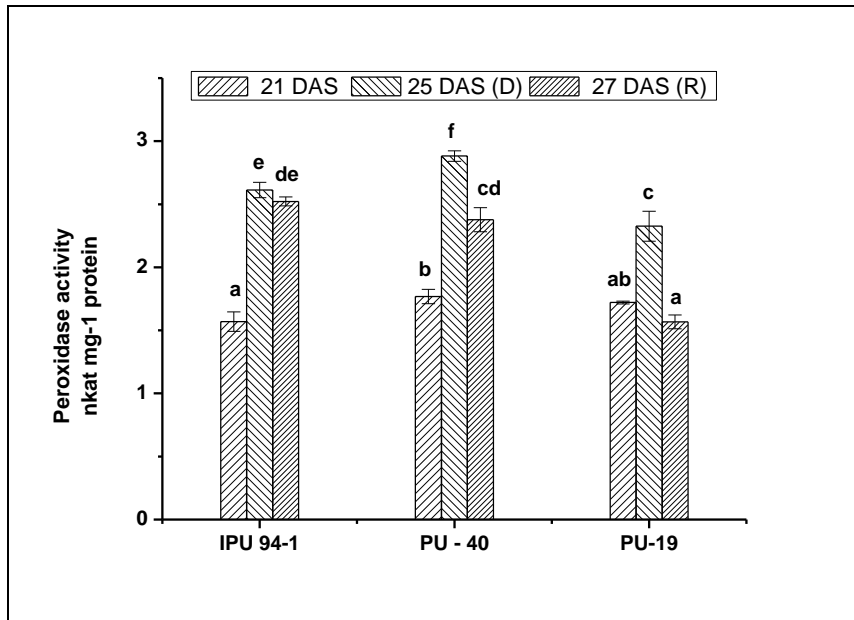


Fig 5: Effect of drought stress on peroxidase activity (nkat mg⁻¹ protein) *V. mungo* leaves. (T9, IPU 94-1 and PU40) Bars are mean ± SD; bars followed by same alphabets are not significantly different at p≤0.05 according to ANOVA and DMRT. DAS: days after sowing, D: 3 days under drought stress, R, recovery.

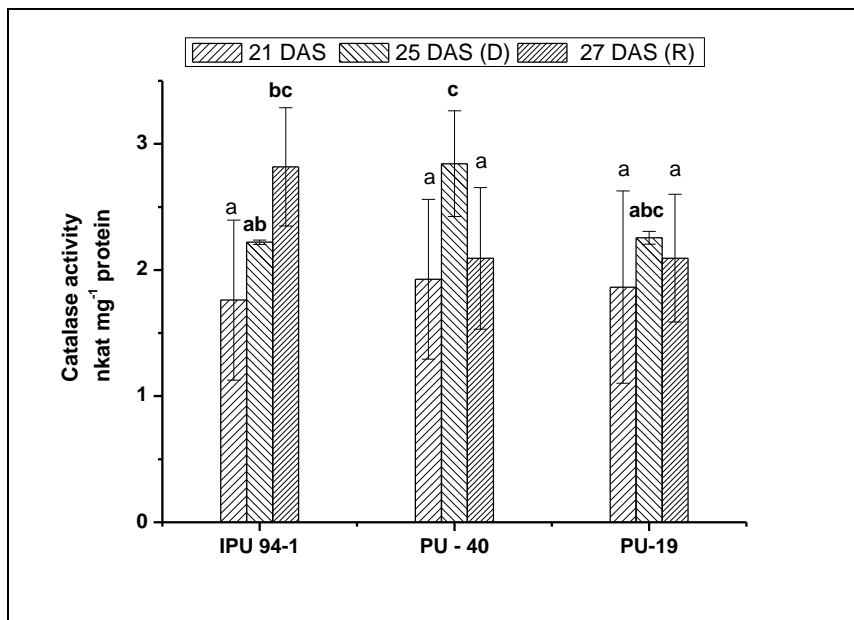


Fig. 6: Effect of drought stress on catalase activity (nkat mg⁻¹ protein) *V. mungo* leaves. (T9, IPU 94-1 and PU40) Bars are mean ± SD; bars followed by same alphabets are not significantly different at p≤0.05 according to ANOVA and DMRT. DAS: days after sowing, D: 3 days under drought stress, R, recovery.

A diverse response in phenolic content was observed under-drought stress condition in the present study (Fig. 2). High phenolic content is observed in PU 40 under stressed condition while in varieties IPU 94-1 and PU 19 content of phenolic compounds decreases. After recovery period from stressed condition, phenolic content increases in IPU 94-1

while it decreases in PU 40 and PU 19. Decreased phenolic content under stress in IPU 94-1 and PU – 19 may be a adaptive mechanism whereby essential metabolites are prevented from entering the secondary metabolite pathway under stress and mitigation maybe through the antioxidant enzymes whereas in PU-40 it provides a direct antioxidant protection. Phenolic

compounds are secondary metabolites and have specific role in plant defence (Mandal et al., 2010). They are also known to have high antioxidant properties and thus, may be involved in reducing stress due to reactive oxygen species (Chakraborty et al., 2008, Jha et al., 2013).

It is observed that lipid peroxidation increases to statistically significant levels in the varieties IPU 94-1 and PU – 19 which indicate membrane damage under drought stress (Fig. 3.). PU – 40 shows a better response to drought stress than the other varieties. Under water stress conditions, a change in lipid composition which is an important constituent of the cell membrane may be vital for membrane integrity (Gigon et al., 2004). Previous studies have shown that lipid peroxidation increases with increasing H₂O₂ concentration (Upadhyaya et al., 2005). ROS induced oxidative stress in plants under drought may increase lipid peroxidation, cause degradation of protein, DNA fragmentation (Apel and Hirt, 2004). Proline accumulation is observed with drought stresses in all varieties under this study and stands higher than control leaves even after recovery (Table 2). The high content of proline under drought in *V. mungo* varieties indicates adaptive response to stress contributing to scavenging free radicals, stabilizing sub-cellular structures and also a part of stress signalling as reported in several studies (Verbruggen and Hermans, 2008).

Antioxidant defense mechanism plays an important role in the tolerance mechanism of plant to drought stress is evident from the observation of antioxidant enzyme activity in the present study. A statistically significant decrease in superoxide dismutase enzyme activity was noted under drought stress in all varieties under experiment which increased at recovery but not to the levels of the control plant (Fig. 4). It is to be noted that SOD generates H₂O₂ which as discussed may act as a signaling compound, but also increases cell damage, which could explain the decrease in SOD activity as drought induced lipid peroxidation and oxidative damage already results in high H₂O₂ concentration in the plant as indicated. Peroxidase activity of drought stressed plants enhanced significantly in all varieties of *V. mungo* under experiment (Fig. 5). Peroxidase activity remains increased even after recovery period of two days. Owing to increase in peroxidase activity drought stressed *V. mungo* plants are able to remove the excess H₂O₂ and maintain a balance between the ROS and antioxidant machinery which is essential to provide a proper biochemical environment within the cell. Previous studies have also indicated increased peroxidase activity as an adaptive response to tolerate environmental stress conditions (Bindschedler et al., 2006; Nerkararyan et al., 2013). Under drought stress,

the activity of CAT is found to increase in all varieties thus activating the detoxification mechanism (Fig. 6). It functions to catalyze the decomposition of hydrogen peroxide to water and oxygen and this reaction is important because if the cells did not break down the hydrogen peroxide, they would be poisoned and die. The activity of the catalase enzyme remains increased after recovery period. The activities of superoxide dismutase, peroxidase and catalase are well known as protective mechanism in both biotic and abiotic stress response (Arora et al., 2002; Noctor et al., 2002). The modulations of the antioxidant response in the different varieties of *Vigna* under study effectively work towards mitigation of the drought stress.

4. CONCLUSION

In the present study, it was observed that the pulse cultivars studied significantly affected by drought stress in terms reduction in chlorophyll and carbohydrate content, increase in lipid peroxidation which indicates membrane damage and is most likely to be triggered by H₂O₂. The modulation of antioxidant enzymes in an effort to combat this stress was also observed. Increased phenolic compounds may also indicate a protective mechanism as they act as antioxidants in plant tissue. During recovery, variations were noted in the different varieties and the variety PU 40 performed better than the other plants. The present study would facilitate application of optimal methods for increasing stress tolerance in the genotypes like application of salicylic acid or plant breeding interventions (Hajizadeh and Aliloo, 2013; Kundu et al. 2011).

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