International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2019; 7(2): 1864-1868

Received: 25-01-2019
Accepted: 27-02-2019

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Biochemical analysis of pearl millet (*Pennisetum glaucum* L.) under heat stress condition during flowering stage

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Abstract
Heat is considered as one of the environmental stress, which decreases crop productivity greatly compared with other environmental stress in India. Mechanisms of pearl millet plants in response to heat stress remain largely unknown. The experiment was conducted mainly in two parts. Short-term heat-induced biochemical responses were monitored in two pearl millet (*Pennisetum glaucum* L.) genotypes contrasting in their tolerance to heat stress. The seeds of two genotypes selected from first experiment, namely J-2454 (heat tolerant) and J-2433 (heat sensitive), were sown in pots containing soil and sand. The pots are irrigated every alternate day up to 50 (flowering) days after germination when each. RWC was found significantly highest 77.61% in heat tolerant genotype J 2454. The heat susceptible genotype J 2433 recorded the lowest (60.88 %) RWC. All the control plants of heat tolerant J 2454 and susceptible J 2433 genotypes showed lower protein content and free proline compared to treated plants.

Keywords: Heat stress, relative water content, protein, proline

Introduction
Pearl millet (*Pennisetum glaucum* L.) is a monocot with cross pollinating crop belonging to the family Poaceae and sub family Penicedae, having relatively small diploid genome (2n = 2x = 14) with DNA content of 1C of 2.36 pg (Budak *et al*., 2003) [1], with a genome size of 2350 Mb. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields in majority of the crop plants by more than 50%. Abiotic stress leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang *et al.*, 1995) [2]. High temperature is one of the most important constraints for agriculture. There is a risk that increased global temperature will change the optimum sites and conditions for crop production and thus affect agriculture. This condition is more tragic in the arid and semi-arid environments like in the Thar desert where crop production is highly unstable and unsustainable due to inhospitable climate and poor soil fertility status. Moreover, under hot arid situations high temperatures are associated with water stress and make abiotic stresses more compound consequently physiological and biochemical changes occur in as adaptive strategies. As accumulation of low-molecular-weight chaperones, compatible solutes such as proline, sugars, polyols are often regarded as a basic strategy for the protection and survival of plants under abiotic stress (Chen *et al.*, 2007) [8].

Materials and Methods
Plant growth condition, sampling description and heat treatments
One heat tolerant and heat susceptible pearl millet genotypes were selected. They were grown in pot filled with soil. One set grown up to flowering stage (50 days) and divided into two groups, control and heat treatments. Heat treatments (45°C and 50°C for 4h) were given using handmade Heating House at stages and samples were collected in ice bag. After heat treatments the leaf samples were taken out and leaves were weighed and then transferred immediately to the respective extracting medium for various analysis. The samples were extracted and stored in deep freeze refrigerator until analysis.

Relative water content
0.2g fresh leaves were weighed to record fresh weight (FW), followed by dipping half of their portion in Petri dish containing 30 ml distilled water for 12 h.

The leaves were blotted to wipe off excess water, weighed to record fully turgid weight (TW), Subject to oven drying at 70°C for 8 h to record the dry weight (DW) Turner (1986) [4].

$$\text{Relative Water Content (\%)} = \frac{\text{Fresh weight (g) – Dry weight (g)}}{\text{Turgid weight (g.) – Dry weight (g.)}} \times 100$$

Extraction and Estimation of Protein

One gram leaves was ground into 2 ml of 0.1M phosphate buffer (pH 7.0) with the help of mortar and pestle and transferred in to centrifuge tube and kept at room temperature for 24hrs. The content was centrifuged at 8,000 rpm for 15 min at 4 °C. Supernatant was collected and was used for protein estimation.

Reagents

1. Solution-A: 2% Na_2CO_3 in 0.1N NaOH.
2. Solution-B: 0.5% $CuSO_4$. $5H_2O$ in 1% sodium potassium tartrate (freshly prepared).
3. Solution-C: Alkaline copper solution (Mix solution A and B in the 49:1 ratio).
4. Std. protein solution: Bovine Serum Albumin (0.1 mg/ml).
5. Commercially available Folin - Ciocalteau reagent (dilute in 1:1 ratio with distilled water.)

True protein was estimated by the method, suggested by Lowry *et al.* (1951). The samples (0.1 ml) and standard BSA (0.5 - 3 ml from 0.1 mg/ml BSA stock) were taken in a series of test tubes and the volume was made up to 3.0 ml with distilled water. Then added 5 ml solution-C, mixed well and incubated at room temperature for 10 mins. Then added 0.5 ml Folin - Ciocalteau reagent, mixed immediately and incubated in dark for 30 mins. A reagent blank was prepared by taking only reagents and volume made up with distilled water. Light blue color was observed after incubation which measured at 660 nm in a spectrophotometer. The protein content was calculated by taking Bovine serum albumin as standard and value expressed as $mg.g^{-1}$.

Extraction and estimation of Free Proline

0.2 gm leaves was ground into 2 ml of 3% sulphosalicylic acid solution with the help of mortar and pestle and transferred in to centrifuge tube and kept at room temperature for 24 hrs. The content was centrifuged at 8,000 rpm for 15 min. Supernatant was collected and was used for estimation of free proline (Bates *et. al.* 1973) [5].

Reagents

1. Acid Ninhydrin reagent was prepared by mixing 1.25 g of ninhydrin in a solvent by 30 ml glacial acetic acid, 8 ml ortho phosphoric acid and 12 ml distilled water.
2. Pure glacial acetic acid,
3. Pure toluene
4. Std. proline solution: Proline (0.05 mg/ml).

Proline was estimated by using acid ninhydrin method described by Bates *et al.* (1973) [5]. The samples (0.3 ml) and standard proline (0.1 – 0.6 ml from 0.05 mg/ml proline stock) were taken in a series of test tubes and the volume was made up to 1.0 ml with distilled water. Then 2 ml gacial acetic acid and 2 ml acid ninhydrin reagent were added. Then tubes were kept in boiling water bath for 1 hour. The tubes were cooled in running water at room temperature. After that 4 ml toluene was added. The absorbance was recorded from toluene phase at 520 nm in spectrophotometer. The free proline was calculated by taking Proline as standard and expressed as $\mu g.g_{-1}$.

Results and Discussion

Relative water content (RWC)

The data on effect of heat stress and duration of heat stress on relative water content of heat tolerant and heat susceptible pearl millet genotypes are presented in Fig.1 and Table 1.

The mean difference of RWC for pearl millet genotypes was found to be significant. RWC was found significantly higher (82.22 %) in heat tolerant genotype J 2454 than that of in heat susceptible genotype J-2433 (66.77 %).

Table 1: Changes in relative water content (%) of pearl millet leaves at flowering stage in response to heat stress.

Genotype/ Treatment	Control	45 ºC	50 ºC	Mean
J 2454	87.42	81.64	77.61	82.22
J 2433	74.36	65.08	60.88	66.77
Mean	80.89	73.36	69.25	
		S.Em.±	C. V. %	
Genotype (G)		0.37	1.73	
Treatment (T)		0.46		
G X T		0.64		

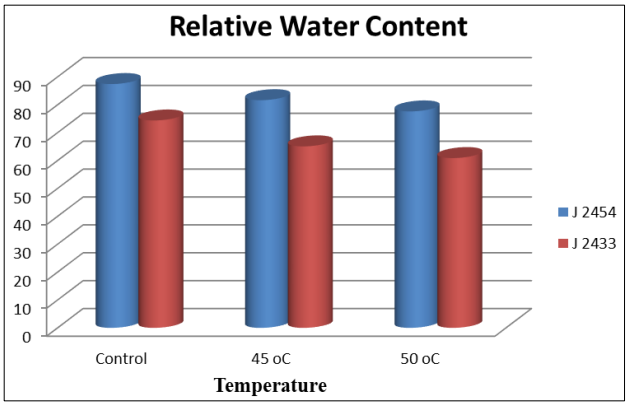

Fig 1: Effect of heat stress on relative water content (%) of pearl millet genotypes.

Between the heat treatments significant difference was found. The control plants (without heat treatment) contained the highest 80.89 % RWC and the heat treatment at 45 °C showed 73.36 % RWC and lowest 69.25 % RWC was observed at 50°C treatment. Irrespective of genotypes and heat treatments, mean value of duration of heat treatments showed significant difference.

Combined effect of genotypes and heat treatments was found to be significant. The highest (87.42%) RWC was recorded in control plants of heat tolerant genotype J 2454 followed by 81.64 % and 77.61 % at 45 °C and 50°C treatments respectively. The heat susceptible genotype J-2433 recorded 74.36 %, RWC in control plants while it was 65.08 % and 60.88 % at 45 °C and 50°C treatments respectively. The RWC was found to be the lowest (60.88%) in J-2433 susceptible genotype at 50°C treatment.

The effects of the genotypes, heat treatment and duration of heat treatment were significantly differed (Table 4.8). All the control plants of heat tolerant J-2454 and susceptible genotypes J-2433 showed higher RWC compared to treated plants. The highest (87.42 %) RWC was recorded in control plants of heat tolerant genotype J-2454 and lowest RWC (60.88%) found in J-2433 heat susceptible genotype when it was treated at 50°C. An increase in heat stress and duration of heat treatment resulted into reduction of RWC.

Manzer *et al.* (2015) [6] screened the tolerant and sensitive genotype(s) on the basis of morpho-physiological and biochemical characteristics of ten *Vicia faba* genotypes. The experimental work was undertaken to study the effects of different levels of temperature (control, mild, and modest) on plant height (PH) of plant, fresh weight (FW) and dry weight (DW) of plant, area leaf, content of leaf relative water (RWC), proline content (Pro) and total chlorophyll (Total Chl). Physiological parameter was decreased as compared to control.

Protein content

The data on effect of heat stress and duration of heat stress on Protein content of heat tolerant and heat susceptible pearl millet genotypes are presented in Fig. 2 and Table 2.

The mean difference of protein content for pearl millet genotypes was found to be significant. Protein content was found significantly higher (13.68 mg/ g Fr. Wt.) in heat tolerant genotype J 2454 than that of heat susceptible genotype J 2433 (10.14 mg/ g Fr. Wt.).

Between the heat treatments significant difference was found. The control (without heat treatment) plants of J 2454 genotype contained the 9.79 mg/ g Fr. Wt. protein content and the heat treatment at 45 °C showed 12.57 mg/ g Fr. Wt. and the 18.69 mg/ g Fr. Wt. protein content was observed at 50°C treatment. The control plants (without heat treatment) of J 2433 genotype recorded 5.95 mg/ g Fr. Wt. protein content but as temperature increased the protein content increased. It was recorded 9.19 mg/ g Fr. Wt. at 45 °C and 15.30 mg/ g Fr. Wt. at 50°C treatment.

Table 2: Effect of heat stress on protein content (mg /g. fr. Wt.) of heat tolerant and heat susceptible pearl millet genotypes at flowering stage.

Genotype/Treatment	Control	45 ºC	50 ºC	Mean
J 2454	9.79	12.57	18.69	13.68
J 2433	5.95	9.19	15.30	10.14
Mean	7.87	10.88	16.99	
		S.Em.±	C. V. %	
Genotype (G)		0.15	4.32	
Treatment (T)		0.18		
G X T		0.26		

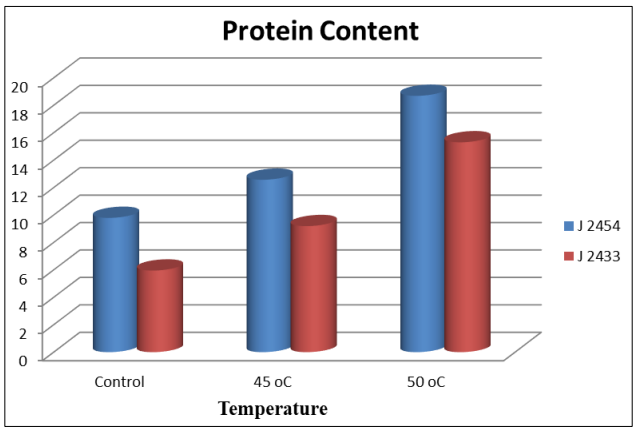

Fig 2: Effect of heat stress on protein content (mg /g. fr. Wt.) of pearl millet genotypes.

Irrespective of genotypes and heat treatments, mean value of duration of heat treatments showed significant difference. Mean value of duration of heat treatment showed that pearl millet plants had significantly higher 13.68 mg/ g Fr. Wt. in control as compared to treatment.

Combined effect of genotypes and heat treatments was found to be significant. The lowest (9.79 mg/ g Fr. Wt.) protein content was recorded in control plants of heat tolerant genotype J 2454 followed by 12.57 mg/ g Fr. Wt. and 18.69 mg/ g Fr. Wt. at 45 °C and 50°C treatments respectively. The heat susceptible genotype J-2433 recorded 5.95 mg/ g Fr. Wt. in control plants while at 45 °C and 50°C the values were 9.19 mg/ g Fr. Wt. and 15.30 mg/ g Fr. Wt. respectively.

The effects of the genotypes, heat treatment and duration of heat treatment were significantly differed. All the treated plants of heat tolerant J-2454 and susceptible genotypes J-2433 showed higher protein content compared to control plants control. The highest (18.69 mg/ g Fr. Wt.) protein content was recorded in 50°C plants of heat tolerant genotype J-2454 and lowest protein content (5.95 mg/ g Fr. Wt.) found in plant of control J-2433 plant of heat susceptible genotype. An increase due to of heat treatment resulted into increase of protein content

Heat shock proteins are molecular chaperones which help the plant to tolerate the extreme heat shock condition by protecting the native protein from denaturation. Plants cope with heat stress in a complex manner, where heat shock proteins (HSPs) might play a central role in the complex cellular network (Baniwal *et al.*, 2004) [7]. Differential expression of carbohydrate biosynthesis and metabolic pathway enzymes, it was also inferred that plants need high energy to cope with heat stress. Our finding supported by Han *et al.*, (2009) where they studied that the responses of rice seedlings to different high-temperature stresses, at 35 °C, 40 °C and 45 °C for 48h. At 35°C, some protective mechanisms were activated to maintain the photosynthetic capability. At 40°C, antioxidative pathways were also active.

When seven day old rice seedlings encountered high-temperature stress at 45°C, in addition to those induced at 35°C and 40°C, heat shock proteins were effectively induced so the protein content also increased as temperature increased. Khalil *et al.*, (2009) [9] carried out two successive seasons to alleviate the harmful effects of high temperature stress (35°C ± 2) on wheat cultivar (Giza 168) by the application of arginine or putrescine (0.0, 1.25 and 2.5 mM). They found that the appearance of new proteins in wheat shoots subjected to the high temperature stress are heat shock proteins of molecular weights 111, 90, 70, 45, 32, 24 and 8 KD a and they were accumulated under high temperature.

Free Proline content

The data on effect of heat stress and duration of heat stress on proline content of heat tolerant and heat susceptible pearl millet genotypes are presented in Fig.3 and Table 3.

The mean difference of proline content for pearl millet genotypes was found to be significant. Proline content was found significantly higher (4.27 mg %) in heat tolerant genotype J 2454 than that of The heat susceptible genotype J 2433 recorded the lowest (3.54 mg %).

Between the heat treatments significant difference was found. The control (without heat treatment) plants of J 2454 genotype contained the 1.56 mg % proline content and the heat treatment at 45 °C showed 3.05 mg % and the 8.19 mg % proline content was observed at 50°C treatment. The control plants (without heat treatment) of J 2433 genotype recorded 1.22 mg % but as temperature increased the proline content increased. It was recorded 2.57 mg % at 45 °C and 6.82 mg % at 50°C treatment.

Irrespective of genotypes and heat treatments, mean value of duration of heat treatments showed significant difference.

Mean value of duration of heat treatment showed that pearl millet plants had significantly lower 1.39 mg % in control as compared to treatment.

Combined effect of genotypes and heat treatments was found to be significant. The lowest (1.56 mg %) proline content was recorded in control plants of heat tolerant genotype J 2454 followed by 3.05 mg % and 8.19 mg % at 45 °C and 50°C treatments respectively. The heat susceptible genotype J-2433 recorded 1.22 mg % in control plants while at 45 °C and 50°C the values were 2.57 mg % and 6.82 mg % respectively.

The effects of the genotypes, heat treatment and duration of heat treatment were significantly differed (Table. 3). All the treated plants of heat tolerant J-2454 and susceptible genotypes J-2433 showed higher proline content compared to control plants. The highest (8.19 mg %) proline content was recorded in 50°C plants of heat tolerant genotype J-2454 and lowest protein content (1.22 mg %) found in control plant of J-2433 heat susceptible genotype. An increase in heat stress of heat treatment resulted into increase of proline content.

Table 3: Effect of heat stress on proline (mg %) of heat tolerant and heat susceptible pearl millet genotypes at flowering stage.

Genotype/Teatment	Control	45 ºC	50 ºC	Mean
J 2454	1.56	3.05	8.19	4.27
J 2433	1.22	2.57	6.82	3.54
Mean	1.39	2.81	7.51	
		S.Em.±	C. V. %	
Genotype (G)		0.06	5.56	
Treatment (T)		0.08		
G X T		0.11		

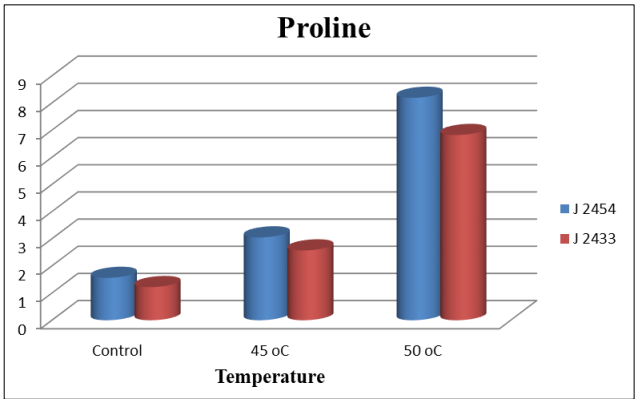

Fig 3: Effect of heat stress on Proline (mg %) of pearl millet genotypes

Harsh *et al.* (2016) [10] Heat stress leads to an array of physiological, biochemical, and molecular changes in plants affecting its growth and development. An experiment was conducted to find out the effect of short-term heat stress on osmo-protectant and antioxidants in 37 genotypes (32 mutants and five varieties) of moth bean (*Vigna aconitifolia*). Seeds were grown in plastic pots containing sterilized vermiculite. Heat stress conditions were created by exposing seven days old seedlings at 42 °C for one hour in hot air oven. Analysis of various parameters was carried out at three days after heat stress. A significant over-accumulation of total sugar and proline along with an increased activity of CAT, GPOX and SOD was observed in most of the genotypes under heat stress.

Summary and Conclusion

Some abiotic constraints such as high temperature, cold, drought, high concentrations of toxic minerals and salinity results in severe loss of crop yield and quality. Heat stress becomes major constraint in loss of crop production due to global warming. The total area of wheat production affected by some form of heat stress is estimated to be 65 to 70 million ha. Of these, 7 million ha are grown under continual heat stress.

RWC was found significantly highest 77.61% in heat tolerant genotype J 2454. The heat susceptible genotype J 2433 recorded the lowest (60.88 %) RWC. The highest (87.42 %) RWC was recorded in control plants of heat tolerant genotype J 2454 followed by 81.64 % and 77.61 % at 45 °C and 50°C treatments respectively. The heat susceptible genotype J2433 recorded 74.36 %, RWC in control plants while it was 65.08% and 60.88 % at 45 °C and 50°C treatments respectively. All the control plants of heat tolerant and susceptible genotypes J 2454 and J 2433 showed higher RWC compared to treated plants.

Protein content was found significantly highest (18.69 mg/ g Fr. Wt.) in heat tolerant genotype J 2454. The lowest protein content was of (9.79mg/ g Fr. Wt.) in control plants of heat tolerant genotype J 2454 followed by 12.57 mg/ g Fr. Wt. and 18.69 mg/ g Fr. Wt. at 45 °C and 50°C treatments respectively. The heat susceptible genotype J 2433 recorded 5.95 mg/ g Fr. Wt. protein content in control plants while it was 9.19 mg/ g Fr. Wt. and 15.30 mg/ g Fr. Wt. at 45 °C and 50°C treatment respectively. All the control plants of heat tolerant J 2454 and susceptible J 2433 genotypes showed lower protein content compared to treated plants.

Proline content was found significantly highest 8.19 mg % in heat tolerant genotype J 2454. The heat susceptible genotype J 2433 recorded the lowest (1.22 mg %) proline content. The lowest proline content was of (1.56 mg %) in control plants of heat tolerant genotype J 2454 followed by 3.05 and 8.19 mg % at 45°C and 50°C testaments respectively. The heat susceptible genotype J 2433 recorded 1.22 mg % proline content in control plants while it was 2.57 and 6.89 mg% at 45 °C and 50°C treatments respectively. All the control plants of heat tolerant genotype J 2454 and heat susceptible genotype J 2433 showed lower proline content compared to treated plants.

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