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Temperature Induction Response and Multivariate Analysis Approaches to Screen Blackgram (*Vigna mungo* L.) Genotypes for Thermotolerance

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ABSTRACT

Background: In the current climate change scenario, high temperature stress is one of the major constraints limiting the yield of blackgram severely. The increasing global temperature is targeting the yield of blackgram by causing severe reproductive failures. In order to combat this problem, it is important and inevitable to screen blackgram genotypes that can withstand to high temperature and produce better yield even under adverse climatic conditions. Keeping this in view, the present investigation was carried out.

Methods: Hundred blackgram genotypes were subjected to Temperature induction response technique, in which lethal temperature was standardized as 54°C for 3 hours and optimum induction temperature as 36-46°C. The selected genotypes from temperature induction response technique were further evaluated for biochemical traits using hierarchial clustering and principal component analysis.

Result: The cellular level tolerance was assessed in blackgram genotypes using standardized lethal and optimum induction temperature. Based on the percent reduction in the seedling growth of induced over control and survival percentage, 27 blackgram genotypes were categorized as highly tolerant, 54 genotypes as moderately tolerant and 19 genotypes as susceptible. Biochemical characters such as antioxidant defence activity, lipid peroxidation and free radicals were analyzed in TIR induced 27 thermotolerant and 3 susceptible genotypes. The results indicated that all the traits such as total seedling length and antioxidant defence enzymes except malondialdehyde and free radicals showed a prominent increase under heat stress. Based on hierarchial clustering, 30 genotypes were clustered as 5 groups: tolerant (cluster-3,4), moderately tolerant (cluster-1,2) and susceptible (cluster-5). Principal component analysis showed that first five components showed 75.8% of total variation in control, whereas 63.3% of the total variance was covered by the first two PCs in heat induced conditions. The information generated from the study would help the breeders in developing heat tolerant varieties that perform better even under extreme high temperatures.

Key words: Antioxidant defense, Blackgram, Hierarchial clustering, Multivariate analysis, Principal component analysis, Temperature induction response, Thermotolerance.

INTRODUCTION

Blackgram is a short duration pulse crop which is grown in all the seasons all over India. It accounts for about 10% of total pulse production in India (Modgil *et al*., 2019). Despite a significant growth in area and productivity of blackgram in India over the past 20 years, the production is not still satisfactory. One of the major reasons for this yield gap in blackgram is due to extreme weather events such as heat waves, drought and unpredicted rainfall etc. due to climate change. In particular, heat stress caused by elevated temperatures is strongly affecting plant growth and development, which leads to drastic reduction in the economic yield (Partheeban and Vijayaraghavan, 2017). Global air temperature is predicted to rise by 0.2°C per decade, which will lead to 1.8-4.0°C higher temperatures than the current level by 2100 (Hasanuzzaman *et al*., 2013). Blackgram being a thermosensitive crop, its yield is sensitive to high temperature above 35°C which leads to massive flower and pod drop resulting in low or no yield (Anitha *et al*., 2015). Hence, there is a need to screen the blackgram genotypes for thermotolerance to reduce the effects of high temperature stress.

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At field level, various screening methods are being used to screen crops on physiological basis for thermotolerance. However, all the physiological parameters at field level are greatly influenced by environmental conditions, which is the major drawback (Selmani and Wasson, 1993). In addition to this, breeding for heat tolerance also became complicated due to lack of efficient screening methods. Therefore, it became very important to develop an *in vitro* screening method for precise screening of crops for thermotolerance. Under these circumstances, Temperature Induction Response technique (TIR) has been evolved as an effective screening method to screen various crops for thermotolerance. Numerous studies have indicated that thermotolerance gained through the TIR approach ensures thermo-protection at the mature level.

Heat stress leads to the production of Reactive Oxygen Species (ROS). The ROS causes oxidative degradation of polyunsaturated fatty acids further leading to deterioration of membranes. Malondialdehyde (MDA) is the product of oxidative degradation. MDA can be used as a reliable marker to measure oxidative damage. Among all the enzymatic antioxidants, Superoxide Dismutase (SOD) acts as a first line of defence against ROS which catalyses O_2^- to H_2O_2 and O_2, whereas Peroxidase (POX) is able to scavenge H_2O_2 through various mechanisms (Gill and Tuteja, 2010).

Genotypes vary in their ability to withstand changing environmental extremities. Assessment of genetic variability among the genotypes can be possible through multivariate analysis approaches. Multivariate analysis is one of the effective method in assessing the degree and nature of divergence of functional characters in the available germplasm. It also helps in the identification of desirable traits governing heat stress tolerance in the thermotolerant genotypes. A set of advance statistical procedures including multivariate analysis techniques such as principal component analysis (PCA) and cluster analyses have been utilized for the further grouping of heat tolerant and susceptible genotypes using morphological and biochemical traits. The main aim of the experiment is to screen the blackgram genotypes for thermotolerance using TIR technique and multivariate approaches.

MATERIALS AND METHODS

An invitro experiment was conducted during 2021-22 at the Department of Crop Physiology, Agricultural College, Bapatla in Acharya N.G. Ranga Agricultural University. Hundred blackgram genotypes were screened using TIR technique for acquired thermotolerance. The seed material of 100 blackgram genotypes was procured from AICRIP (pulses), Regional Agricultural Research Station (RARS), Lam, Guntur, Andhra Pradesh, India. TIR technique involves in the identification of lethal temperature followed by standardization of sublethal temperature for screening blackgram genotypes for thermotolerance. It was followed in accordance with Partheeban *et al.* (2017) with slight modifications.

Identification of lethal temperature

Three days old blackgram seedlings were exposed to various lethal temperatures, such as 48, 49, 50, 51, 52, 53, 54, 55 and 56°C for 3 hours in the temperature controlled heat chamber to determine the lethal temperature for TIR technique. Following the heat treatment, the seedlings were left to recover for 72 hours in the same chamber at 30°C with 60% relative humidity.

The survival percentage of seedlings was recorded at the end of recovery period. The temperature at which more than 90% mortality of seedlings occurred was identified as lethal temperature. At 54°C, 94% mortality of seedlings was observed, hence, 54°C was considered as the lethal temperature in our study. For standardizing TIR technique, at each temperature three replications were maintained with 25 seedlings per petriplate.

Survival per cent (%) =

$$\frac{\text{Number of seedlings survived}}{\text{Total number of seedlings}} \times 100$$

Standardization of optimum induction temperature

Three days old uniform seedlings of blackgram were exposed to different induction temperatures for 3 hours at the rate of 2°C per every half an hour following which they were transferred to identified lethal temperature of 54°C for 3 hours. The sets of different induction temperatures are as follows: 32-42°C, 36-46°C, 38-48°C and 42-52°C. Following the heat treatment, the same sets of seedlings were transferred to 30°C for 72 hours for acclimation to heat stress. Observations such as survival percentage, total seedling length (TSL), per cent reduction in the seedling length over control were recorded. A separate set of seedlings were maintained at 30°C which was served as absolute control.

Then the blackgram genotypes were categorized as highly tolerant (0-30%), Moderately tolerant (30-50%) and Suceptible (50-90%) based on the per cent reduction in growth of seedlings over absolute control.

Growth during recovery (GDR) =

Growth at the end of recovery (GER)- Growth at the end of induction (GER)

Per cent reduction in growth =

$$\frac{\text{GDR of control - GDR of induced}}{\text{GDR of control}} \times 100$$

Measurement of biochemical traits in TIR seedlings

Various biochemical traits such as proline, SOD, POX, CAT, APOX, MDA, Superoxide radical and hydrogen peroxide were analyzed in the selected TIR induced blackgram seedlings both under control and heat induced conditions. After exposure of seedlings to TIR technique, biochemical parameters were measured in young leaves both under control and heat induced conditions.

Estimation of proline and antioxidant defence enzymes at cellular level for intrinsic heat tolerance

Proline content

The amount of proline was assayed according to Bates *et al.* (1973). Leaf samples (500 mg) was homogenized in 3% aqueous sulfosalicylic acid (10 mL) and filtered through Whatman No. 42 filter paper. Two millilitres of acid ninhydrin (1.25 g ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 M phosphoric acid) and 2 mL of glacial acetic acid were heated for 1 h at 100°C. The reaction mixture was extracted with 4 mL of toluene and mixed vigorously for 15-20 s and the absorbance of the toluene layer measured spectrophotometrically at 520 nm using toluene as the blank.

Superoxide dismutase (SOD) activity

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) using the method of Dhindsa *et al.* (1981). 0.2 g of leaf sample was homogenized in 10 ml of 0.5M phosphate buffer containing 1% NEDD. The homogenate was centrifuged at 4°C for 30 min at 10000 rpm. The 3 mL reaction mixture contained 1.5 mL phosphate buffer, 0.2 mL of methionine solution. 0.1 mL of riboflavin was added at the end and the tubes were shaken and placed 10 cm below a light source consisting of two 15 W fluorescent lamps. The reaction was started by switching on the light and was allowed to run for 15 min. The reaction was stopped by switching off the light and the tubes were covered with a black cloth. The absorbance of the reaction mixture was read at 560 nm. A non irradiated reaction mixture did not develop colour and served as control. One unit SOD activity was defined as the amount of enzyme required to 50 per cent inhibition of the rate of NBT reduction at 560 nm.

Peroxidase (POX) activity

The extraction and assay of POX was carried out as per the method described by Putter (1974). The reaction mixture contained 3 mL 0.1 M phosphate buffer (pH 7.0), 0.05 mL 20 mM guaiacol, 0.03 mL H_2O_2 and 0.5 mL enzyme. Enzyme activity was detected by increase in the absorbance at 436 nm min^{-1}. The activity of POX was expressed in terms of the enzyme units g^{-1} fwt.

Catalase (CAT) activity

Catalase (CAT) activity was determined spectrophotometrically by measuring the rate of H_2O_2 disappearance at 240 nm (Aebi, 1974). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) and 10.5 mM H_2O_2. The reaction was run at 25°C for 2 min, after adding the enzyme extract and rate of decrease in absorbance at 240 nm. Catalase activity was calculated by decrease in H_2O_2 absorbance at 240 nm and expressed in µg H_2O_2 $min^{-1}g^{-1}$.

Ascorbate peroxidase (APX) activity

Ascorbate peroxidase (APX) was assayed by the method as described by Nakano and Asada (1981). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid and 0.25 mM H_2O_2. The reaction was started at 25°C by the addition of H_2O_2 after adding the enzyme extract. The decrease in absorbance at 290 nm for 1 min was recorded and the amount of APX was calculated from the extinction coefficient 2.8 mM^{-1} cm^{-1}.

Determination of lipid peroxidation

The amount of MDA derived from unsaturated fatty acid peroxidation of membrane lipids was measured according to the method of Sese and Tobita (1998). 250 mg leaf sample was weighed and homogenized with 5 ml of 0.1% TCA. 1mL supernatant was taken and 4 mL of 20% TCA containing 0.5% TBA was added. The mixture was heated at 95°C for 30 min. The content was cooled in an ice bath and again centrifuged at 10000 rpm for 10 min. The absorbance of was measured at 532 nm and the result was expressed in nmol g^{-1}.

Quantification of free radicals

Superoxide free radical was quantified by its capacity to reduce nitroblue tetrazolium chloride (NBT) by following the method of Chaitanya and Naithani (1994). 0.1 g of leaf sample was homogenized in 2 mL of precooled phosphate buffer (0.2 M, pH 7.2). The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. A 3 mL reaction mixture contained 100 µL supernantant in 0.75 mM NBT, 25 mM sodium carbonate, 0.1 mM EDTA and 13.3 mM L-Methionine. Reaction mixture was incubated at 30°C in a waterbath for 10 min and absorbance was recorded at 540 nm.

Hydrogen peroxide was estimated by formation of titanium-hydroperoxide complex (Mukherjee and Choudhari, 1983). Leaf samples were ground in 3 mL cooled acetone in a chilled mortar and pestle. The homogenate was filtered through Whatsman No. 1 filterpaper followed by addition of 2 mL of titanium reagent and 2.5 mL of ammonium hydroxide solution to precipitate the titanium hydroperoxide complex. The reaction mixture was centrifuged at 10,000 rpm for 10 min. The precipitate was dissolved in 5 mL of 2 M concentrated sulphuric acid and recentrifuged. The supernatant was read at 415 nm aganist blank and H_2O_2 was expressed as µmol $H_2O_2g^{-1}$fwt.

Statistical analysis

The data generated from screening experiments was statistically analyzed by using completely randomized block design (CRD) (Gomez and Gomez, 1984). The data collected from the biochemical traits was subjected to multivariate analysis, including cluster analysis and principal component analysis. Cluster analysis was performed using hierarchial clustering approach with two way clustering to generate a tree diagram based on Euclidean distances by Wards method. Cluster analysis was done using statistical package SAS-JMP whereas PCA was performed using XLSTAT software.

RESULTS AND DISCUSSION

Standardization of lethal and optimum induction temperatures

The lethal temperature in this experiment was standardized as 54°C for 3 hours at which 90% mortality of seedlings

Table 1: Standardization of sub-lethal temperature using TIR technique.

Treatments	Standardization of optimum induction temperature		
	Shoot and root length (cm)		
	Growth at the end of induction (GEI)	Growth at the end of recovery (GER)	Growth during recovery (GDR)
Control			
Shoot	3.30	13.68	10.38
Root	1.95	4.38	2.43
32-42°C			
Shoot	3.25	7.92	4.67
Root	1.90	2.50	0.60
36-46°C			
Shoot	3.28	8.78	5.50
Root	1.95	2.95	1.00
38-48°C			
Shoot	3.28	8.66	5.38
Root	1.93	2.91	0.98
42-52°C			
Shoot	3.24	8.21	4.97
Root	1.90	2.62	0.72

Table 2: Influence of TIR on seedling growth and per cent reduction in seedling growth over control.

Treatments	Seedling growth (cm)		Per cent reduction in seedling growth over control
	Growth at the end of recovery (GER)	Growth during recovery (GDR)	
Control	18.06	12.81	NA
32-42°C	10.42	5.27	58.86
36-46°C	11.73	6.5	49.26
38-48°C	11.57	6.36	50.35
42-52°C	10.83	5.69	55.58

was observed. The optimum induction temperature was standardized as 36-46°C at which 49.26% reduction in seeding growth over control was observed (Table 1 and 2).

Genotypic variability was observed among the genotypes with respect to seedling length and percent survival. Based on percent reduction in seedling growth over control all the blackgram genotypes were categorized. Among the hundred blackgram genotypes, 27 genotypes such as LBG-1016, LBG-1004, LBG-752, TBG-129, PU-1804, LBG-1015, LBG-918, LBG-997, PUSA B-58, LBG-904, LBG-1009, GBG-1, TBG-141, VBN-8, LBG-989, LBG-995, OBG-48, PU-31, LBG-1006, Tutiminumu, LBG-1010, LBG-932, PU-1822, TBG-104, GBG-645, LBG-999 and LBG-996 recorded higher survival percentage and less percent reduction in seedling growth over control indicating their tolerance to high temperature. Nineteen genotypes showed lowest survival percentage and highest per cent reduction in seedling growth indicating their sensitivity to high temperature. Among these 19 heat sensitive genotypes, 3 genotypes such as TBG-125, PU-1801 and LBG-1023 showed extremely lowest survival percentage and highest percent growth reduction indicating higher susceptibility to high temperatures (Fig 1 and 2).

Similar method of standardization of challenging and induction temperature has been practiced in blackgram by (Partheeban *et al*., 2017). The report of Senthil kumar, 2001 stated that exposure to optimum induction temperatures facilitate the expression of stress responsive genes. Our results are in conformity with Partheeban *et al*. (2017).

Effect of heat stress on biochemical activities

Proline, antioxidant activity, lipid peroxidation and free radicals were estimated in the blackgram seedlings both under control and heat induced conditions. From the present study, it was revealed that all the tolerant genotypes exhibited a significant increase in proline and all the antioxidant defence enzymes under heat induction conditions in order to cope up with heat stress induced ROS injury. Under heat

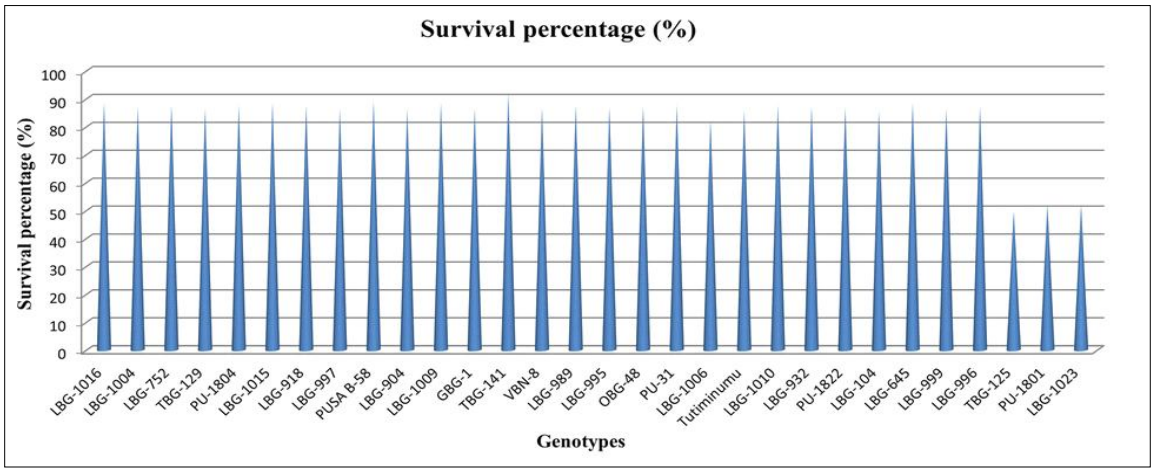


Fig 1: Effect of 36-46°C temperature induction cycle on survival percentage of selected contrasting blackgram genotypes.

induced conditions, the per cent increase in proline accumulation was highest in PU-1804 (50.38%) whereas lowest in TBG-125 (22.60%). The per cent increase in SOD activity over control was highest in PU-1804 (74.48%), whereas lowest in PU-1801(20.00%). The percent increase over control with respect to peroxidase activity was highest in LBG-1004 (76.38%) followed by TBG-141 (76.12%), whereas lowest in TBG-125 (19.06%). The CAT activity was highest in LBG-1004 (62.99%) whereas it was lowest in PU-1801 (9.84%). APOX activity was increased by 56.78% in TBG-141. On the other hand, decrease in APOX activity was noticed in all the susceptible genotypes (Fig 3).

All the tolerant genotypes showed a marginal rise in free radicals which might be due to scavenging activity of antioxidant defence enzymes. The highest rise in free radicals was noticed in susceptible genotypes under heat stress indicating higher oxidative damage in the susceptible genotypes. MDA accumulation was also more in the susceptible genotypes *i.e*., TBG-125 (26.04%), LBG-1023 (22.55%) and PU-1801 (23.98%) which might be due to increased ROS production which caused lipid peroxidation leading to membrane damage (Fig 4).

Thus, from the above results, it can be concluded that heat stress caused more pronounced accumulation

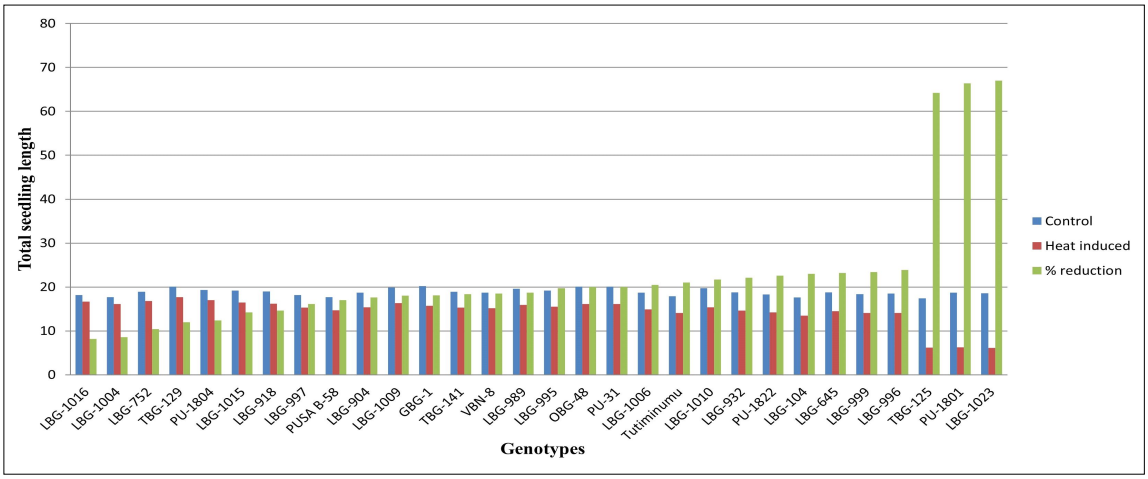


Fig 2: Effect of 36-46°C temperature induction cycle on total seedling length of selected contrasting blackgram genotypes under both control and heat induced conditions.

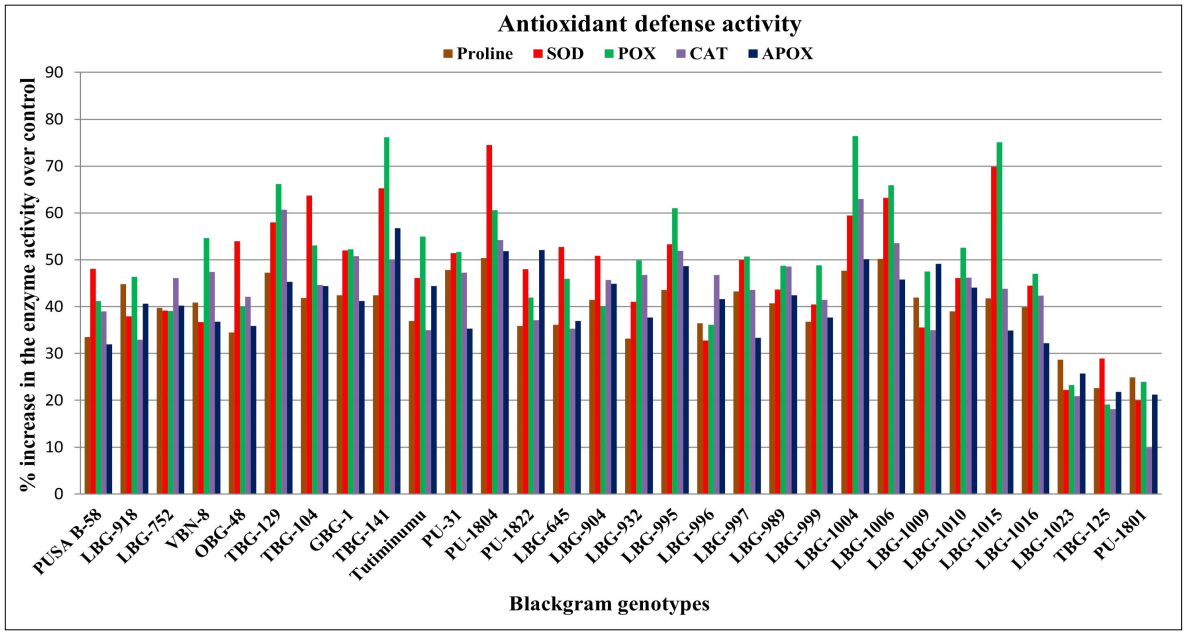


Fig 3: Per cent increase in proline and antioxidant defence enzyme activity in the blackgram genotypes under heat induced conditions.

of antioxidant defence enzymes in thermotolerant genotypes than susceptible genotypes which might be the reason behind the thermotolerance. The current results concur with the published reports of Partheeban *et al*. (2017), who reported the positive accumulation of proline, SOD and POX activity in heat tolerant blackgram genotypes.

Cluster analysis

Hierarchial clustering analysis was performed to cluster the selected 27 thermotolerant and 3 susceptible blackgram genotypes based on total seedling length and biochemical traits under control and heat induced conditions. Under

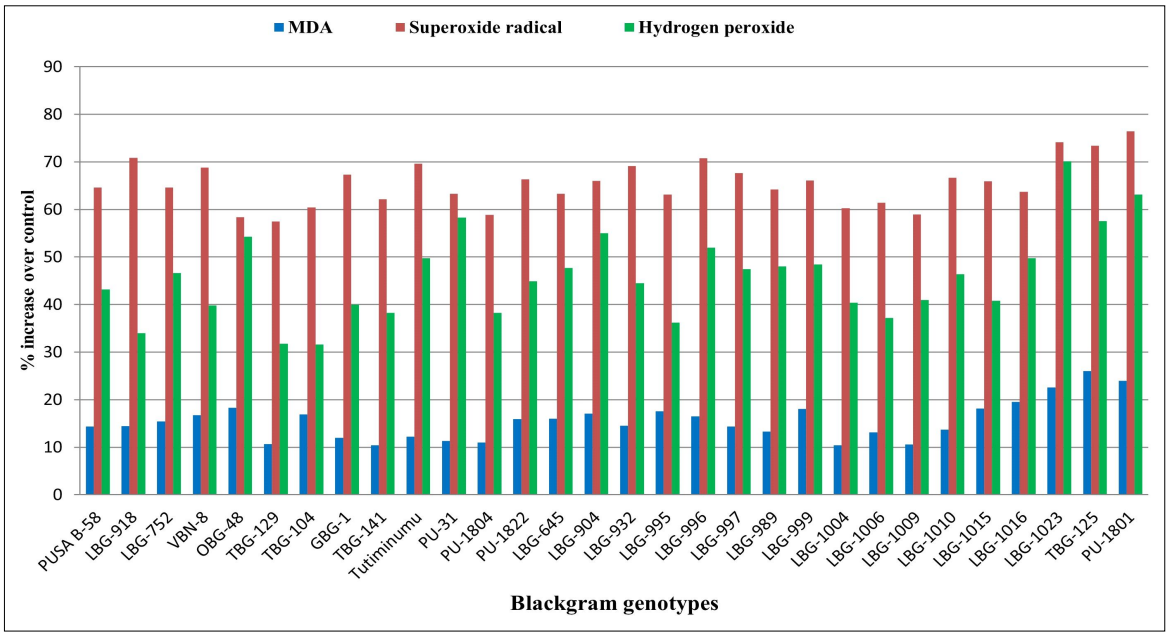
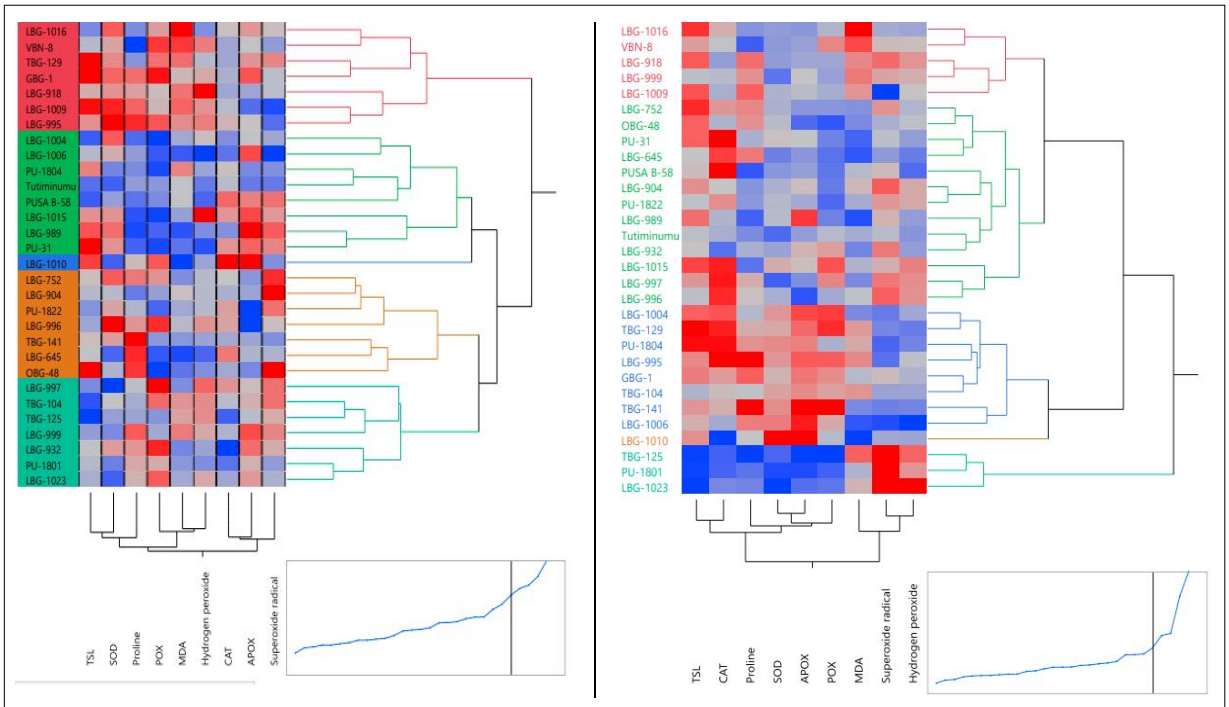


Fig 4: Per cent increase in MDA and free radicals accumulation in the blackgram genotypes under heat induced conditions.



Different colours representing genotypes based on performance. Distance graphs below the cluster diagrams are representing the threshold to decide number of possible clusters.

Fig 5: Hierarchial clustering of 30 blackgram genotypes for biochemical traits under control (left) and heat induced conditions (right).

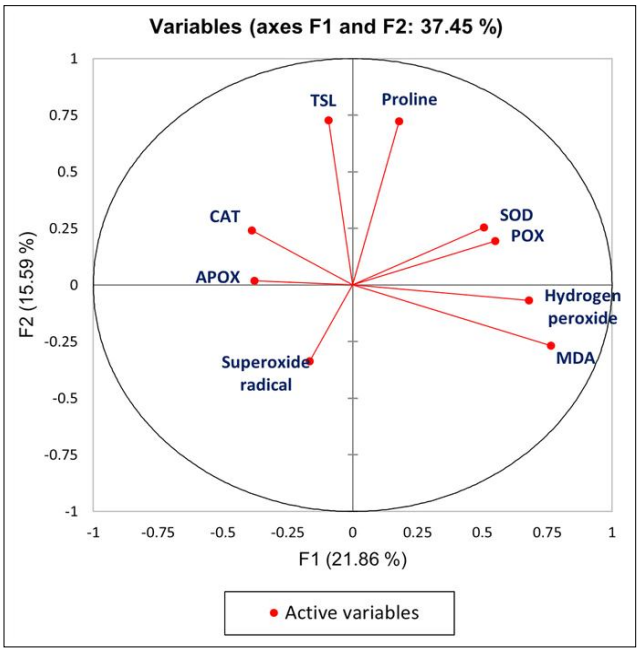


Fig 6: Principal component analysis (PCA) of various biochemical traits in blackgram genotypes under control conditions.

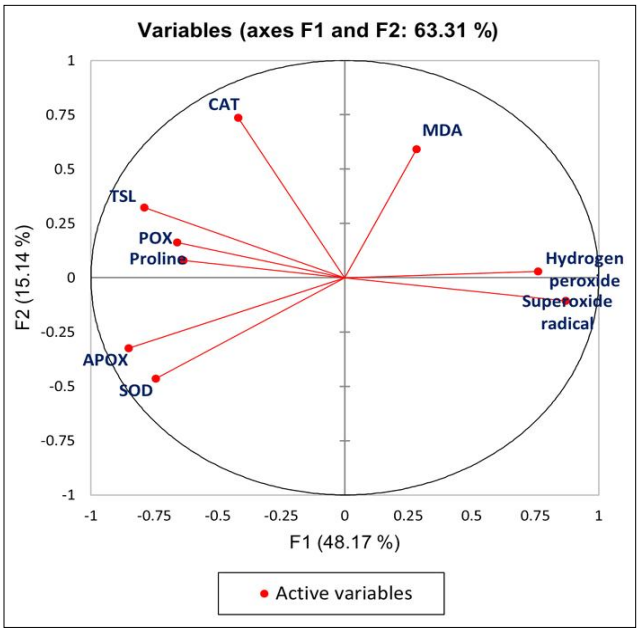


Fig 7: Principal component analysis (PCA) of various biochemical traits in blackgram genotypes under heat induced conditions.

control conditions, 30 genotypes were clustered into 5 groups. Groups 1,2,3,4 and 5 consist of 7,8,1,7 and 7 genotypes, respectively. Under heat induced conditions, 30 genotypes were clustered into 5 groups. Group 1,2,3,4 and 5 contained 5, 13, 8, 1, 3 genotypes, respectively (Fig 5). The genotypes of cluster 3 and 4 showed higher TSL, antioxidant defence activity, osmolyte accumulation and lower lipid peroxidation and lower ROS accumulation under heat induced conditions have been designated as heat tolerant genotypes. The genotypes in cluster 1 and 2 have shown a slight decrease in biochemical activity have been designated as moderately tolerant. The fifth cluster with green colour based on the declined biochemical performance under heat stress has been designated as susceptible. Based on clustering, under heat stress conditions, the genotypes LBG-1004, TBG-129, LBG-995, PU-1804, GBG-1, TBG-104, TBG-141, LBG-1006 and LBG-1010 were superior for most of the traits studied. Our results of clustering were in accordance with the reported research previously by Zafar *et al*. (2021) in cotton.

Principal component analysis

The PCA was performed in TIR induced blackgram genotypes based on biochemical traits under control and heat induced conditions. In control conditions, the first five PCs with more than 1 eigan value explain 75.8 % of total variance whereas 63.3% of total variance was explained by first two PCs in heat induced conditions. Biplots of the investigated traits in TIR induced blackgram genotypes in control and heat induced conditions are depicted in Fig 6 and 7, respectively. The biplot of control conditions represents a strong positive correlation of POX with SOD. Under heat induced conditions, TSL and all the antioxidant defence enzymes such as POX, SOD, APOX and proline are positively correlated with each other whereas antioxidant defence enzymes such as proline, APOX, POX, SOD and CAT showed negative correlation with free radicals. SOD showed a strong negative correlation with superoxide radical indicating that increased SOD activity scavenged the superoxide radicals. Similarly, CAT, POX and APOX showed a negative correlation with hydrogen peroxide indicating the detoxification of hydrogen peroxide by antioxidant defence enzymes. The occurrence of negative correlation of antioxidant defence enzymes with free radicals under heat induced conditions indicating that strong antioxidant defense activity might be the reason for heat tolerance at cellular level. Moreover, the free radicals such as superoxide radical and hydrogen peroxide were positively correlated with MDA reflecting increase in the membrane damage due to lipid peroxidation leading to MDA accumulation.

By employing this principal component analysis genetic variability of blackgram genotypes was successfully exploited and conserved by dividing into different principal components. This information could be used for improving specific traits contributing heat tolerance in breeding programmes. Our results were in accordance with the published reports of Manivannan *et al.* (2007) who reported that an increase in CAT and SOD activity are directly linked to an increase in stress tolerance, since it is associated with scavenging H_2O_2 and superoxide radical, respectively.

CONCLUSION

From the present investigation, it is more evident that TIR technique is the most precise and reliable method to screen

large number of blackgram genotypes for thermotolerance within short period of time. Cluster analysis was performed in the selected 30 blackgram genotypes for biochemical traits to explore genetic variability both under control and heat induced conditions. Under heat induced conditions, the genotypes in the cluster 3 and 4 such as LBG-1004, TBG-129, PU-1804, LBG-995, GBG-1, TBG-104, TBG-141, LBG-1006 and LBG-1010 showed higher antioxidant activity, osmolyte accumulation and lower lipid peroxidation and ROS accumulation. The identified thermotolerant genotypes can be used in hybridization programme to develop superior transgressive segregants. The results of PCA revealed that antioxidant enzymes showed a negative correlation with free radicals indicating tolerance at cellular level. For further confirmation, the expression analysis of stress responsive proteins in thermotolerant blackgram genotypes would be needed. The blackgram genotypes will be further assessed for reproductive efficiency and yield under field conditions during summer.

It was further concluded that our findings have paved the way for the identification thermotolerant genotypes based on their intrinsic tolerance to high temperatures at cellular level. Biochemical basis for thermotolerance of blackgram genotypes at cellular level was analyzed thoroughly in our study. Further work can be focussed on assessing molecular basis of thermotolerance in the identified thermotolerant and susceptible genotypes which help in identifying genes responsible for thermotolerance.

Conflict of interest

All authors declared that there is no conflict of interest.

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