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The Rapid and Reliable Method to Assess the Blackgram (*Vigna mungo* (l.) Hepper) Genotypes for Thermotolerance

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Abstract

High temperature stress in plants reduces crop yield because it negatively affects several plant physiological processes, including photosynthesis, respiration, growth, development and partitioning. Pulses are more sensitive to high temperature stress at reproductive stage. Blackgram is an important source of protein and widely used in daily diet. In this study, lethal temperature and induction temperature was standardized for blackgram genotypes by using Temperature Induction Response (TIR) technique. The standardization of induction temperature and lethal temperature is based on per cent growth reduction and survival percentage at the end of recovery period. The induction temperature was standardized as 36 to 46 °C for 3 h and the lethal temperature as 52 °C for 3 h. A total of nineteen blackgram genotypes were screened and evaluated for thermo tolerance. By using standardized optimum induction and challenging temperature, cellular level tolerance was assessed in all the blackgram genotypes. Based on root length and shoot length of induced seedlings over control seedlings, the cellular level tolerance in terms of least reduction in growth and highest survival percentage was calculated. Also, the physiological basis of thermo tolerance was assessed by measuring the proline content and antioxidant enzyme activities. Thermotolerant genotypes show higher survival percentage and lower growth reduction. Further the tolerant genotypes identified based on TIR also showed higher antioxidant enzymes activity implying the critical role of antioxidant in cellular thermotolerance. The genotypes VBG-07-001, VBG-06-010 has shown intrinsic heat tolerance and therefore they can be explored as donor source in breeding programme aimed for global warming.

Keywords: Blackgram, thermotolerance, antioxidant enzyme, proline, survival percentage

1. Introduction

Blackgram [*Vigna mungo* (L.) Hepper] is a short duration legume crop grown in India. India ranks first in the world in terms of pulses production (25.5% of total world's production) (FAOSTAT, 2014). But pulses are very sensitive to drought, water logging and high temperature. Recently, high temperature is implicated as a major limiting factor for yield decline particularly when flowering and anthesis coincides with temperature rise (Onoriode Coast et al., 2014).

The main principle in induction response technique is to initially expose seedlings plants^{-1} to a less severe temperature before they are challenged with severe temperature and subsequently recovery growth is measured. The seedling growth and recovery growth is considered as criteria to arrive at optimum acclimation stress levels (Senthil Kumar et al., 2006). Thermo-protection on exposure to acclimation treatment was also observed not only in seedlings but also in mature plant level (Vijayalakshmi et al., 2015). They also reported that acclimated plants showed significantly higher leaf recovery growth compared to plants that were directly exposed to challenging temperature.

Therefore, evaluating the relative performance of blackgram genotypes for high temperature tolerance using TIR technique is the main objective.

2. Materials and Methods

2.1. Experimental details

The experiment was conducted during the month of June, 2014 at the Department of Crop Physiology, Tamil Nadu (India) Agricultural University, Coimbatore. Nineteen blackgram genotypes were used for the study and screened for thermo-tolerance. This approach of TIR involves first the identification

of challenging temperature and induction temperature and later standardizing them before being used for screening the germplasms for intrinsic tolerance. The protocol used in this study is described here.

2.2. Identification of lethal temperature (Challenging temperature)

To identify the lethal temperature for Temperature Induction Response Technique, three day old blackgram seedlings were exposed to different challenging temperatures such as 48, 50 and 52 °C for 3 hrs in the temperature controlled heat chamber. After the heat treatment, the seedlings were allowed to recover at 30 °C with 60% relative humidity for 72 hr in the same chamber.

At the end of recovery period, survival per cent of the seedlings was measured to arrive at the challenging temperature. The minimum temperature at which 90% mortality of seedlings occurs was taken as lethal or challenging temperature. Three replications of 25 seedlings petriplate-1 were maintained for standardizing TIR technique. Figure 1 explains the step wise

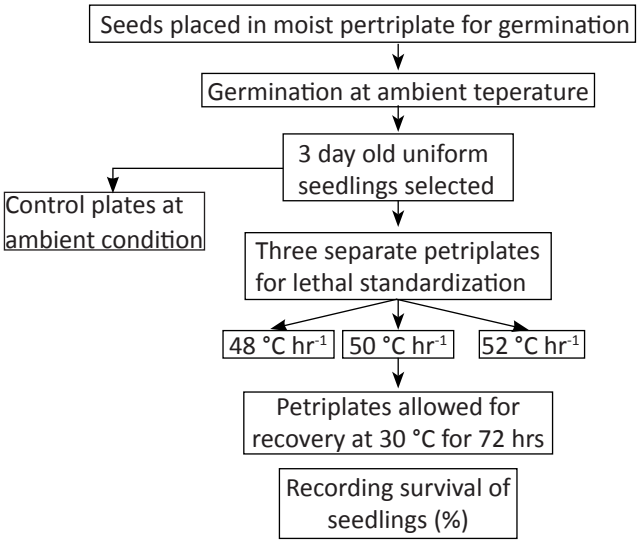

Figure 1: Flow chart for standardizing lethal temperature

protocol for arriving at the lethal temperature.

$$\text{Survival per cent (\%)} = \frac{\text{No. of seedlings survived}}{\text{Total number of seedlings}} \times 100$$

2.3. Standardization of optimum induction temperature for TIR

Three day old blackgram seedlings were subjected to different induction temperature gradually for 6 hrs at the rate 2 °C increment hr^{-1} following which they were transferred to a standardized challenging temperature. The different induction temperatures set for standardization were as follows:

32–42 °C; 36–46 °C; 38–48 °C

After exposure to these induction temperatures, the seedlings were exposed to a lethal temperature of 50 °C for three hours. Again the same sets of seedlings were subjected to 30 °C for 72 hrs for acclimation process of heat stress. After this, observations on seedling survival per cent, shoot length and root length were measured for assessing the seedling growth.

Based on the decline in growth of seedlings under TIR treatment, the blackgram genotypes could be categorized as Susceptible (50–90%), Moderately Tolerant (30–50%) and Highly Tolerant (0–30%) for high temperature stress based on the overall seedling growth. The seedlings which were maintained at 30 °C served as the absolute control. For standardization of induction and lethal temperature, the blackgram genotype COBG-759 was used as the test variety. The protocol for temperature induction response is explained in Figure 2. In addition, the following parameters were computed to assess the induction response.

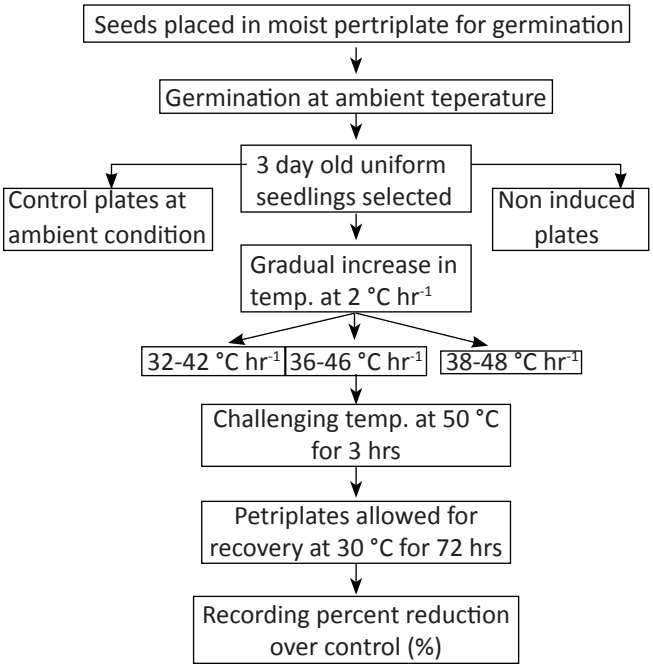

Figure 2: Flow chart for standardizing optimum induction temperature

(a) Growth during recovery (GDR)

Growth at the end of recovery-Growth at the end of induction

(b) Per cent reduction of growth

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

(c) Per cent germination of the seedling

$$\frac{\text{No. of seedlings germinated at the end of the recovery}}{\text{Total no. of seedlings taken for the experiment}} \times 100$$

2.4. Measurement of cellular level tolerance using TIR

By using standardized optimum induction and challenging temperature, cellular level tolerance using TIR protocol was carried out in all the blackgram genotypes. Based on root

length and shoot height of induced over control seedlings, the cellular level tolerance in terms of least reduction in growth and highest survival percentage was calculated.

2.5. Estimation of proline in leaves at plant level

The estimation of proline content was adopted from Bates et al. (1973) with slight modifications. After lethal temperature treatment, the seedlings (1 g) were homogenized with 10 ml of 3% sulphosalicylic acid and centrifuged at 3000 rpm for 10 minutes. Two mL of the supernatant was taken and 2 ml of glacial acetic acid and 2 ml of acid ninhydrin mixture were added. The contents were allowed to react at 100 °C for 1 hr and then it is incubated on ice for 10 minutes to terminate the reaction. The reaction mixture was mixed vigorously with 4 mL toluene for 15–20 seconds. The chromophore containing toluene was aspired from the aqueous phase, warmed to room temperature and optical density was read at 520 nm. The proline content was determined from the standard graph prepared using commercially available proline in the concentration range of 20–100 µg.

2.6. Estimation of antioxidant enzymes

Peroxidase activity (change in OD value at 430 nm g^{-1} min^{-1}) was determined according to Angelini et al. (1990). Catalase activity was assayed from the rate of H_2O_2 decomposition extinction coefficient of 39.4 mmol as measured by the decrease in the absorbance at 240 nm, following the procedure of Aebi (1974).

2.7. Estimation of malondialdehyde (MDA) content

The amount of MDA derived from unsaturated fatty acid peroxidation of membrane lipids was measured according to the method of Sese and Tobita (1998). The result was expressed in nmol g^{-1}.

2.8. Statistical analysis

The data on various parameters were analyzed statistically as the procedure^{-1} suggested by Gomez and Gomez (1984). Wherever the treatment differences were found significant, critical differences were worked out at five per cent probability level and the values were presented in the relevant tables

3. Results and Discussion

3.1. Standardization of lethal and optimum induction temperature

The challenging lethal temperature was standardized as 50 °C at which 98% of the seedling mortality was noticed. The induction temperature was standardized as 36 to 40 °C at which 46.4% of growth reduction over control was noticed (Table 1 and 2).

The biometric parameters such as seedling growth and survival percent (%) indicated genotypic variability. The per cent growth reduction was observed to be very minimal such as 13.1 and 14.5 in VBG-06-002 and VBG-07-001. In contrast to this VBG-10-024 showed highest growth reduction of 53.9% indicating the sensitivity towards heat stress. The survival per cent under heat treatment showed a mean value of 95 irrespective of the genotypes. It is seen that almost 100% survival was noticed in the heat tolerant genotypes namely VBG-06-002, VBG-06-005, VBG-10-008, COBG-11-02, COBG-759 (Table 3). Several studies have indicated that TIR technique would be a useful tool to identify the thermo tolerance even at the seedling stage. Attempts to identify heat tolerance from TIR indicated that, the so called heat tolerant genotypes identified both in the field experiment as well as under controlled environment studies produced the same genotypes possessing inherent heat tolerance mechanisms. The report by (Sira et al., 2016) suggest that genetic variability cannot be visualized when the plants are directly exposed to severe stress but only upon acclimation to prior severe stress (Sapna Harihar et al., 2014).

Table 1: Impact of Temperature Induction Response on growth of seedling (Shoot length and root length in cm) and per cent reduction over control

Treatments	Growth of seedling (Shoot length and root length in cm)		Per cent reduction over control
	Growth at the end of recovery	Growth during recovery	
Control	14.4	9.77	NA
32–42 °C	8.4	3.90	60.1
36–46 °C	9.92	5.24	46.4
38–48 °C	9.61	4.97	49.2

Table 2: Standardization of temperature induction response (Shoot height and root length in cm)

Temp. Regimes (Treatments)	Parameter		
	Growth at the end of Induction	Growth at the end of recovery	Growth during recovery
T_1 Control			
Shoot	2.93	10.73	7.81
Root	1.70	3.67	1.97
T_2 32–42 °C			
Shoot	2.81	6.47	3.66
Root	1.69	1.93	0.24
T_3 36–46 °C			
Shoot	2.93	7.37	4.44
Root	1.75	2.55	0.80
T_4 38–48 °C			
Shoot	2.93	7.10	4.17
Root	1.71	2.51	0.80

Table 3: Measurement of cellular level tolerance of blackgram genotypes using Temperature Induction Response (TIR) technique

Sl. No.	Genotypes	Root length (cm)		Shoot length (cm)		Seedling growth (RL+SL) (cm)		Survival per cent		Per cent growth reduction
		Control	Induced	Control	Induced	Control	Induced	Control	Induced	
1.	VBG-04-003	3.5	3.0	8	4.1	11.5	7.1	100	96	38.2
2.	VBG-04-005	3.2	2.9	7.5	4.0	10.7	6.9	100	96	35.5
3.	VBG-06-002	3.2	2.7	8.2	7.2	11.4	9.9	100	100	13.1
4.	VBG-06-003	6.9	3.4	13.9	7.4	20.8	10.8	100	92	47.6
5.	VBG-06-005	5.1	3.9	12.2	9.3	17.3	13.2	100	100	23.6
6.	VBG-06-009	5.5	3.4	13.5	5.6	19.0	9.0	100	92	52.3
7.	VBG-06-010	3.8	3.2	10.8	8.0	14.6	11.2	96	92	22.8
8.	VBG-07-001	3.9	3.0	9.2	8.2	13.1	11.2	100	99	14.5
9.	VBG-08-003	3.7	2.1	9.8	4.4	13.5	6.5	100	90	51.8
10.	VBG-10-008	3.9	3.2	9.5	8.2	13.4	11.4	100	100	14.9
11.	VBG-10-024	4.7	3.0	11.5	4.4	16.2	7.4	100	95	53.9
12.	VBN-6	3.4	2.9	9.7	5.4	13.1	8.3	100	96	36.6
13.	VBN-7	2.0	2.0	7.6	4.2	9.6	6.2	100	96	35.4
14.	CO-6	3.7	2.0	9.3	4.2	13.0	6.2	100	88	52.3
15.	COBG-11-02	3.1	2.3	6.6	4.7	9.7	7.0	100	100	27.7
16.	COBG-11-03	3.0	2.1	12.4	5.1	15.4	7.2	100	88	53.2
17.	COBG-10-05	4.9	3.8	14.8	9.4	19.8	13.2	100	90	33.3
18.	COBG-10-06	3.1	3.1	15.2	7.4	18.3	10.5	100	95	42.7
19.	COBG-759	3.7	3.2	10.4	7.4	14.1	10.7	100	100	24.1
	Mean	3.9	2.9	10.5	6.2	14.4	9.2	99.8	95.0	35.4
		G	T	G×T	G	T	G×T	G	T	G×T	G	T	G×T	
SEd±		0.03	0.01	0.05	0.09	0.03	0.13	0.13	0.04*	0.18	0.92	0.29	1.30	
CD (*p*=0.05)		0.07*	0.02*	0.10*	0.18*	0.06*	0.26*	0.26*	0.08*	0.37*	1.82*	0.59*	2.58*	

G: Genotype; T: Treatment; G×T: Genotype and treatment interaction; *: Significant

Henceforth, an attempt to standardize TIR for blackgram has culminated in isolation and identification of six promising genotypes which were earlier designated as heat tolerant genotypes. Hence, the methodology described for standardizing TIR for blackgram can be followed for identifying heat tolerant blackgram genotypes.

3.2. Effect of temperature induction response on proline content (µg of proline g^{-1} of FW) in blackgram genotypes at seedling stage

Proline is a free amino acid that has been implicated as an osmolyte leading to the accumulation under any stress situation. Proline content was estimated in nineteen blackgram genotypes (Table 4) that has been subjected to TIR studies. It is seen that the heat treatment invariably led to the accumulation of proline and there is a significant genotypic variability for this character. Heat treatment showed a 0.4 µg g^{-1} of increment when compared to untreated control irrespective of the genotypes. The proline content under control ranged from 0.53 µg g^{-1} in VBG-06-003 to as much as 0.96 in VBG-06-010. Under heat treatment, the proline content ranged from 0.71 in VBG-06-009 to 1.80 in VBG-06-002 registering 46.2% and 47.2% increase over control. On the other hand VBG-10-024 showed the lowest per cent increase over control in the proline content. In general there was an increase of mean (37.3%) over control under heat treatment irrespective of the genotypes. It was further noticed that the accumulation of proline was much more pronounced in the case of heat tolerant genotypes. In seven day old, TIR seedlings proline accumulation was more in quantity in the tolerant genotypes when compared to susceptible ones. Accumulation of proline was reported to be associated with stress tolerance (Zhong, 2013). The present investigation was

thus is in conformity with the findings of Harsha et al. (2016).

Table 4: Effect of temperature induction response on proline content (µg g^{-1}) of blackgram seedlings

Sl. No.	Genotypes	Control	Induced	% change over control
1.	VBG-04-003	0.88	1.30	32.3
2.	VBG-04-005	0.72	1.19	39.5
3.	VBG-06-002	0.95	1.80	47.2
4.	VBG-06-003	0.53	0.89	40.4
5.	VBG-06-005	0.94	1.70	44.7
6.	VBG-06-009	0.49	0.71	31.0
7.	VBG-06-010	0.96	1.70	43.5
8.	VBG-07-001	0.57	1.06	46.2
9.	VBG-08-003	0.77	1.10	30.0
10.	VBG-10-008	0.91	1.50	39.3
11.	VBG-10-024	0.88	1.24	29.0
12.	VBN-6	0.67	0.99	32.3
13.	VBN-7	0.69	1.05	34.3
14.	CO-6	0.82	1.20	31.7
15.	COBG-11-02	0.79	1.40	43.6
16.	COBG-11-03	0.71	1.02	30.4
17.	COBG-10-05	0.76	1.19	36.1
18.	COBG-10-06	0.80	1.25	36.0
19.	COBG-759	0.54	0.93	41.9
Mean		0.8	1.2	37.3
		G	T	G x T
SEd±		0.008	0.002	0.011
CD (*p*=0.05)		0.016*	0.005*	0.023*

3.3. Effect of temperature induction response on antioxidant enzymes in blackgram genotypes at seedling stage

Any biotic stress invariably showed an increased activity of Reactive oxygen Species and heat stress is no more an exception. Two genotypes each from heat tolerant, moderately heat tolerant and heat susceptible groups were evaluated for peroxidase, catalase and also malondialdehyde content in the seedlings under TIR. It seen that there is a general increased activity of peroxidase and catalase due to high temperature. The mean peroxidase activity ranged from 16.9 mg g^{-1} FW min^{-1} in COBG-11-03 to as like as 24.4 in VBG-07-001 irrespective of the treatments (Table 5).

The high peroxidase activity in response to high temperature was recorded in VBG-07-001 of 27.03 mg g^{-1} FW min^{-1}. Coming to the catalase activity VBG-08-003 registered 13.3 mg g^{-1} FW min^{-1} as the lowest value and VBG-07-001 showed high catalase activity of 19.5 irrespective of the treatments (Table 5). Under heat stress, again VBG-07-001 recorded the highest value of 20.54 mg g^{-1} FW min^{-1}.

Malondialdehyde is a compound resulting from lipid peroxidation. It is known that abiotic stress particularly heat stress is expected to disorganize the cell wall components. Due to this malondialdehyde will accumulate depending upon the degree of heat. It is seen that the heat stress has shown an increment of 2.6 µ mol g^{-1} of malondialdehyde irrespective of the genotypes. The highest accumulation of 20.22 µ mol g^{-1} of MDA was recorded in COBG-11-03 which is designated as heat susceptible genotype. On the other hand the heat stress has caused MDA accumulation of 12.23 in VBG-06-010 which was the lowest value among the six genotypes evaluated.

Thus, it is seen that the heat tolerant genotypes have recorded higher values of free radicals enzymes such as catalase, peroxidase in the seedling. On the other hand the malondialdehyde has shown accumulation in response to heat stress and interestingly it is evident that the degree of

Table 5: Effect of temperature induction response on POX, CAT and MDA in blackgram genotypes at seedling stage

Sl. No.	V. No	Genotype	Peroxidase (POX) activity (mg g^{-1} FW min^{-1})			Catalase (CAT) activity (mg g^{-1} FW min^{-1})			Malondialdehyde (MDA) content (µmol g^{-1})		
			Control	Induced		Control	Induced		Control	Induced	
1.	V10	VBG-06-010	20.33	25.32		16.11	20.54		11.54	12.23	
2.	V9	VBG-07-001	21.74	27.03		18.00	21.09		12.69	13.55	
3.	V23	VBN-6	18.66	20.20		13.65	18.15		13.23	15.64	
4.	V26	COBG-11-02	19.25	22.56		14.21	18.97		14.11	17.31	
5.	V27	COBG-11-03	14.38	19.45		10.73	14.55		16.89	20.22	
6.	V17	VBG-08-003	15.27	20.11		11.25	15.28		18.35	23.76	
		Mean	18.3	22.4		14.0	18.1		14.5	17.1	
			G	T	G×T	G	T	G×T	G	T	G×T
SEd±			0.193	0.111	0.272	0.077	0.044	0.108	0.183	0.105	0.258
CD (*p*=0.05)			0.391*	0.226*	0.553*	0.156*	0.090*	0.221*	0.371*	0.214*	0.525*

malondialdehyde accumulation is much more pronounced in the case of heat susceptible genotypes. The antioxidant profile of the representation group indicates that the catalase and peroxidase enzymes had invariably positive accumulation while MDA showed drastic reduction. Again the heat tolerant genotypes showed more pronounced accumulation of antioxidant enzymes while decline in the rate of enzyme in case of MDA. Possibly the accumulation of antioxidant enzyme is indicative of more response towards heat while there is a less degeneration of cell wall components as indicated by MDA contents. This was well documented by Patrice et al. (2013) in support of this investigation.

In our study, the genotype VBG-06-010, VBG-07-001 has shown less malondialdehyde accumulation even under heat stress indicating the superiority of these genotypes for thermo tolerance.

4. Conclusion

Present investigation has paved way for identifying superior genotypes tolerant to high temperature. The genotype VBG-06-002 is planned to use as a donor, the other genotype VBG-07-001 will be evaluated further in multi-location trials and form a new variety for heat tolerance especially for the hot spot areas not only for Tamil Nadu and also for neighbouring states.

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