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Histochemical Staining and SDS-PAGE Analysis of Blackgram Genotypes Subjected to Temperature Induction Response Technique

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The study was conducted at Department of Crop Physiology, Agricultural College, Bapatla in Acharya N.G Ranga Agricultural University. The results of the histo-chemical staining revealed that the genotypes TBG-129, PU-1804 and TBG-104 showed lesser free radical accumulation on the surface of the leaves indicating tolerance to high temperatures. The protein profile as evidenced by SDS page analysis indicated distinct expression of band between 23-30 KDa protein in tolerant genotypes such as TBG-129, PU-1804, LBG-995, PU-31, TBG-104, TBG-141, LBG-1004, LBG-1015 and LBG-996. Moreover, the band between 23-30 KDa was denser in TBG-129 and PU-1804. This expression of protein might be an indicative feature of thermotolerance.

Keywords: Blackgram; histochemical staining; SDS-PAGE; thermotolerance; temperature induction response.

1. INTRODUCTION

"Blackgram (*Vigna mungo* L.) is cultivated as short duration fallow crop after rice cultivation in India" [1]. "India currently represents the largest producer of blackgram which accounts for more than 70 % of global production. In India, blackgram is cultivated in an area of 46.33 lakh hectares with an annual production of 27.75 lakh tonnes and productivity of 4.24 lakh tonnes and 1059 kg ha^{-1}" [2]. "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" [3]. Development of suitable varieties and genotypes of black gram with adaptation to local agro-climatic conditions is an important factor for its increase and improved production.

"At field level, it is difficult to screen large number of genotypes for thermotolerance as it is influenced by environmental factors. In addition to this, breeding for heat tolerance also became complicated due to lack of efficient screening methods. Keeping this in view, a laboratory protocol called temperature induction response (TIR) technique was used to screen blackgram genotypes for thermotoerance. In response to heat stress, plants upregulate several heat inducible genes which are commonly referred as "heat shock genes" (HSGs). HSGs encode heat shock proteins (HSPs) and these active products are very much necessary for plant's survival under fatal HT" [4]. "These HSPs act as molecular chaperones and imparts thermotolerance to the plants. They protect intracellular proteins from being denaturation and preserve their stability and function through proper protein folding" [5]. "The expression of HSPs is restricted to certain developmental stages of plant like seed germination, embryogenesis, microsporogenesis and fruit maturation" [6]. In plants, HSPs can be grouped into five different families *viz.,* HSP 100, HSP 90, HSP 70, HSP 60 and HSP 20 (or small HSP, sHSP). Therefore, the expression of different classes of HSPs can be observed by protein profiling of thermotolerant and thermosusceptible blackgram genotypes identified in TIR technique.

2. 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. This technique involves in the identification of lethal temperature followed by standardization of sublethal temperature for screening blackgram genotypes for thermotolerance. The TIR technique was followed in accordance with Partheeban *et al.* [7] with slight modifications. The lethal temperature in this experiment was standardized as 54℃ for 3 hours at which 90% mortality of seedlings was observed. The optimum induction temperature was standardized as 36 – 46 ℃ at which 49.26% reduction in growth over control was observed. Based on the per cent reduction in the seedling length over control of the seedlings exposed to the induction cycle of 36-46 ℃, hundred blackgram genotypes were categorized as highly tolerant (0-30 %), moderately tolerant (30-50 %) and susceptible (50-90 %). Histochemical staining was performed in the twenty thermotolerant blackgram genotypes such as LBG-645, TBG-129, PU-1804, PU-1822, LBG-752, LBG-932, PUSA B-58, LBG-997, LBG-995, GBG-1, PU-31, TBG-104, VBN-8, LBG-1006, LBG-1010, LBG-999, TBG-141, LBG-1004, LBG-1015 and LBG-996 and

three susceptible genotypes such as TBG-125, LBG-1023 and PU-1801 identified through TIR technique.

2.1 Histochemical Staining for Free Radicals

ROS was detected in the leaves of blackgram genotypes subjected to TIR technique by histochemical staining for hydrogen peroxide and superoxide radicals.

2.2 Hydrogen Peroxide

Leaves of blackgram genotypes subjected to TIR technique and control were stained in sodium phosphate buffer (10 mM; pH 7.0) containing DAB (1 mg mL^{-1}) at 25 ℃ in dark for 1 h. Stained leaves were bleached in ethanol until the complete elimination of chlorophyll, and examined under the stereomicroscope (LEICA 9i stereomicroscope).

2.3 Superoxide Radical

Leaves of blackgram genotypes subjected to TIR technique and control were stained in 10 mM potassium phosphate buffer, pH 7.8, containing 0.1 % NBT and 10 mM sodium azide at 25 ℃ in dark for 1 h. Stained leaves were bleached in ethanol until the complete elimination of chlorophyll, and examined under the stereomicroscope (LEICA 9i stereomicroscope).

2.4 SDS-PAGE Profiling in TIR Seedlings

Immediately after the temperature induction response, the heat induced blackgram seedlings were finely powdered with the help of liquid nitrogen and to it 2 mL of extraction buffer (1M Tris-HCl, pH-6.8) was added. The content was centrifuged at 6000 rpm for 10 min at 4 °C. After centrifugation, supernatant was collected. 5 ml of pre-chilled solution containing 50 % acetone, 10 % TCA and 60 µL ß-mercaptoethanol was added to it, kept at 4 °C overnight and again centrifuged at 6000 rpm for 10 min. The pellet was collected, washed three times with acetone containing 0.07 % ß-mercaptoethanol, dried and dissolved in extraction buffer for overnight. The quantification of protein was done by using Lowry's method. To analyze the heat stress induced changes in protein profiles of blackgram genotypes, protein analysis was done by the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as per the method reported by Laemmli [8]. The gel was submerged in staining solution and kept overnight (12–16 h) at room temperature. Destaining was carried out by placing the gel in destaining solution (50% methanol, 10% glacial acetic acid) in water until clear bands are visible. After complete destaining, the banding pattern was analyzed.

3. RESULTS AND DISCUSSION

3.1 Superoxide Radical

Nitro Blue Tetrazolium (NBT) dye was used for the detection of the accumulation of superoxide radicals on the surface of leaves of blackgram genotypes subjected to TIR technique. A significant enhancement in the level of superoxide radicals was observed in all the blackgram genotypes after exposure to heat treatment compared to control. The blackgram genotypes TBG-129, OBG-48, LBG-1009, PU-1804 and TBG-104 showed less blue color staining zones on the leaf surface indicating lesser accumulation of superoxide radicals, and higher antioxidant defence activity in these genotypes. Whereas, the susceptible genotypes TBG-125, LBG-1023 and PU-1801 showed more intense blue crystal patches over the leaf surface indicating higher accumulation of superoxide radicals, and lower antioxidant defense activity (Fig 1 and 2). Similar findings of more accumulation of superoxide radicals on the leaf surface of 12 days old blackgram seedlings subjected to drought was previously reported in blackgram by Singh *et al.* [9] and Gupta *et al.* [10].

3.2 Hydrogen Peroxide Radical

Diaminobenzidine dye was used to detect the H_2O_2 accumulation on the surface of leaves of blackgram genotypes subjected to TIR technique. A substantial increment in the H_2O_2 accumulation was observed on the surface of leaves of all the blackgram genotypes after exposure to heat treatment. The blackgram genotypes TBG-104, TBG-129, LBG-918, LBG-995 and PU-1804 showed less dark brown staining zones on leaf surface indicating lesser accumulation of hydrogen peroxide radicals and higher antioxidant defense activity. Whereas, the susceptible genotypes TBG-125, LBG-1023 and PU-1801 showed more dark brown staining over the leaf surface indicating higher accumulation of superoxide radicals and lower antioxidant defense activity (Fig 3 and 4). Similar findings of more accumulation of hydrogen peroxide radicals on the leaf surface on exposure to high

temperatures was previously reported in blackgram by Rakavi and Sritharan [11].

3.3 SDS-PAGE Analysis in TIR Induced Seedlings

Small heat shock proteins (sHSPs) are a class of heat shock proteins which are ubiquitously produced both in prokaryotic and eukaryotic cells in response to heat stress, having size ranging from 15 to 42 kDa Sun *et al.,* [12].

SDS-PAGE analysis was performed in 20 tolerant and 3 susceptible blackgram genotypes selected from TIR technique revealed variations in protein profiles (Fig 5, 6 and 7). Protein bands were observed at 7 KDa, 23-30 KDa and 80-175 KDa. Among the different protein bands observed, a band at 7 KDa was observed in LBG-645, TBG-129, PU-1804, PU-1822, LBG-752, LBG-932, LBG-997, PUSA B-58 and LBG-995. A band between 23-30 KDa was observed in genotypes such as TBG-129, PU-1804, LBG-995, PU-31, TBG-104, TBG-141, LBG-1004, LBG-1015 and LBG-996 which might be due to the enhanced expression of heat protective genes during induction period, promoted the production of heat shock proteins (HSPs) and enzymatic antioxidants such as SOD, CAT, POX and APOX for adaptation of plant to high temperature stress. The band between 23-30 KDa was denser in TBG-129 and PU-1804. In addition to this, a conspicuous band between 80-175 KDa was observed in GBG-1, PU-31, TBG-104, VBN-8, LBG-1006, TBG-125, LBG-999, TBG-141, LBG-1023, LBG-1004, LBG-996 and PU-1801.

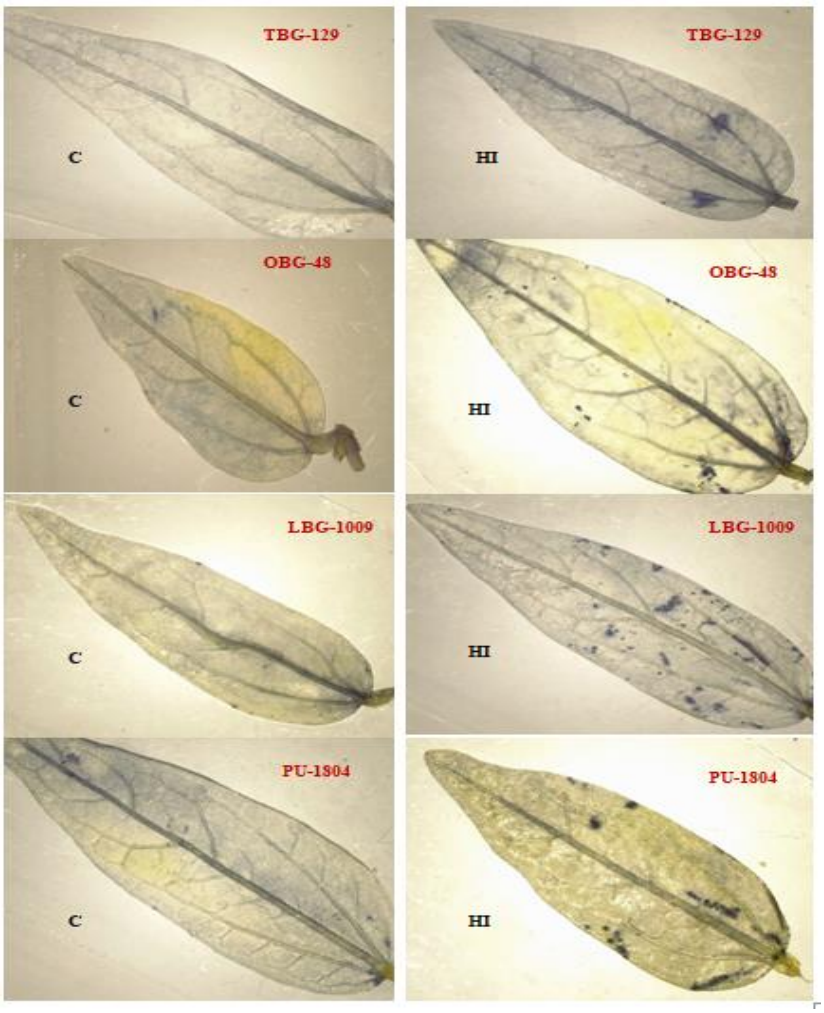

Fig. 1. Histochemical staining for detecting superoxide radical accumulation in the control and TIR induced seedlings of thermotolerant blackgram (TBG-129, OBG-48, LBG-1009 and PU-1804) genotypes. (Photographs were taken with digital camera mounted on stereomicroscope at 10X magnification)

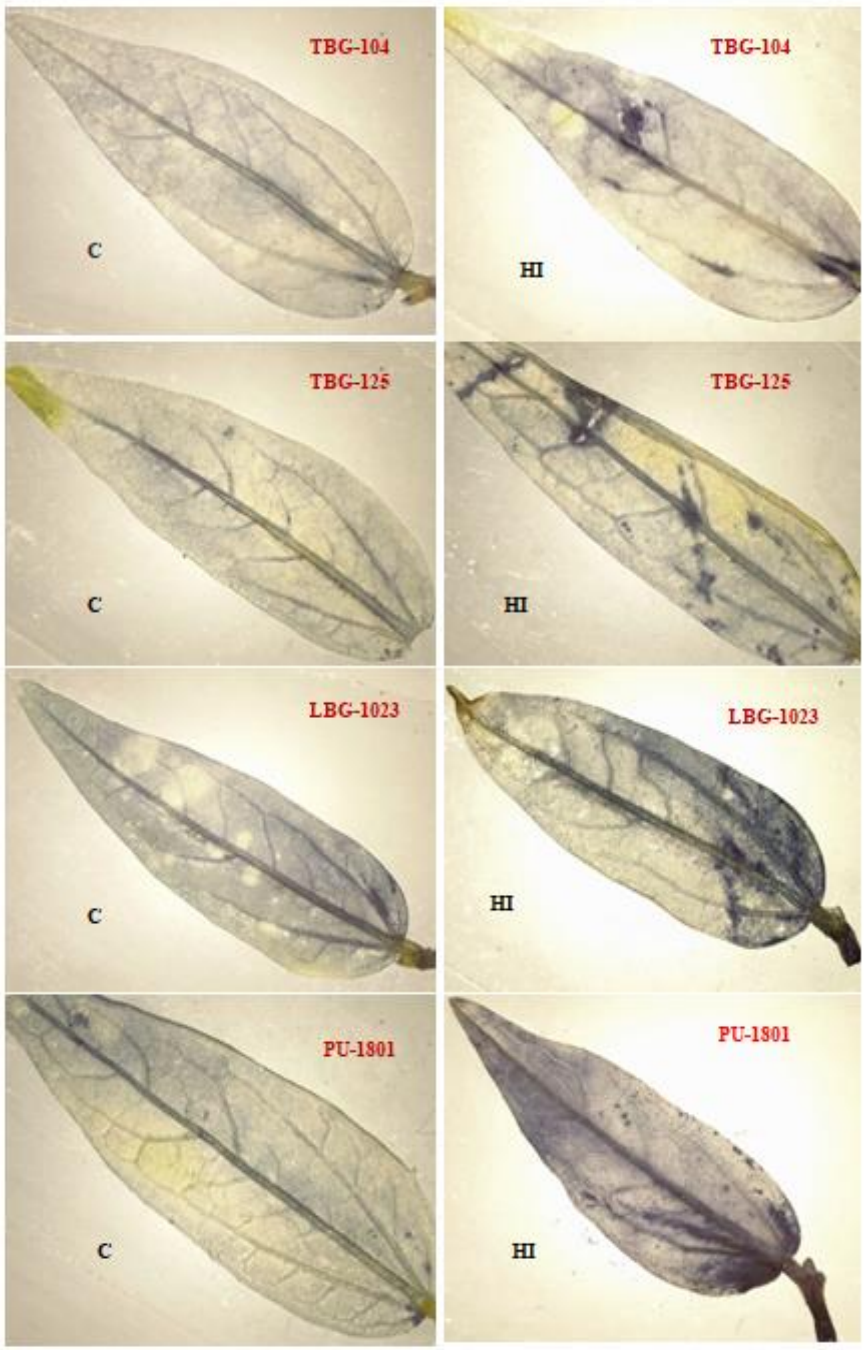

Fig. 2. Histochemical staining for detecting superoxide radical accumulation in the control and TIR induced seedlings of thermotolerant (TBG-104) and susceptible (TBG-125, LBG-1023 and PU-1801) genotypes. (Photographs were taken with digital camera mounted on stereomicroscope at 10X magnification)

The protein profile as evidenced by SDS page analysis indicated distinct expression of band between 23-30 KDa protein in tolerant genotypes such as TBG-129, PU-1804, LBG-995, PU-31, TBG-104, TBG-141, LBG-1004, LBG-1015 and LBG-996 which is not expressed in the other genotypes. Probably, the association of 23-30 KDa protein band would be the indicative feature of thermotolerance in these genotypes. Reports are available on accumulation of specific protein due to heat stress. Similar results were previously reported by Partheeban *et al.* [13] who demonstrated the presence of the additional band at 40 KDa in the blackgram genotypes

subjected to TIR technique. Our results also concur with the published reports of Meena *et al.* [14] in chickpea and Sujatha et al. [15] in blackgram. Reports are available on accumulation of specific protein due to heat stress. Bansod and Malode [16] demonstrated accumulation of specific protein accumulation in *Vigna mungo.* Tourinho-dos-Santos *et al.* [17] reported similar results of the specific protein accumulation.

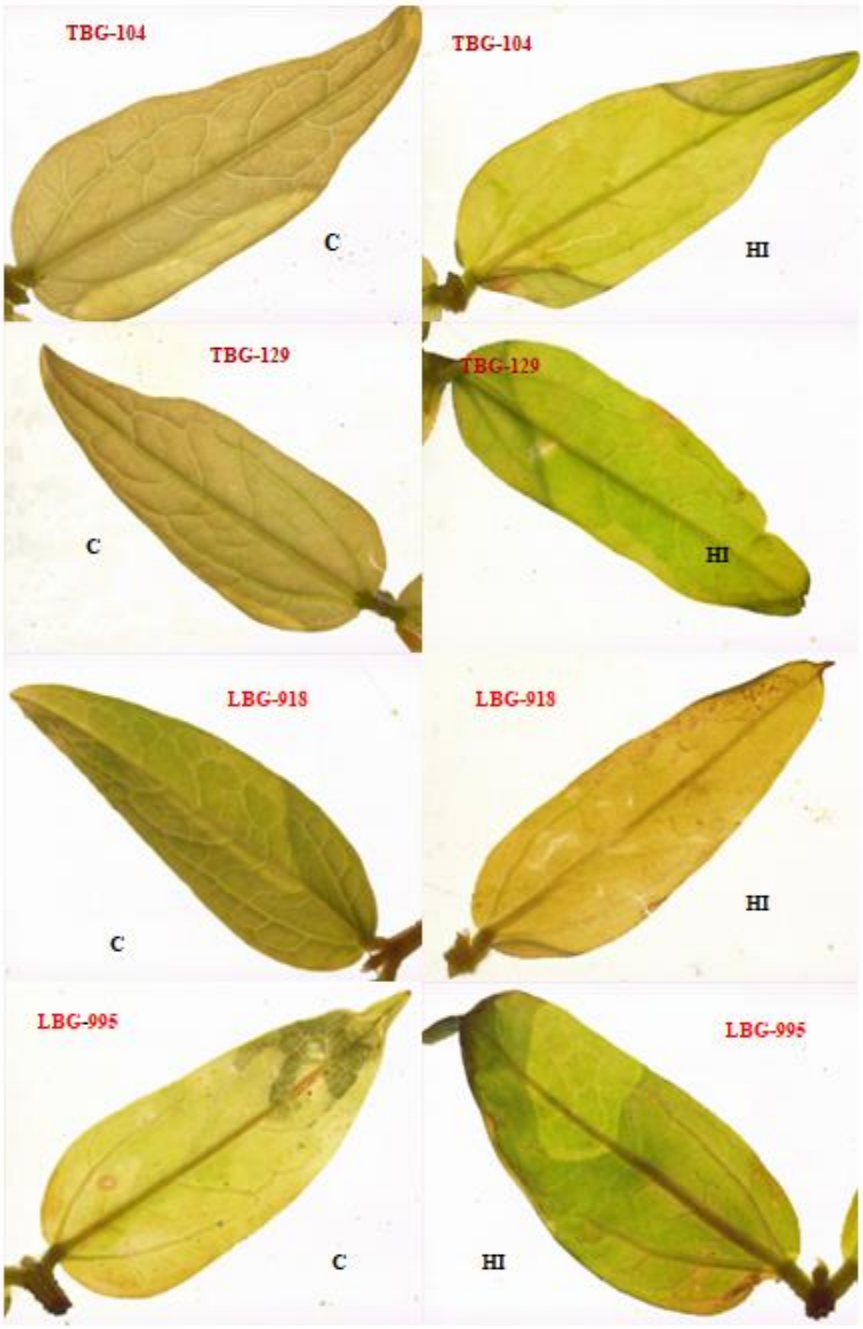

Fig. 3. Histochemical staining for detecting hydrogen peroxide radical accumulation in the control and TIR induced seedlings of thermotolerant (TBG-104, TBG-129, LBG-918 and LBG-995) blackgram genotypes. (Photographs were taken with digital camera mounted on stereomicroscope at 10X magnification)

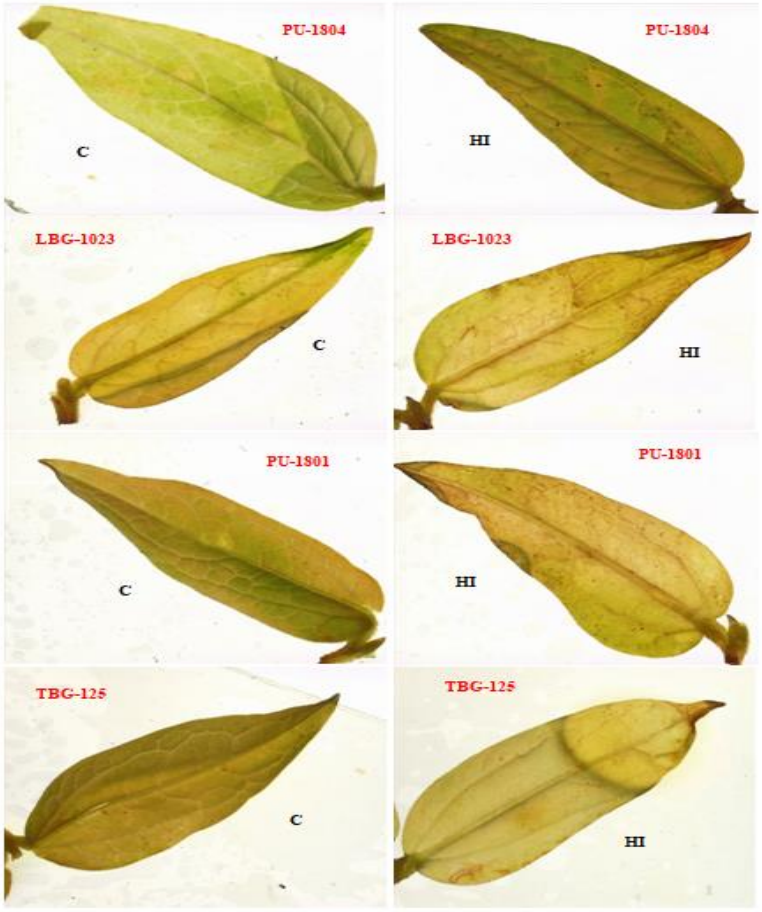

Fig. 4. Histochemical staining for detecting hydrogen peroxide radical accumulation in the control and TIR induced seedlings of thermotolerant (PU-1804) and susceptible (LBG-1023, PU-1801 and TBG-125) genotypes. (Photographs were taken with digital camera mounted on stereomicroscope at 10X magnification)

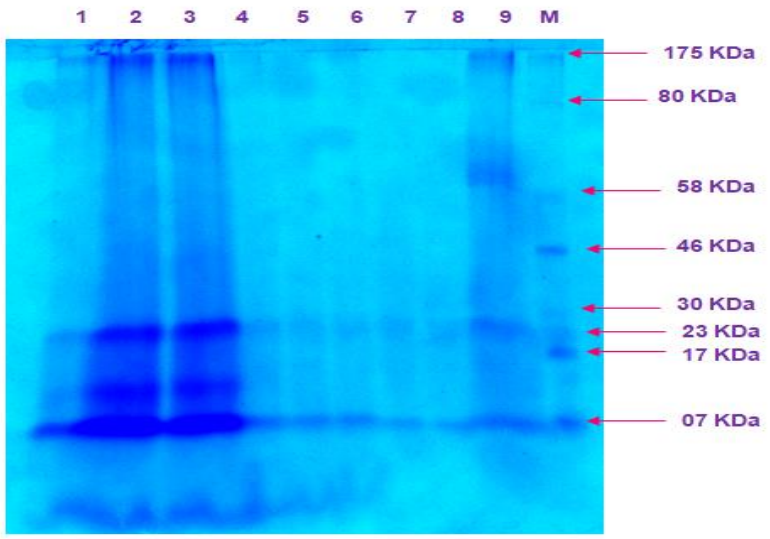

Fig. 5. SDS-PAGE banding pattern in TIR induced blackgram genotypes Lanes 1) LBG-645 2) TBG-129 3) PU-1804 4) PU-1822 5) LBG-752 6) LBG-932 7) PUSA B-58 8) LBG-997 9) LBG-995

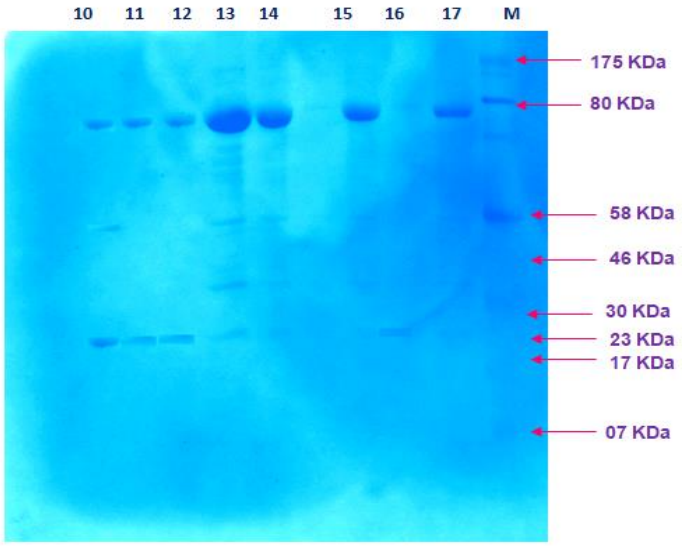

Fig. 6. SDS-PAGE banding pattern in TIR induced blackgram genotypes Lanes 10) GBG-1 11) PU-31 12) TBG-104 13) VBN-8 14) LBG-1006 15) TBG-125 16) LBG-1010 17) LBG-999

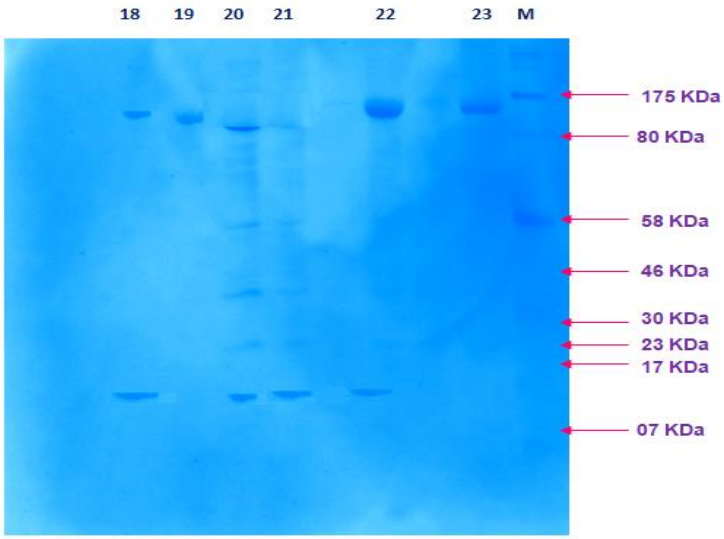

Fig. 7. SDS-PAGE banding pattern in TIR induced blackgram genotypes Lanes 18) TBG-141 19) LBG-1023 20) LBG-1004 21) LBG-1015 22) LBG-996 23) PU-1801

4. CONCLUSION

This induction technique helps the plants to produce heat shock protein slowly in early stages of the crop growth and also can able withstand in the critical situation. The expression of heat shock protein may be a useful parameter for selecting tolerant genotypes. The results of the histo-chemical staining revealed that the genotypes TBG-129, PU-1804 and TBG-104 showed lesser free radical accumulation on the surface of the leaves indicating tolerance to high temperatures.The results of SDS-PAGE analysis revealed the presence of band between 23-30 KDa in the genotypes TBG-129, PU-1804, LBG-995, PU-31, TBG-104, TBG-141, LBG-1004, LBG-1015 and LBG-996 might indicates the enhanced expression of heat protective genes during induction period which promoted the production of HSPs for adaptation to high temperatures. Moreover, blackgram genotypes will be further assessed for reproductive efficiency and yield under field conditions during summer.

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