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Morphological and Ultra-Structural Characterization of Blackgram [*Vigna mungo* (L.) Hepper] Genotypes Grown under High Temperature Stress

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ABSTRACT

Background: In this decade, increasing temperatures and adverse environmental factors are expected to influence the crop yield, seed quality and germination ability. Prolonged heat stress affects the pod setting, seed morphology and alters the starch granules. **Methods:** In this study, Scanning Electron Microscopy (SEM) and Light microscope was used to investigate the variations of morphological and ultra-structural characteristics in blackgram genotypes grown under high temperature (38+2°C) stress (HTS). Nineteen blackgram genotypes were evaluated under open top temperature controlled chamber (OTC) and the genotypes were classified into three categories such as heat tolerant, moderately heat tolerant and heat susceptible genotypes based on the Temperature Induction Response (TIR) and yield reduction percentage under HTS. Two genotypes were selected and examined the seed size, surface pattern, starch granule size and hilum from the each category.

Result: Seed coat morphology was captured using SEM and light microscope showed that the tolerant genotype VBG-07-001 had a clear shiny surface, thin and reduced cotyledon fissures, compact surface devoid of surface deposits and pits whereas rest of the genotypes (VBG-06-001, VBN-6, CoBg-11-02, CoBg-11-03 and VBG-08-003) had a rough surface. A bold and well-structured starch granule was observed in heat tolerant lines whereas, unstructured and pleated granules were observed in moderately tolerant and heat stress lines. These morphological and ultra-structural studies revealed remarked diversity among different genotypes that can be regarded as useful to screen the genotypic tolerant characters for HTS.

Key words: Blackgram, Heat stress, High temperature stress, Hilum, SEM, Starch granule.

INTRODUCTION

Blackgram [*Vigna mungo* (L.) Hepper] is an important pulse crop preferred in India due to its daily dietary component. Also, pulses are an important component of human nutrition as sources of proteins, carbohydrates and minor nutrients such as vitamins and minerals. Often the productivity is limited under abiotic stresses, such as heat stress, drought, salinity, radiation and heavy metal stress *etc*. In particular, transitory or constantly high temperatures cause an array of morpho-anatomical, physiological and biochemical changes in plants, which strongly affect the plant growth and development and may lead to a drastic reduction in economic yield.

Partheeban and Vijayaraghavan (2017) reported that the vulnerability of pulses to heat stress causes reproductive failure and substantial yield loss. Plant responses to heat stress are mediated by both their inherent ability to survive and their ability to acquire tolerance (acclimation). These mechanisms in pulses can be attributed to activation of different genetic systems such as modification of membrane fluidity, expression of stress associated genes and altered sugar metabolism. Although mechanisms underlying thermo tolerance are not yet completely understood, correlations of physiological and biochemical changes under heat stress have been reported. Heat stress is reported to impair pod setting and pod filling which leads the pseudo-seed setting problems that adversely affect the yield by inhibiting the deposition of storage components such as starch and protein (Kumar *et al.,* 2013). Prolonged heat stress affects the seed filling stages alters the starch granule structure (Liu *et al.,* 2011).

Seed morphology, vegetative and reproductive characters has been employed to provide useful characters for the analysis of cultivars or taxonomic relationships. Epidermal characters are only slightly influenced by environmental conditions (Khedia *et al.,* 2017). It has been reported that, spermoderm pattern reflects topographic diversity, to be characteristic of each genotype (Gandhi *et al.,* 2011). However, little is known about the effects of high temperature during the grain-filling period on starch concentration and traits. Light and Scanning Electron Microscopy (SEM) studies on seed micro-morphological and

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morphological features of blackgram genotypes in relevance to high temperature stress have been investigated in this study. The importance of ultra-structural pattern analysis of blackgram seeds observed under SEM reveals a reliable approach for characterization of genotypes for thermotolerance other than the whole plant morphological and physiological adaptations. The objective of the present study was to assess the effects of high-temperature stress on flowering to harvest stages in grain filling of blackgram. Such information will aid understanding how heat affects seed morphology, starch concentration and traits and provide a valuable reference for improving a grain yield under high-temperature stress. This study may further be exploited to develop tolerant blackgram cultivars with high quality seeds.

MATERIALS AND METHODS

The study was conducted to evaluate and compare the variation in seed morphological features of blackgram genotypes grown under high temperature stress. Nineteen blackgram genotypes were taken for the study. The genotype description is given below:

Genotype	Source
VBG-04-003	NPRC, Vamban, Tamil Nadu, India
VBG-04-005	NPRC, Vamban, Tamil Nadu, India
VBG-06-002	NPRC, Vamban, Tamil Nadu, India
VBG-06-003	NPRC, Vamban, Tamil Nadu, India
VBG-06-005	NPRC, Vamban, Tamil Nadu, India
VBG-06-009	NPRC, Vamban, Tamil Nadu, India
VBG-06-010	NPRC, Vamban, Tamil Nadu, India
VBG-07-001	NPRC, Vamban, Tamil Nadu, India
VBG-08-003	NPRC, Vamban, Tamil Nadu, India
VBG-10-008	NPRC, Vamban, Tamil Nadu, India
VBG-10-024	NPRC, Vamban, Tamil Nadu, India
VBN-6	NPRC, Vamban, Tamil Nadu, India
VBN-7	NPRC, Vamban, Tamil Nadu, India
Co-6	TNAU, Coimbatore, Tamil Nadu, India
COBG-11-02	TNAU, Coimbatore, Tamil Nadu, India
COBG-11-03	TNAU, Coimbatore, Tamil Nadu, India
COBG-10-05	TNAU, Coimbatore, Tamil Nadu, India
COBG-10-06	TNAU, Coimbatore, Tamil Nadu, India
COBG-759	TNAU, Coimbatore, Tamil Nadu, India

*NPRC (National Pulses Research Centre); TNAU (Tamil Nadu Agricultural University).

Based on the Temperature Induction Response (TIR) (Vijayalakshmi *et al*., 2015; Partheeban *et al*., 2017) and yield reduction percentage under heat stress, the genotypes were classified into three categories such as heat tolerant, moderately heat tolerant and heat susceptible. Blackgram genotypes were grown in open top temperature controlled chamber (OTC). The genotypes were exposed to high temperature 38+2°C from flowering stage to harvest. A similar set of pot culture was maintained in the OTC without heat to serve as control for comparison.

Blackgram seeds harvested from stress imposed plants and seeds were examined using light microscope and SEM to study the differences in starch granule size, surface pattern and characteristics nature of hilum among the blackgram genotypes.

Scanning Electron Microscopy (SEM)

Micro-morphological features, hilum characteristics and starch granule morphologies were examined under SEM at the Department of Nano Science and Technology, Tamil Nadu Agricultural University, Coimbatore and photographed at defined magnifications. Seed samples were washed with absolute alcohol or acetone for 1 to 2 minutes to remove any debris present in the surface of the seeds. They were further subjected to ultrasonic cleaning by changing absolute alcohol repeatedly and then directly mounting over carbon conducting tape mounted on brass stubs. Seeds were placed on the stub with their dorsal, ventral, lateral side upwards and cross section so that characteristic features of all the different sides could be scanned and photographed using QUANTA 250 - FEI. To achieve better resolution the accelerating voltage varied up to 15 kV.

Light microscopic studies

Light microscopic studies were carried out by using LEICA 6SD. Mature, dry seeds were thoroughly cleaned with alcohol to avoid any alteration in the morphological features and examined for diagnostic features of shape, size and colour.

RESULTS AND DISCUSSION

As described above, transitory or constantly high temperatures cause an array of morpho-anatomical, physiological and biochemical changes in plants, which strongly affect the plant growth and development and may lead to a drastic reduction in economic yield. Hence, based on the yield reduction percentage the genotypes were classified into three groups, i) Heat tolerant genotypes (0-10% yield depression), ii) Moderately heat tolerant (11-20% yield depression) and iii) Heat susceptible (>21% yield depression) (Table 1).

From the above classification, two genotypes from each group were examined using SEM to study the difference in starch granule size, surface and hilum characteristics. The highlighted genotypes were chosen for the microstructural analysis, based on the per cent yield decrease.

The average size of the blackgram seed was ranged from 6.46 to 8.24 mm in length (L) and 4.47 to 5.45 mm in breadth (B). The mean value of 1.47 was recorded for LB ratio. The seed size was reduced due to high temperature stress in irrespective of the genotypes (Table 2 and 3; Fig 1). The high temperature damages the natural functions of plant metabolism, subsequently decreases the crop yield. Temperature plays crucial role in plant growth such as germination, seedling growth, flowering, fertilization, pod formation and seed filling (Hasanuzzaman *et al*., 2013; Chaves *et al*., 2009). Similar genotypic variability in response

Table 1: Classification of blackgram genotypes based on the decline in yield due to heat stress.

Heat tolerant genotypes (0-10%)	Moderately heat tolerant genotypes (11-20%)	Heat susceptible genotypes (> 21 %)
1. VBG-06-002	1. VBG-04-003	1. VBG-06-009
2. VBG-06-005	2. VBG-04-005	2. VBG-08-003
3. VBG-06-010	3. VBG-06-003	3. VBG-10-024
4. VBG-07-001	4. VBN-6	4. Co-6
5. VBG-10-008	5. VBN-7	5. COBG-11-03
6. COBG-759	6. COBG-11-02	
	7. COBG-10-05	
	8. COBG-10-06	

to temperature stress was also reported in chickpea (Krishnamurthy *et al*., 2011).

Microstructure information using SEM in blackgram seeds and their changes under heat stress revealed significant variations in their seed coat surface, cross section, hilar region and starch granule integrity.

Seed coat surface

The studied seeds of six blackgram genotypes differed appreciably in the seed coat surface. In the present work, both light microscopic and SEM studies were used, that complemented each other in obtaining a clear differentiation between genotypes.

Seed coat morphology, captured using light microscopic showed that the high temperature stress tolerant genotype VBG-07-001 had a clear smooth, shiny surface whereas rest of the genotypes (VBG-06-001, VBN-6, CoBg-11-02, CoBg-11-03 and VBG-08-003) had a rough surface. SEM images of seed surface also reveal that the genotype, VBG-07-001 has a compact surface devoid of surface deposits and pits. In the genotype COBg-11-02, the seed surface captured using SEM showed scattered and surface deposits that are loosely distributed over the surface (Fig 1). The genotype COBG-11-03 and VBG-06-010 showed similar seed coat surface with clear surface deposits fewer than the genotype of VBG-08-003 and VBN-06. The SEM images of blackgram genotypes 08-003 and VBN-06 depicted a highly rough surface with denser seed coat. The surface reveals pitted deposits with undulation that are polygonally arranged. The surface showed prominent reticulate uneven areas with pits and depressions (Sahai, 2001).

These ultra-structural study revealed remarked diversity among different genotypes that can be regarded as useful characters for genotypic classification. This also showed that variations in the seed coat morphology are genetically linked (Gulgan *et al.,* 2016) and also affected by heat stress (Bhandari *et al.,* 2016; Sharma *et al.,* 2016; Sehgal *et al*., 2017).

Microstructure of cotyledons

Observations on the SEM images of blackgram seed cotyledons revealed notable differences among the genotypes. The cross section of cotyledons showed seed coat (SC), double palisade (Dp), layers of cells at the hila, tracheid bars (t), vascular tissue in the sub-hilar region. Also

Table 2: Impact of high temperature on seed dimension in blackgram genotypes.

Genotype	Length (mm)	Breadth (mm)	L/B ratio
VBG-04-003	7.64	5.00	1.53
VBG-04-005	6.85	4.60	1.37
VBG-06-002	7.08	4.47	1.42
VBG-06-003	7.64	5.06	1.53
VBG-06-005	7.53	4.59	1.51
VBG-06-009	6.88	5.15	1.38
VBG-06-010	7.43	5.21	1.49
VBG-07-001	6.82	4.72	1.36
VBG-08-003	8.24	5.25	1.65
VBG-10-008	6.89	4.98	1.38
VBG-10-024	6.46	4.53	1.29
VBN-6	6.96	4.73	1.39
VBN-7	7.11	4.65	1.42
CO-6	7.76	5.26	1.55
COBG-11-02	7.69	4.75	1.54
COBG-11-03	7.44	4.92	1.49
COBG-10-05	7.59	5.31	1.52
COBG-10-06	7.64	4.82	1.53
COBG-759	7.74	5.45	1.55
Mean	7.34	4.92	1.47
	G	T	G X T
SEd	0.175	0.090	0.027
CD (P=0.05)	0.382*	0.196*	0.060*

G : Genotype; T : Treatment; G x T : Genotype and treatment interaction; * : Significant.

visible in the images are the cotyledons fissures (F). The structure that covers the hilar region of seeds may be remnants of funiculi (Fn).

The tolerant genotypes, VBG-07-001 and VBG-06-001, showed a thin and reduced cotyledon fissures, whereas the heat susceptible genotypes VBG-08-003, COBG-11-03 shows deeper and larger cotyledonary fissures (Fig 1). Clear, distinct differences in moderately tolerant genotypes were observed, that depicted less deep and smaller fissures compared to the susceptible genotypes. This shows the fracture- resistant ability of the cells to any external stress. The cotyledon of heat susceptible genotype exhibited fractures penetrating or fissures into hilar region disturbing the vascular tissues at the sub hilar region. High temperature

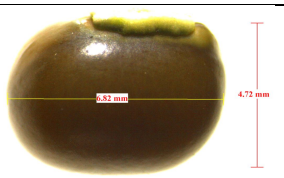
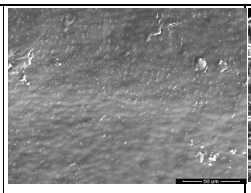
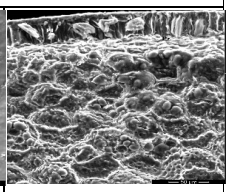
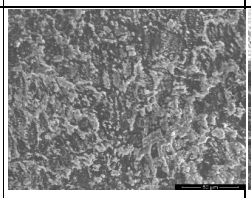
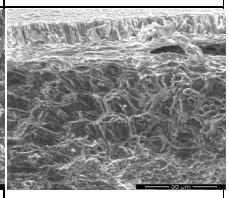
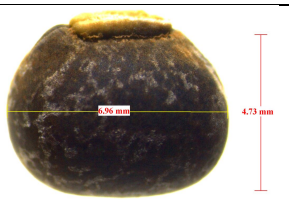
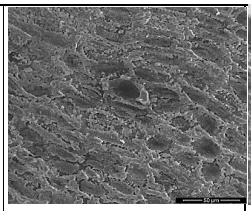
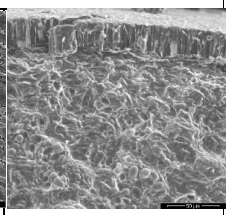
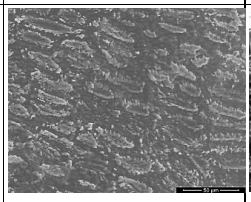
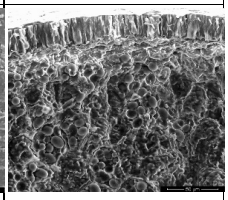
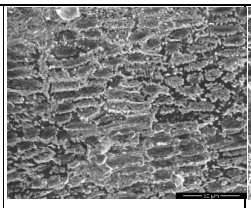
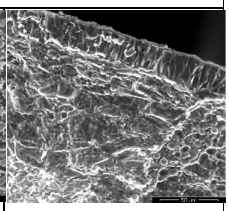
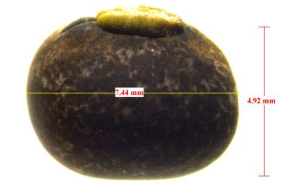
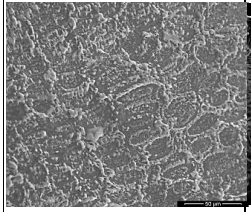
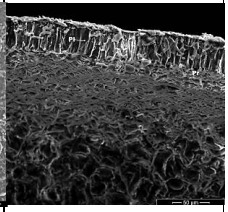
Genotypes	1) Seed pattern	2) Seed Surface	3) Seed coat
HEAT TOLERANT			
A) VBG-07-001			
B) VBG-06-010			
MODERATELY HEAT TOLERANT			
C) VBN-6			
D) COBG-11-02			
HEAT SUSCEPTIBLE			
VBG-08-003			
COBG-11-03			

Fig 1: Continue...

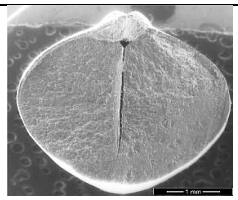
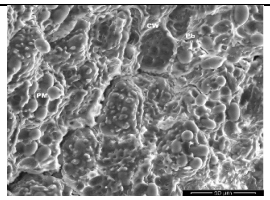
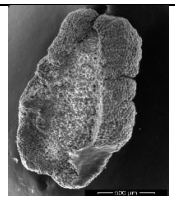
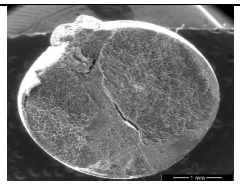
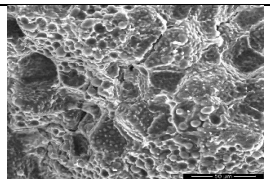
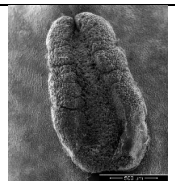
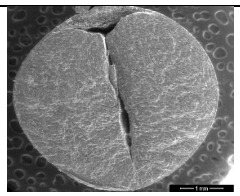
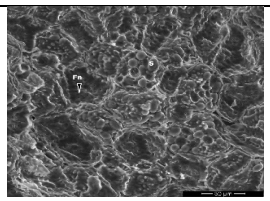
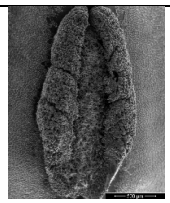
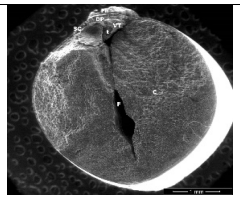
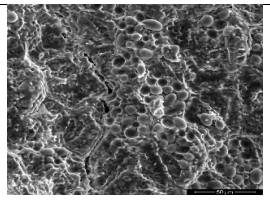
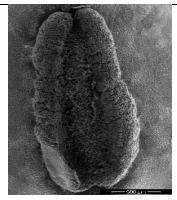
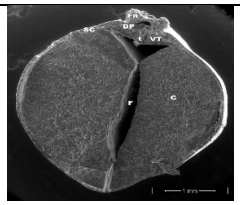
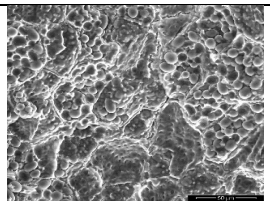
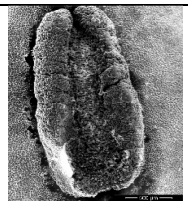
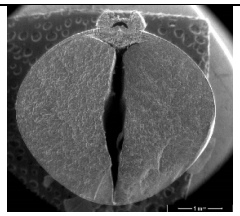
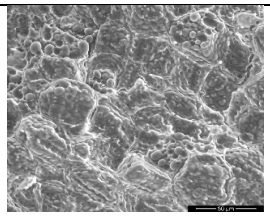
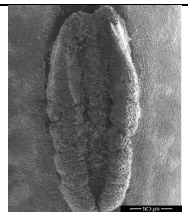
Genotypes	1) Cotyledon	2) Starch granules	3) Hilum
HEAT TOLERANT			
A) VBG-07-001			
B) VBG-06-010			
MODERATELY HEAT TOLERANT			
C) VBN-6			
D) COBG-11-02			
HEAT SUSCEPTIBLE			
VBG-08-003			
COBG-11-03			

Fig 1: Light microscope and scanning electron microscopic view of blackgram genotypes.

stress during seed filing stage may have undesirably affects cell expansion, cotyledon cell number and thus seed filling rate, resulting in the lowered weight per seed (Munier- Jolain and Ney, 1998).

Cross section of the seed coat

The following tissues were clearly distinguished in the seed coat SEM images- cuticle, macrosclerid layer, osteosclerid layer, palisade parenchyma cells. The SEM images showed higher variation in the seed sculpture of blackgram genotypes with different sizes of macroscleroid layers, various patterns of cuticle racks and deposits of cuticle remnants and starch granules embedded in the cells.

The seed coat contained an elongated layer of palisade cells and randomly organized irregular shaped parenchyma layer. The palisade layer was thicker than the parenchyma layer. The seed coat cross section was examined at the non-hilar regions. All the six blackgram genotypes showed a thick column of macroscleroid layers and thin lining of osteosclereid layer which together form the palisade layer.

Taking a closer look at the cross section, the heat tolerant genotypes VBG-06-001 and VBG-07-001, shows intact structures with globules distributed throughout the cross section which is seen to be absent in the heat susceptible genotypes (shows a shrunken structure) CO-Bg-11-03 and VBG-08-003. The moderately heat tolerant genotypes (COBg-11-02 and VBN-06) are seen with fewer globules like structures and less intact cell wall material (Fig 1). These globule like structures, are the starch granules which are reported to be altered under heat stress (Julio *et al*., 2018).

SEM of starch structure

SEM of starch granules was carried out in seeds (at dough stage) of blackgram grown under heat stress conditions. A bold and well-structured starch granule was observed in heat tolerant lines whereas, unstructured and pleated granules were observed in moderately tolerant and heat stress lines (Fig 1).

The damage due to exposure of heat stress was more pronounced in the susceptible genotypes. The images showed a well- structured integrate and compact compartments of tolerant genotypes and vice versa in moderately heat tolerant and heat susceptible lines.

SEM images showed the disintegrated starch granules with empty compartments and less endoscopic cells of wheat grains under heat stress has been documented by Kumar *et al.* (2013). The study showed that, under heat stress in susceptible wheat cultivars, aleuronic layer was defractured and had disintegrated compartments; the starch granules were flat and pleated.

Studies showed that, this could be attributed to altered source to sink relation. The poor sucrose synthesis and transportation decrease in the activities if the key enzymes like ADP-glucose phosphorylation starch synthase and starch branching enzymes due to thermal denaturation might be responsible for the low starch granules synthesis under heat stress condition.

Hilar region

With regards to hilar region, being the less investigated, is characteristic with a very special organization. The shape of the hilum is the main distinguishing feature. The present SEM studies showed that, in blackgram seeds, the hilum is concave and orate. A thick and raised rim is also present along the hilum (Serrato *et al*., 1995). The hilum region is also shown to have some matted reticulate storage cells. The hilar microstructure of pulses has been well documented by Joseph *et al* (1993) and Swanson *et al* (1985). With regards to genotypes under heat stress, the tolerant genotype VBG-07-001 has shown ovate and concave hilum with thick and raised hilar rim with highly porous surface. The hilum region of VBN-6 showed relatively deep concave hilum with raised rim surface. The hilum region of heat susceptible variety COBG-11-03 shows simple concave depression with a raised rim (Fig 1). The rim portion appears to be having more fissures when compared to the tolerant genotype.

The spermoderm characteristics are genetically determined and are the main source of intra and inter specific variations. It has been reported that, spermoderm patterns reflect remarkable topographic diversity, to be characteristic of each genotype (Gandhi *et al*., 2011).

CONCLUSION

Indian legumes such as black, green and red gram seeds are microstructurally similar to legume seeds consumed in other parts of the world. Little was known about the effects of high temperature during different grain-ûlling periods on the morphology of starch granules. The present study observed that the shape and structure of starch granules were visibly different from the control. The microstructure of blackgram seed coat surfaces, cross sections of the seed coats, hilum region and cotyledons will be useful to study the properties of the cultivars and relationships between structural and functional of legumes. It is obvious that improved ûlling and tighter compaction of starch granules would result in higher starch concentration, whereas changes in morphology and volume of granules caused by high-temperature stress could lead to lower starch concentration and starch viscosity. The present results revealed that high-temperature stress altered the morphology and reduced the volume of type A starch granules and consequently starch deposition was retarded and starch concentration and starch viscosity were decreased.

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