Plant, Cell & Environment

Plant, Cell and Environment (2017) doi: 10.1111/pce.12931

Original Article

Quantifying pearl millet response to high temperature stress: thresholds, sensitive stages, genetic variability and relative sensitivity of pollen and pistil

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ABSTRACT

The objectives were to (1) quantify high temperature (HT) stress impacts at different growth stages (season long, booting to seed-set and booting to maturity) on various yield components; (2) identify the most sensitive stage(s) to short episodes of HT stress during reproductive development; (3) understand the genetic variations for HT stress tolerance based on cardinal temperatures for pollen germination; and (4) determine relative sensitivity of pollen and pistil to HT stress and associated tolerance or susceptible mechanisms in pearl millet. High temperature stress (≥36/26°C) imposed at different stages and durations caused decrease in number of seeds, individual seed weight and seed yield. Two periods (10–12 days and 2–0 days before anthesis) were identified as most sensitive to short episodes of stress, causing maximum decreases in pollen germination percentage and seeds numbers. HT stresses of ≥36/26°C results in floret sterility. Pistils were relatively more sensitive than pollen grains, causing decreased number of seeds and seed yield. HT stress increased the reactive oxygen species contents and decreased the activity of the antioxidant enzymes in both pollen and pistils. Under HT stress, pistils had relatively higher reactive oxygen species and lower antioxidant enzymes activity compared with pollen grains, which explains greater susceptibility of pistils.

Key-words: High temperature stress; pearl millet; pollen; pistil; reciprocal cross; sensitive stage.

INTRODUCTION

High temperature (HT) stress is an important abiotic stress, which limits productivity of food crops. Several climate models project that the ambient air temperature will rise between 1°C and 3.4°C by the end of this century, depending on population growth and greenhouse gas emissions scenarios (IPCC 2014). In addition, it is expected that crops in future climates will be exposed not only to higher mean air temperatures but also to more frequent short episodes of HT stress. Arid and semi-arid regions are particularly vulnerable to changes in climate and extreme temperature events. Pearl millet (*Pennisetum glaucum* L. R. Br.) is an important dryland nutritional coarse cereal crop in arid and semi-arid regions providing food and nutritional security. The optimum daily mean temperature for vegetative and reproductive growth of pearl millet is 26°C and 22–25°C, respectively (Ong 1983a, 1983b). However, in the arid and semi-arid regions, current daily mean air temperatures are >25°C during reproductive stages of crop development, which is already above the optimum for growth and development of pearl millet (Gupta et al. 2015). Temperature in Africa is projected to rise faster than the rest of the world, which could exceed 2°C by mid-21st century and 4°C by the end of 21st century (Niang et al. 2014). Butt et al. (2005) projected the impact of climate change scenarios (Hadley Centre Coupled Model and Coupled General Circulation Model) in Mali and showed that grain yield of pearl millet will decrease between 6% and 12%, respectively, without adoption of HT tolerant genotypes; however, with adoption of HT tolerant genotypes, the decreases was smaller and ranged between 0.7% and 8%. Millet yields are likely to decrease by about 0–40% in the 21st century over west Africa because of HT, irrespective of the levels of rainfall (Sultan et al. 2013). Climate models projects that the pearl millet yield in sub-Saharan Africa will decrease from 7% to 17% by 2050 (Nelson et al. 2009; Schlenker and Lobell 2010). These findings are similar to those reported by Knox et al. (2011), who reported a 10% yield losses across south Asia and Africa in pearl millet. However, the extent to which HT stress decrease grain yield will depend on the timing of HT stress during reproductive development, intensity and duration of stress.

Plants are relatively more sensitive to HT during reproductive than vegetative stages (Farooq et al. 2011; Prasad et al. 2017). High temperature stress during the reproductive periods decreased number of seeds panicle^{-1} and individual seed weight, leading to reduced seed yield (Prasad et al. 2017). The number of seeds panicle^{-1} is a consequence of the percent seed-set, which depends upon pollen and ovule fertility, successful pollination, pollen tube growth and formation of embryo (Bita and Gerats 2013). Failure of any of these processes decreases fertilization and/or increase early embryo abortion,

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causing decreased crop yield. High temperature stress affects both microsporogenesis and megasporogenesis (occurs prior to flowering stage), resulting in decreased pollen viability (Prasad et al. 2006) or ovule function (Gross and Kigel 1994). High temperature stress during periods of sporogenesis causes abnormal pollen exine wall, membrane damage and tapetal cell degradation leading to pollen sterility in sorghum (*Sorghum bicolor* L. Moench; Djanaguiraman et al. 2014), wheat (*Triticum aestivum* L.; Prasad and Djanaguiraman 2014) and soybean (*Glycine max* L. Merr.; Djanaguiraman et al. 2013). Deformities of pistil (stigma, style and ovaries) along with desiccation and flaccidity of stigma was observed under HT stress in wheat (Prasad and Djanaguiraman 2014). Exposure to HT during flowering (anthesis) stage significantly decreased seed-set in rice (*Oryza sativa* L., Jagadish et al. 2010), wheat (Pradhan et al. 2012), sorghum (Prasad et al. 2006) and groundnut (*Arachis hypogaea* L.; Prasad et al. 2001). Among the cereals, pearl millet is more resilient to HT stress. However, critical sensitive stages and temperature thresholds and duration of HT stress have not been quantified adequately. Such knowledge will improve our understanding of HT stress impacts and opportunities for genetic improvement.

Relative reproductive tissues (male, pollen; female, pistil) sensitivity to HT stress is not well understood in grain crops. Sensitivity of pollen to HT was documented in several studies, but there was no direct comparison with pistil (Djanaguiraman et al. 2013, 2014; Prasad and Djanaguiraman 2014; Prasad et al. 2015). Saini and Aspinall (1982) observed that a level of HT stress causing male sterility in wheat had no damaging effect on the functions of female sexual tissue generation, indicating that female gametophyte had greater tolerance to HT stress. However, susceptibility of the female gametophyte to HT has been observed in rapeseed (*Brassica napus*; Polowick and Sawhney 1988), tomato (*Solanum lycopersicum* L.; Sato et al. 2002) and arabidopsis (Whittle et al. 2009). Hence, a detailed study to understand relative susceptibility/tolerance of male and female reproductive tissues in pearl millet to HT stress would be critical. Well-established cytoplasmic male sterility system and protogynous flowering nature of pearl millet makes the crop as an excellent model plant to study the relative sensitivity of pollen and pistil to HT stress. High temperature stress evokes the reactive oxygen species (ROS) generation, which includes hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), singlet oxygen ($^1O_2$) and hydroxyl radical (OH^-) causing oxidative damage in the tissue (Potters et al. 2007; Djanaguiraman et al. 2014). However, plants possess an array of defense mechanisms against these ROS through enzymatic and non-enzymatic antioxidant systems. The antioxidant enzymes include superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), while the non-enzymatic antioxidants include ascorbate and glutathione (Apel and Hirt 2004). SOD is capable of scavenging O_2^-, and H_2O_2 can be degraded into H_2O by CAT or by POX in the presence of a reductant (Noctor and Foyer 1998). High temperature stress is known to cause oxidative damage in vegetative leaf tissues. However, it is not clear the extent of oxidative damage in reproductive tissues and its relation to fertility and sensitivity or tolerance.

The objectives of this research were to (1) quantify HT stress impacts at different growth stages (season long, booting to seed-set and booting to maturity) on various yield components; (2) identify the most sensitive stage(s) to short episodes of HT stress during reproductive development; (3) understand the genetic variations for HT stress tolerance based on cardinal temperatures for pollen germination; and (4) determine relative sensitivity of pollen and pistil to HT stress and associated tolerance or susceptible mechanisms.

MATERIALS AND METHODS

A series of controlled environment experiments using the facilities available at Agronomy Department, Kansas State University, Manhattan, KS, USA, were conducted.

Experiment I: impact of temperature stress from emergence to maturity

Seeds of hybrid pearl millet (Tifgrain 102) were sown at 5 cm depth in pots (pot diameter at the top and bottom was 15 and 12 cm, respectively) containing commercial Sun Grow Metro Mix 200 potting soil (Hummert International, Topeka, KS, USA). After emergence, plants were thinned to two plants per pot and maintained until maturity. A systemic insecticide, Marathon 1% G (granules) (active ingredient: Imidacloprid 1-((6-chloro-3-pyridinyl) methyl)-*N*-nitro-2-imidazolidinimine, Hummert International), was applied to each pot at 4 g pot^{-1}. The medium was fertilized with Osmocote (Hummert International) at 5 g pot^{-1} (controlled release fertilizer, 14:14:14%, N:P_2O_5:K_2O, respectively) before sowing. To avoid water stress, all pots were watered daily up to pot water capacity from sowing to maturity.

Plants were grown at temperature (32/22°C; daytime maximum and nighttime minimum temperature) from sowing to emergence (three leaf stage) in seven growth chambers. Thereafter, each growth chamber was maintained at seven different temperatures (32/22°C, 34/24°C, 36/26°C, 38/28°C, 40/30°C, 42/32°C and 44/34°C) until physiological maturity. The pots were randomly arranged within each growth chamber to avoid positional effects. In all the temperature regimes, daytime maximum and nighttime minimum were held for 4 h, and transition period between the daytime maximum and nighttime minimum was 8 h, and *vice versa*. Relative humidity in all growth chambers was maintained between 70% to 80% to avoid any confounding effects of dry air (drought stress). The maintained photoperiod was 12 h (from 0800 to 2000 h). In all growth chambers, the canopy level photon flux density (400 to 700 nm) about 900 μmol m^{-2} s^{-1} was provided by cool white fluorescent lamps (Philips Lighting Co., Somerset, NJ, USA). Data on days to heading, biomass production, seed yield and individual seed weight were measured from five randomly tagged plants. At maturity, tagged panicles were harvested and dried at 40°C for 10 days, and hand threshed, and the seeds were weighed to determine seed yield $plant^{-1}$, expressed as gram (g). The individual seed weight was determined by dividing seed yield by the number of seeds

and expressed as milligram per seed (mg seed^{-1}). The biomass was dried at 60°C for 10 days, weighed and expressed as gram per plant (g plant^{-1}).

Experiment II: impact of temperature stress from booting to maturity

Pearl millet genotype (KSU 2068B) was grown under optimum temperature (OT: 28/18°C; daytime maximum and nighttime minimum temperature) under fully watered conditions from emergence to booting stage. Methods of plant growth (pot and medium) were similar to those mentioned in experiment I. Thereafter, eight pots were moved to the growth chambers maintained at either OT (28/18°C) or HT (32/22, 36/26, 40/30 and 44/34°C) till physiological maturity (45 days).

In all the temperature regimes, daytime maximum and nighttime minimum temperature were held for 6 h, and transition period between the daytime maximum and nighttime minimum temperatures was 6 h, and *vice versa*. Relative humidity, in all growth chambers was maintained between 70% and 80% to avoid any confounding effects of dry air (drought stress). The photoperiod was 14 h (from 0800 h to 2200 h). In all the growth chambers, the canopy level photon flux density (400 to 700 nm) was about 870 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Philips Lighting Co., Somerset, NJ, USA). During stress, the photosynthetic rate was measured on the tagged flag leaves from day 0 to 12 with an interval of 4 days as described by Djanaguiraman et al. (2014). Before moving the plants into various temperature treatments, the panicles were tagged to record pollen germination percent, number of seeds panicle^{-1}, number of panicles plant^{-1} and seed yield plant^{-1}. Panicles were harvested and dried at 40°C for 10 days, and hand threshed, and number of seeds were counted and expressed as seed number panicle^{-1}. The seeds were weighed to determine seed yield plant^{-1}.

The pollen germination percentage was arrived by dusting pollen grains collected from freshly exerted florets in the pollen germination medium (150 mg H_3BO_3, 500 mg Ca $(NO_3)_2{\cdot}4H_2O$, 200 mg $MgSO_4{\cdot}7H_2O$, 100 mg KNO_3, and 300 g sucrose dissolved in 1 L of deionized water to which 10 g of agar was mixed). The mixture was heated slowly on a hotplate until the agar was completely dissolved. The germinating medium was poured onto clean glass slides and incubated at 25°C. After dusting pollen grains, glass slides were kept in petri dishes and kept in the dark for 45 min. The pollen germination percent was estimated by counting the total number of pollen grains and number of germinated pollen in three random microscopic fields in each glass slide (Prasad et al. 2006).

Experiment III: impact of HT stress from booting to start of grain filling

Pearl millet genotype (Tift 23DBE) was grown under OT (28/18°C; daytime maximum and nighttime minimum temperature) under fully watered conditions (as mentioned in experiment I) from emergence to booting stage. Thereafter, eight pots were moved to the growth chambers maintained at either 28/18°C (OT), 36/26°C (HT stress) or 40/30°C (HT stress) for 18 days (start of grain filling). After imposing the HT treatments, the pots were returned back to OT (28/18°C) and remained until final harvest at maturity. The methods of imposition of stress, environmental conditions and data collection were similar to those as detailed in experiment II. During stress, the photosynthetic rate was measured on the tagged flag leaves from day 0 to 12 with an interval of 4 days as described by Djanaguiraman et al. (2014). Before moving the plants into HT treatment, the panicles were tagged to record the pollen germination percent, number of seeds panicle^{-1}, number of panicles plant^{-1} and seed yield plant^{-1}.

Experiment IV: impact of short episodes of HT stress during reproductive stages of development

Pearl millet genotype (Tift 23DBE) was grown at OT (28/18°C; daytime maximum and nighttime minimum temperature) under fully watered conditions (as mentioned in experiment II) from emergence to about 12 days before anthesis. Thereafter, five pots were moved at every 2 days interval to the growth chambers maintained at 40/30°C (HT stress). After imposing the HT treatment, the pots were returned back to OT (28/18°C) and remained until final harvest at maturity. The plants were transferred to HT early in the morning (just before 0800 h).

The method of imposition of stress, environmental conditions and data collection were similar to those as detailed in experiment II. Before each transfer for HT stress, primary panicles were tagged to record the pollen germination. At anthesis, the pollen grains were collected from tagged panicle and germinated in pollen germination medium incubated at 25°C, and germination percentage was estimated following the procedures detailed in experiment II. At physiological maturity, tagged panicles were harvested and dried at 40°C for 10 days, and hand threshed, and number of seeds were counted and expressed as number panicle^{-1}. The seeds were weighed to determine seed yield plant^{-1}.

Experiment V: genetic variability for HT stress tolerance, cardinal temperatures for pollen germination

Twenty-eight pearl millet genetically diversed lines were collected from the Plant Genetic Resources Conservation Unit, USDA-ARS Georgia, USA, and evaluated for HT stress tolerance using pollen germination assay. Each genotype was planted in pots as detailed in experiment II. Plants were grown under 30/20°C (daytime maximum and nighttime minimum temperature) under fully watered conditions.

At anthesis, pollen grains were collected from three tagged panicles from each genotype. The tagged panicles were cleaned gently with tissue paper to remove the already opened floret before the anthesis time. Pollen grains were

collected from the freshly exerted florets and dusted on the pollen germination medium as detailed in experiment II. In developing temperature response curves, the germinating medium was poured onto clean glass slides and incubated at different temperatures ranging from 10°C to 45°C with an interval of 5°C and kept in the dark for 45 min. The percentage of pollen germination was estimated by counting the total number of pollen grains and number of germinated pollen in three random microscopic fields in each glass slide. These percentages were used to determine pollen temperature response curves and model cardinal temperatures [T_{min} (minimum temperature below which pollen grains did not germinate), T_{opt} (temperature where maximum germination occurred) and T_{max} (temperature above which pollen grains did not germinate)]. The pollen was considered germinated if the pollen tube was longer than the diameter of the pollen grain (Prasad et al. 2006). Pollen germination percentage recorded at each temperature was analysed using regression model to determine cardinal temperatures as described by Kakani et al. (2002).

Experiment VI: relative sensitivity of pollen and pistil to HT stress

Pearl millet genotypes KSU2068A (male sterile) and KSU 2068B (male fertile) were planted in controlled environments and grown as mentioned in experiment II. Plants were grown under OT (28/18°C; daytime maximum and nighttime minimum temperature) under fully watered conditions from emergence to booting stage. Thereafter, eight pots of male plants were moved to each growth chambers maintained at either OT (28/18°C) or HT (36/26 or 40/30°C) from booting to 3 days after anthesis. To have pollination control, we kept female plants in another three growth chambers representing three temperature regimes (28/18°C; 36/26°C; and 40/30°C). The growth conditions were same as explained in experiment II. Before moving the plants into the growth chamber, the panicles were tagged. In pearl millet, anthesis starts from the middle portion of the panicle by showing extruded stigma. The panicle portion (3 cm) showing extruded stigma was marked with a twin on each panicle, and the extruded stigma of rest of the panicle was gently removed using forceps on subsequent days. The crosses between a female and a male plants in each pair with four different combinations (OT female and OT male; OT female and HT male; HT female and OT male; and HT female and HT male) was performed. Pollinations were manually executed using freshly collected pollen from anthers of either the OT or HT treated plants and gently dusted on female plants. The manual crossing process was repeated for two successive days to ensure that all the stigmas of marked portion of tagged panicles are crossed to achieve maximum pollination. As detailed previously, crossing using female and male plants grown at (1) 28/18°C and 40/30°C; (2) 28/18°C and 36/26°C and (3) 36/26°C and 40/30°C was performed. After pollination, the plants remained in either OT or HT conditions for 3 days, and all plants were then returned to OT and maintained until harvest.

At physiological maturity, marked portion of the panicles (3 cm) where crossing is executed were harvested and dried at 40°C for 10 days, and hand threshed, and seed numbers were counted and expressed as number panicle^{-1}. The seeds were weighed to determine seed yield plant^{-1}. The individual seed weight was determined as explained in experiment I.

During anthesis, pollen grains and pistils were also collected from the plants grown at three different temperatures 28/18°C, 36/26°C and 40/30°C and were immediately frozen in liquid nitrogen and stored at −80°C until further biochemical analysis. The frozen samples were quantified for the antioxidant enzymes like xanthine oxidase (XO), superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) activity along with hydrogen peroxide and starch contents using procedures described later. For SOD, CAT and POX antioxidant enzymes assays, frozen pollen grains or pistils were homogenized in 1 mL of ice-cold 0.1 M Tris–HCl buffer, pH 7.8 per gram fresh weight. The homogenate was centrifuged at 20, 000 *g* at 4°C. The supernatant was used for measuring enzyme activity.

XO enzyme activity

The pollen grains or pistils (100 mg) were grounded in 1 mL of phosphate buffer pH 7.5 and centrifuged at 15000 *g* for 10 min at 4°C, and the supernatant was collected and analysed for superoxide production (30 min at 37°C) using xanthine as substrate according to the kit instructions from the Amplex® Red Xanthine Oxidase Assay kit (Molecular Probes, Eugene, OR, USA, catalogue number A22182) expressed as milliunit (Hofer et al. 2008).

Hydrogen peroxide content

The pollen grains or pistils (100 mg) were grounded in 1 mL of cold acetone and centrifuged at 5000 *g* for 10 min at 4°C, and the supernatant was used for H_2O_2 assay. The H_2O_2 content was measured using a molecular probe (Amplex® Red hydrogen peroxide/peroxidase assay kit), which is one-step assay that uses the Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to detect H_2O_2. The Amplex® Red reagent, in combination with horseradish peroxidase (HRP), has been used to detect H_2O_2. In the presence of peroxidase, the Amplex® Red reagent reacts with H_2O_2 in a 1 : 1 stoichiometry to produce the red-fluorescent oxidation product, resorufin, which has the absorption maxima at 560 nm. The background absorbance derived from no H_2O_2 control was subtracted for all samples and expressed as nM g^{-1} on fresh weight basis (Shin et al. 2005).

SOD enzyme activity

Total SOD activity was measured on the supernatant by the superoxide dismutase assay kit (Cayman Chemical, Ann Arbor, MI, USA, catalogue number 706002) according to manufacturer's instruction. This kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The SOD activity was expressed

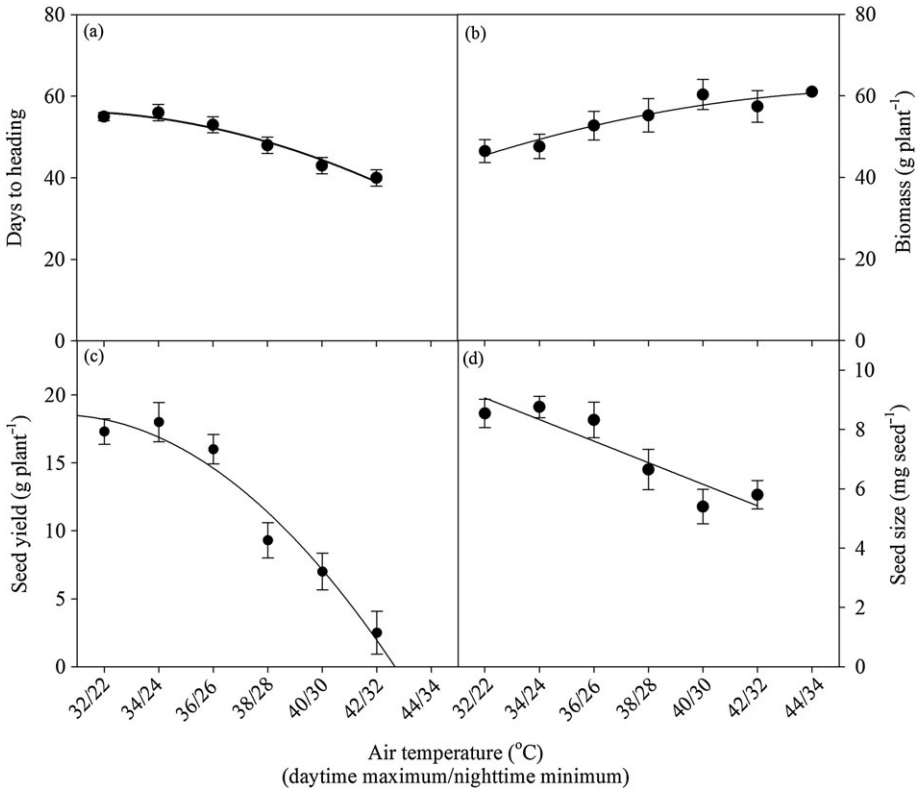

Figure 1. Impact of high temperature stress (32/22°C or 34/24°C or 36/26°C or 38/28°C or 40/30°C or 42/32°C or 44/34°C daytime maximum/nighttime minimum temperature) from emergence to physiological maturity on (a) days to heading, (b) vegetative biomass (g plant^{-1}), (c) seed yield (g plant^{-1}) and (d) individual seed weight (mg). Vertical bars denote ±S.E. of mean.

as enzyme units. One unit of SOD is defined as the amount of enzyme needed to obtain 50% dismutation of superoxide radical (Sebastiani et al. 2007).

CAT enzyme activity

The catalase enzyme activity was measured using Amplex® Red catalase assay kit, as it provides an ultrasensitive, simple assay method for measuring CAT activity. In the assay, CAT first reacts with H_2O_2 to produce water and oxygen [O_2]. Next, the Amplex red reagent (10-acetyl-3,7-dihydroxyphenoxazine) reacts with a 1 : 1 stoichiometry with any unreacted H_2O_2 in the presence of HRP to generate the red-fluorescent oxidation product, resorufin, which has the absorption maxima at 560 nm and expressed as enzyme units. One enzyme unit was defined as the amount of catalase enzyme that decompose 1.0 μM of H_2O_2 per minute at 25°C per gram of tissue on fresh weight basis (Jones and Suggett 1968).

POX enzyme activity

The peroxidase (POX) enzyme activity was measured using an Amplex® Red hydrogen peroxide/peroxidase assay kit, which is one-step assay that uses the Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to detect POX activity. The enzymatic activity of peroxidases was determined following the same procedure as the determination of H_2O_2 except that the Amplex Red reagent contained 2 mM H_2O_2 instead of HRP. The result was expressed as enzyme unit (Liu et al. 2010).

Starch content

The starch content in the pollen and pistil was quantified using a Starch Assay Kit (SA20, Sigma-Aldrich, St Louis, MO, USA) by following the protocol of Thelander et al. (2004).

Seed numbers panicle^{-1}, individual seed weight and seed yield panicle^{-1}

The crossed panicles were hand harvested and threshed to estimate the number of seeds panicle^{-1}, individual seed weight and seed yield per panicle as per procedure mentioned in experiment II.

Data analyses

Data from all experiments were statistically analysed using PROC GLM in the SAS software (SAS Institute 2003). The experimental layout for each experiment was a randomized complete block design. Plants for different treatments were randomly arranged within each growth chamber (treatments) and selected within each treatment. Growth chamber

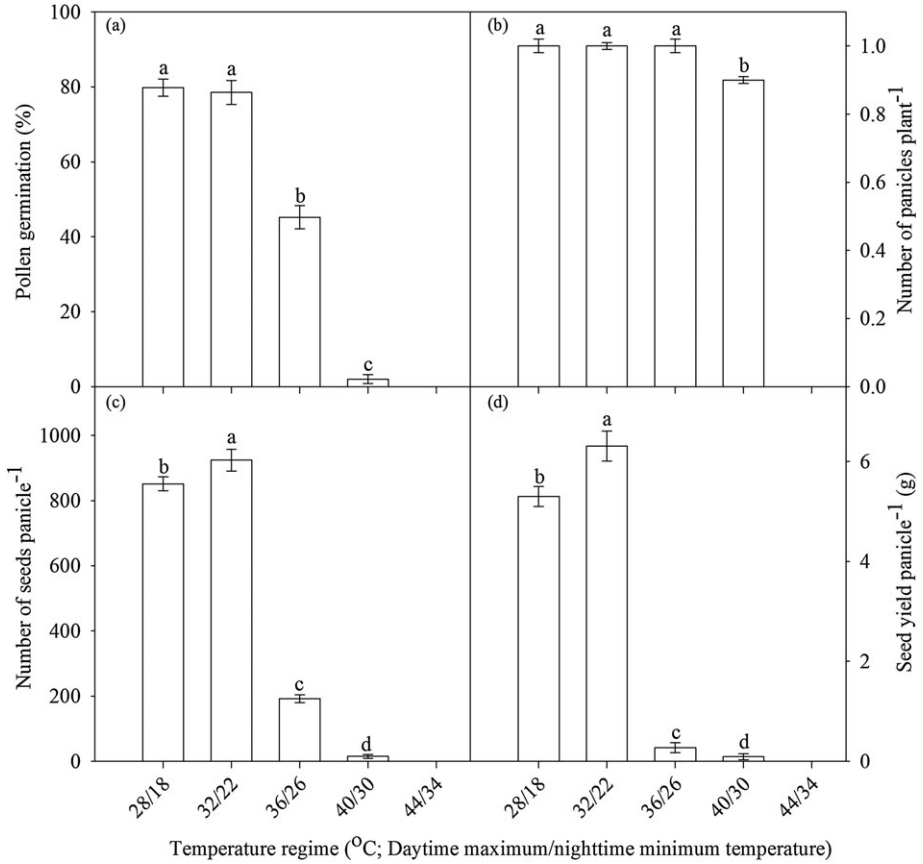

Figure 2. Impact of high temperature stress (32/22°C or 36/26°C or 40/30°C or 44/34°C daytime maximum/nighttime minimum temperature) from booting to physiological maturity (45 days) on (a) pollen germination, (b) number of panicles plant^{-1}, (c) number of seeds panicle^{-1} and (d) seed yield panicle^{-1} (g). Vertical bars denote ±S.E. of mean. Means with different letters are significantly different according to the least significant difference test at $P < 0.05$.

temperatures were also randomly assigned. The number of replications varied among different experiments. Experiments I, II, III and IV were with five replications (pots) for each treatment; experiment V had three replications (panicles), and each replication has two sub-sample; and experiment VI had eight replications. For all measured observations, standard errors were shown as an estimate of variability, and means were separated using least significant difference at probability level of 0.05.

For determination of cardinal temperature for different genotypes (experiment V), different regression equations (linear, broken stick and quadratic) for the response of pollen germination to temperature were compared for variation, determined by calculating the coefficient of determination (R^2) and root mean square deviation (r.m.s.d) for observed and fitted values. A quadratic equation best described the responses of pollen germination for most genotypes was used to estimate cardinal temperatures [(T_{min}), (T_{opt}) and (T_{max})] for pollen germination of all genotypes. The RSREG procedure in SAS (SAS Institute 2003) was used to estimate parameters in the quadratic equation as it uses least squares to fit quadratic response surface regression models. Cardinal temperatures were estimated following a similar procedure as presented by Djanaguiraman et al. (2014).

RESULTS

Quality control of growth chambers

Mean daytime and nighttime temperatures of all the growth chambers were ±0.5°C of the target day and night temperatures, and relative humidity was within ±10% of the target. Quality of the temperature control and growth chamber performance was previously published (Pradhan et al. 2012).

Experiment I: impact of HT stress from emergence to maturity

Increasing growth temperature from 32/22°C to 44/34°C significantly decreased the phenology, individual seed weight and seed yield (Fig. 1a,c,d). However, there was no negative influence of increasing temperature in the range of 32/22°C to 44/34°C on biomass production (Fig. 1b). In contrast, there was a quadratic decrease in seed yield with increasing temperature, and there were no seed formation at about 42.3/33.2°C (Fig. 1c). Individual seed weight and seed yield were decreased with increasing growth temperature; however, greater decrease was observed in seed yield

compared with individual seed weight. Increasing temperature from 32/22°C to 42/32°C decreased seed size by about 50% (Fig. 1d).

Experiment II: impact of HT stress from booting to maturity

High temperature (40/30°C) stress caused all the pollen grains become sterile causing close to zero (3%) pollen germination (Fig. 2a). However, at 36/26°C (HT), the pollen germination percent was significantly ($P < 0.01$) decreased by 43% (Fig. 2a). Similarly, the number of seeds panicle^{-1} and seed yield panicle^{-1} significantly decreased by 77% and 94%, respectively, at 36/26°C, and close to 97% and 98% at 40/30°C, respectively, compared with OT (28/18°C) (Fig. 2c,d). At temperature of 44/34°C, the plants remained at booting stage and did not exsert panicle completely until physiological maturity (Fig. 2b). Leaf photosynthetic rates were not affected by temperature in the range of 28/18°C through 40/30°C, but exposure to 44/34°C decreased it by about 10% when compared with 28/18°C (Fig. 3a).

Experiment III: impact of HT stress from booting to start of grain filling

Among the various temperature regimes, there was no significant difference for photosynthetic rate (Fig. 3b). At HT (40/30°C), the plants were almost 100% sterile (98% sterility). However, at 36/26°C, the pollen germination percent was significantly ($P < 0.05$) decreased by approximately 28% (Fig. 4a). Similarly, the number of seeds panicle^{-1} and seed yield panicle^{-1} was significantly ($P < 0.05$) decreased by 52% and 50%, respectively, compared with OT (28/18°C) (Fig. 4c,d). The HT stress (36/26°C and 40/30°C) significantly ($P < 0.01$) increased (41% and 170%, respectively) the number of panicles plant^{-1} compared with OT (28/18°C) (Fig. 4b).

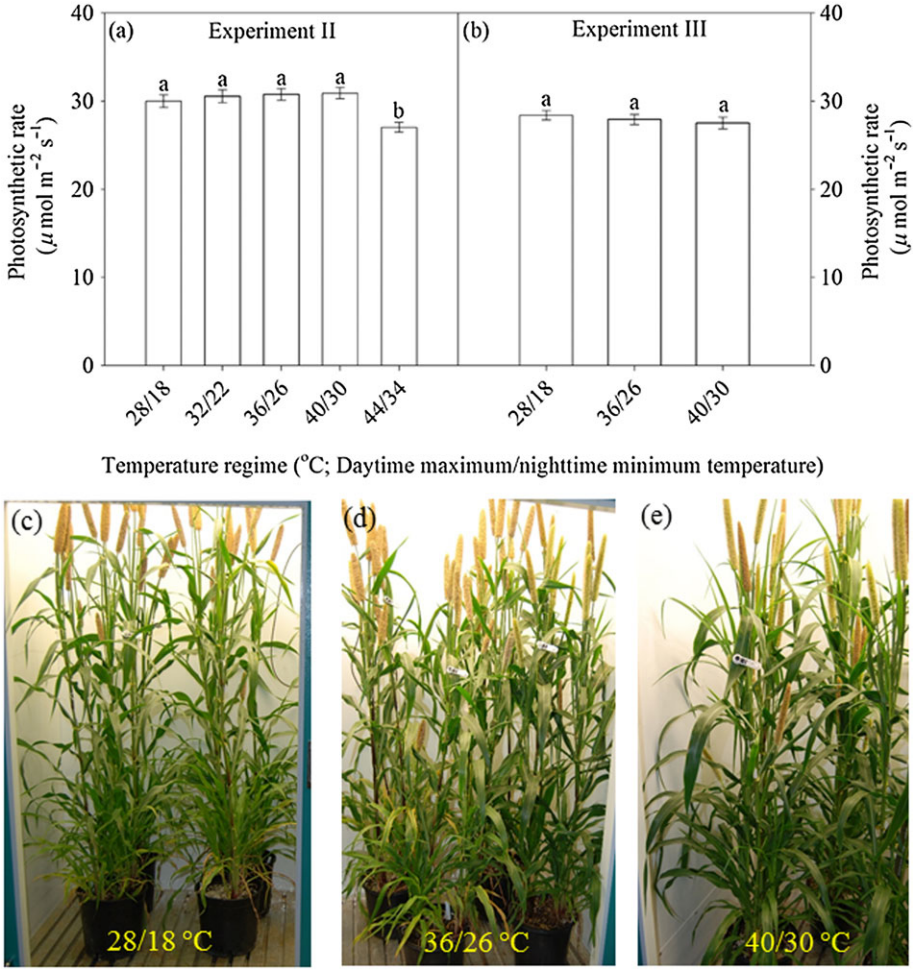

Figure 3. Impact of high temperature stress (32/22°C, 36/26°C, 40/30°C and 44/34°C daytime maximum/nighttime minimum temperature) on (a) photosynthetic rate (μmol m^{-2} s^{-1}) from booting to maturity, (b) photosynthetic rate (μmol m^{-2} s^{-1}) booting to start of grain filling and (c, d, e) photographs indicating new growth, tillers, panicles and biomass. Vertical bars denote ±S.E. of mean of all observations during the treatment period. Means with different letters are significantly different according to the least significant difference test at $P < 0.05$.

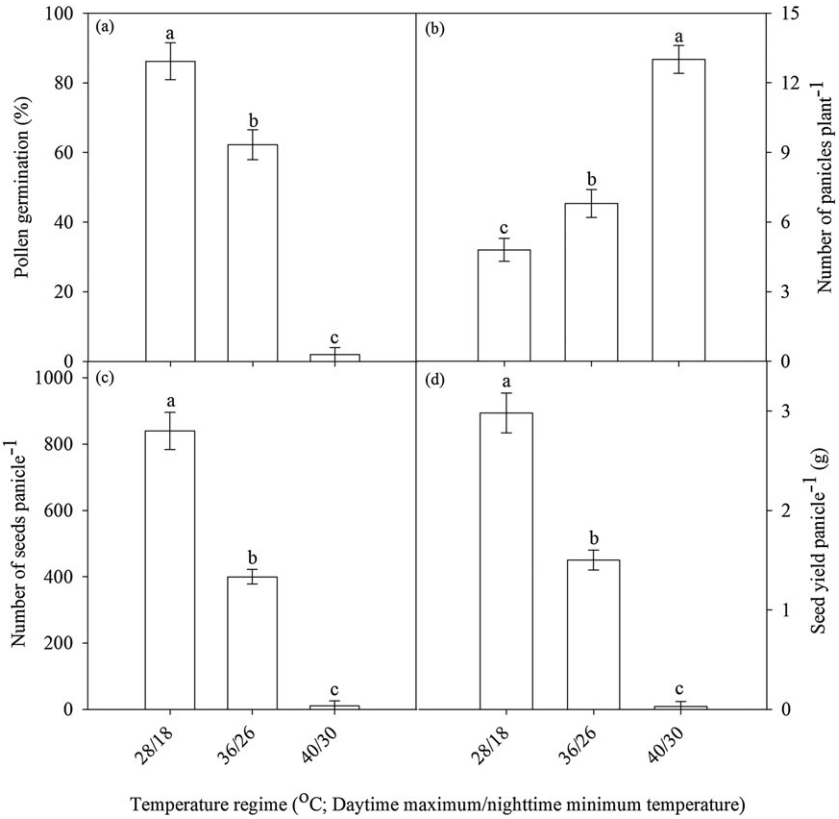

Figure 4. Impact of high temperature stress (36/26°C or 40/30°C daytime maximum/nighttime minimum temperature) imposed from booting to start of grain filling stage (18 days) on (a) pollen germination, (b) number of panicles plant^{-1}, (c) number of seeds panicle^{-1} and (d) seed yield panicle^{-1} (g). Vertical bars denote ±S.E. of mean. Means with different letters are significantly different according to the least significant difference test at $P < 0.05$.

Experiment IV: impact of short episodes of HT stress during reproductive stages of development

Compared with OT (28/18°C), exposure to HT (40/30°C) stress for 2 days significantly ($P < 0.05$) decreased pollen germination percent when imposed at 12, 10, 8, 6 or 0 day before anthesis (Fig. 5a). Maximum decrease in pollen germination occurred when HT stress was imposed at 8 to 10 days or 2 to 0 day before anthesis (Fig. 5a). Similar trend was observed for number of seeds panicle^{-1} (Fig. 5c) and seed yield panicle^{-1} (Fig. 5d). Number of panicles plant^{-1} were not affected by HT stress (Fig. 5b).

Experiment V: genetic variability for HT stress tolerance: cardinal temperatures for pollen germination

The quadratic equation provided the best fit to predict the pollen germination response of different genotypes to temperature (Fig. 6a–d). The quadratic equation gave highest R^2 value and smallest r.m.s.d compared with the cubic, polynomial or bilinear equations (data not shown) for pollen germination. Therefore, a quadratic response was used to estimate cardinal temperatures [T_{min}, T_{opt} and T_{max}]. The result indicated that cardinal temperatures for pollen germination and maximum pollen germination percentage varied significantly among the pearl millet genotypes (Table 1). PI526279 and PI307704 had the highest (73.4%) and lowest (18.5%) pollen germination percentage (Table 1). Among the genotypes, T_{min} ranged from 5.4°C to 12.5°C (Table 1). Overall, the mean T_{opt} was 28.3°C, and the genotype PI526279 had the highest (34.4°C) and PI521642 had the lowest (24.3°C) value (Table 1). Among the genotypes T_{max} ranged from 43.8°C to 52.3°C with an overall mean of 47.6°C. The lines PI526279 and PI307704, respectively, had the highest and lowest T_{max} value (Table 1).

Experiment VI: relative sensitivity of pollen and pistil to HT stress

Pollination of panicles of both male and female plants grown under OT (28/18°C; OT male versus OT female) had significantly ($P < 0.05$) increased number of seeds panicle^{-1} compared with other cross combinations (Fig. 7a). At HT, 40/30°C, all the pollen grains were sterile, leading to no seeds panicle^{-1} (data not shown). Similarly, there was no seeds panicle^{-1} in the crosses when the female plants are exposed to same temperature (40/30°C; data not shown). Crosses that involve OT (28/18°C) and HT (36/26°C) or vice versa showed significant ($P < 0.05$) differences in number of seeds panicle^{-1}, individual seed weight and seed yield panicle^{-1} (Fig. 7a–c).

Cross between OT female (28/18°C) and OT male (28/18°C) had a seed number of 176, seed yield of 1.12 g and seed size of

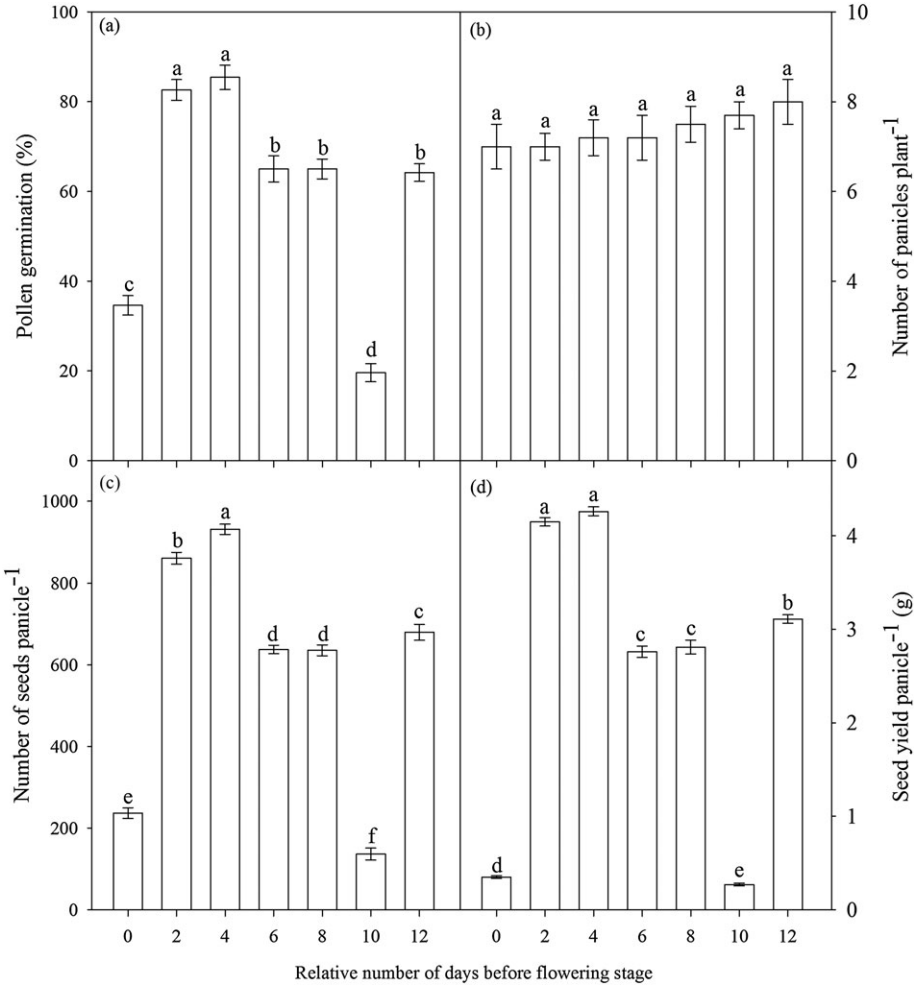

Figure 5. Impact of short episode of high temperature stress (40/30°C daytime maximum/nighttime minimum temperature for 2 days) at different times relative to anthesis on (a) pollen germination, (b) number of panicles plant^{-1}, (c) number of seeds panicle^{-1} and (d) seed yield panicle^{-1} (g). Vertical bars denote ±S.E. of mean. Means with different letters are significantly different according to the least significant difference test at $P < 0.05$.

0.0064 g (Fig. 7a–c). These data are from 3 cm marked portion of the panicle. However, the cross between HT female (36/26°C) and HT male (36/26°C) had a seed number of 33, seed yield of 0.2 g and seed size of 0.006 g (Fig. 7a–c). Cross between OT female (28/18°C) and HT male (36/26°C) had a seed number of 93, seed yield of 0.59 g and seed size of 0.006 g (Fig. 7a–c). However, cross between HT female (36/26°C) and OT male (28/18°C) had a seed number of 49, seed yield of 0.29 g and seed size of 0.006 g (Fig. 7a–c).

High temperature (36/26°C and 40/30°C) significantly ($P < 0.05$) increased XO enzyme activity and H_2O_2 content in both pollen and pistil compared with OT (28/18°C) (Fig. 8a,b). The pistil showed an increased in the XO enzyme activity and H_2O_2 content compared with pollen grains under HT stress (Fig. 8a,b). The antioxidant enzymes like SOD, CAT and POX activity were significantly ($P < 0.05$) decreased by HT stress (36/26 and 40/30°C) compared with OT (28/18°C) (Fig. 8c–e). The decrease in antioxidant enzymes activity is proportional to growth temperature, and maximum decrease was observed in pistil compared with pollen grains (Fig. 8c–e). HT stress did not alter the starch content in both pistil and pollen grains (Fig. 8f).

DISCUSSION

High temperature stress has direct impact on plant reproduction (fertility, seed-set and seed growth). Identification of temperature thresholds provides a basis for quantifying the impacts of HT stress. In most cereals, HT often leads to decreased seed-set, lower seed number, decreased seed filling duration and smaller grain size. Collectively, the effects of HT result in decreased seed yield (Prasad et al. 2017). Quantifying impacts of HT on different crops and different stages of crop development will help to define threshold and sensitive stages. The temperature ceiling (where seed yield was almost zero) for season long stress for dwarf pearl millet hybrid identified in this study was 42/22°C, which is relatively higher than other crops (rice: 40/30°C; Boote et al. 2005; temperate sorghum hybrid: 39/29°C; Prasad et al. 2006; wheat and dry bean: 36/26°C;

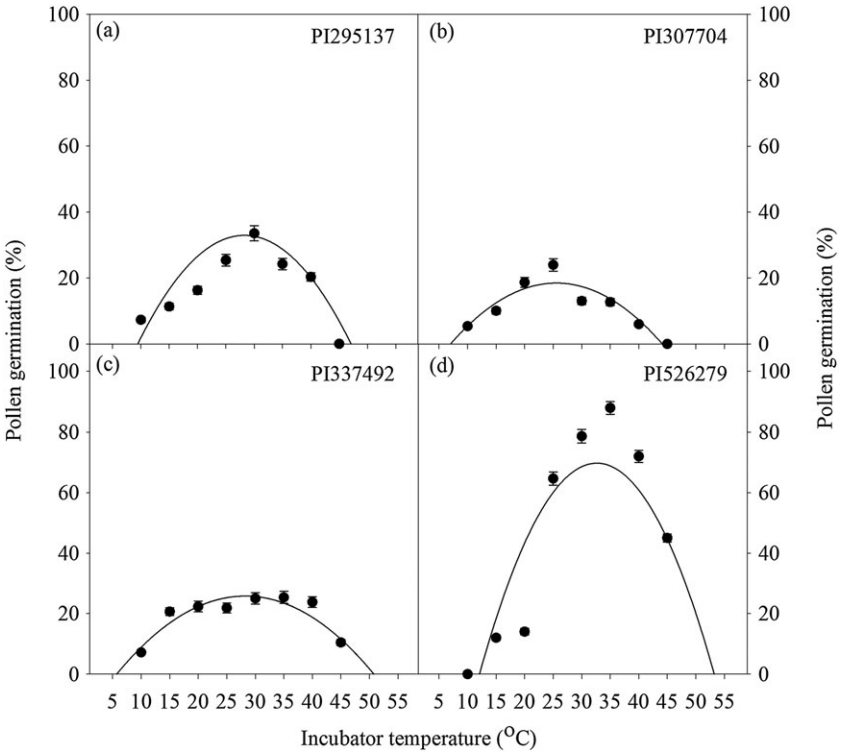

Figure 6. Pollen germination in response to temperature and fitted lines based on quadratic equation of contrasting pearl millet genotypes (PI295137, PI307704, PI337492 and PI526279) to determine cardinal temperatures [T_{min} (minimum temperature below which pollen grains did not germinate), T_{opt} (temperature where maximum germination occurred) and T_{max} (temperature above which pollen grains did not germinate)]. Vertical bars denote ± S.E. of means.

Prasad et al. 2002; Prasad and Djanaguiraman 2014; Prasad et al. 2017). Slightly lower ceiling temperatures in season long HT stress and flowering and entire reproductive period HT stress could be due to genotypic differences, and also, the season long experiment had 4 h maximum and minimum temperature compared with others, which had 6 h of maximum or minimum temperatures.

Temperature is one of the most important environmental factors affecting reproductive processes. High temperature stress when imposed during panicle development and flowering decreased floret fertility and seed-set percent. In the present study, *in vitro* pollen germination was severely decreased under both high and low temperature conditions. All the 28 pearl millet genotypes had defined T_{opt}, T_{max} and T_{min}, for the pollen germination. The quadratic model best described the response of pollen germination to temperature (Fig. 6a–d). Similar response was previously observed in grain sorghum (Djanaguiraman et al. 2014), groundnut (Kakani et al. 2002) and soybean (Salem et al. 2007). Among pearl millet genotypes PI307704 and PI521646 had the lower T_{max} (~44 °C) compared with genotype PI295158, PI337492 and PI526279 (~50°C). The observed T_{max} value for pearl millet was higher than most of the field crops (sorghum, peanut or soybean: T_{max} < 45°C; cotton: T_{max} [*Gossypium hirsutum*] >46°C; Kakani et al. 2005). Pearl millet is generally grown in areas where the maximum air temperatures range from 40°C to 48°C; and during reproductive stages, the air temperature would reach >40°C (Gupta et al. 2015). Recent pearl millet field studies in north western India (one of the hottest regions during summer season in India) showed genetic variability for seed-set at maximum air temperature of ≥42°C during flowering stage (Gupta et al. 2015). Hence, our observed T_{max} value >45°C is possible. Some genotypes had ceiling temperature close 48°C to 50°C for pollen germination, which is relatively higher as compared with other crops such as sorghum, cotton, groundnut or soybean. The cardinal temperatures are based on incubator temperatures (the actually tissue or medium temperatures could be about 1°C to 2°C cooler because of air circulation and location of the sensor that controls incubator temperature).

In sorghum, there was a strong positive correlation between *in vitro* pollen germination and seed-set (Djanaguiraman et al. 2014), indicating that pollen germination could be a useful tool for testing cultivar tolerance to HT. Hence, the ability of pollen grains to germinate and grow well at temperatures ~48°C under *in vitro* condition could be used as a tool to identify genotypes that can set seed at HT. However, the differences in cardinal temperatures did not reflect the tolerance or susceptibility of a genotype to HT because genotypes that had a higher OT (T_{opt}) did not always have a higher T_{max} or vice versa. Similarly, genotypes that had higher T_{max} did not have greater overall pollen germination percentages. Similar observations were reported in cotton (Kakani et al. 2005) and groundnut (Kakani et al. 2002). Thus, percent seed-set or seed

Table 1. Maximum pollen germination percentage, quadratic equation constants ($y = ax^2 + bx + c$) and cardinal temperatures for pollen germination for 28 pearl millet genotypes in response to temperature

Genotype	Maximum pollen germination (%)	Equation constant			R^2	RMSE	Cardinal temperature		
		a	b	c			T_{min}	T_{opt}	T_{max}
PI279663	26.0	−0.08	4.5	−37.7	92.2	3.4	10.3	27.9	45.7
PI295137	27.7	−0.08	4.4	−34.0	81.0	5.8	9.3	27.9	46.6
PI295146	55.9	−0.19	10.7	−95.7	89.2	9.8	11.1	28.4	45.6
PI295155	33.4	−0.11	5.8	−45.7	88.2	5.9	9.6	27.3	45.1
PI295158	61.0	−0.13	7.9	−53.3	64.8	15.3	7.7	29.1	50.5
PI295166	28.6	−0.08	4.7	−42.8	81.7	6.1	11.1	30.3	49.5
PI295168	28.0	−0.10	5.2	−43.8	77.5	7.4	10.4	27.8	45.1
PI307698	36.1	−0.10	5.7	−46.4	78.4	7.9	9.8	29.2	48.6
PI307703	35.6	−0.09	4.8	−27.0	70.4	8.2	6.4	25.8	46.1
PI307704	18.5	−0.05	2.8	−16.6	81.5	4.1	7.0	25.5	43.8
PI307713	48.4	−0.11	5.8	−30.9	81.9	6.1	5.9	27.3	48.5
PI337492	26.4	−0.05	3.1	−19.0	86.4	3.0	6.8	29.0	50.2
PI338000	26.5	−0.06	3.2	−15.8	91.3	2.7	5.5	26.3	47.2
PI511047	43.8	−0.10	6.3	−31.6	91.7	4.7	6.6	26.3	48.6
PI521612	46.0	−0.14	8.5	−83.5	65.3	17.7	12.3	30.4	48.6
PI521638	23.0	−0.06	3.4	−28.4	70.9	5.8	9.9	29.7	49.5
PI521642	59.5	−0.14	7.5	−37.1	68.2	15.3	5.4	24.3	45.8
PI521646	35.4	−0.11	6.1	−45.9	87.0	6.8	9.1	26.8	44.4
PI526275	34.6	−0.12	4.4	−58.6	85.0	7.0	10.9	28.0	45.2
PI526279	73.4	−0.23	15.6	−183.3	91.0	5.6	12.1	34.4	52.3
PI526282	36.8	−0.12	6.7	−55.9	89.0	6.1	10.2	27.6	45.0
PI526283	43.7	−0.14	8.3	−73.7	87.2	8.9	11.1	28.4	45.7
PI526287	43.5	−0.13	8.0	−77.2	63.5	16.4	12.0	30.2	48.3
PI526293	44.1	−0.15	9.1	−91.5	65.4	17.7	12.5	29.8	46.7
PI526308	27.9	−0.06	3.4	−19.8	79.5	4.5	6.4	27.9	49.5
PI526312	38.5	−0.13	7.9	−70.3	90.7	9.0	10.6	27.5	48.9
PI526335	19.9	−0.22	3.8	−33.6	84.1	4.3	11.1	28.3	45.6
PI526341	55.1	−0.16	9.5	−89.1	70.6	16.1	11.6	30.3	49.1
Mean	38.5	−0.11	6.1	−50.2	80.1	8.7	9.4	28.3	47.6
LSD	4.8	—	—	—	—	—	1.2	1.2	2.6

Data are based on incubator temperature and not medium temperature.
LSD, least significant difference.

numbers under HT stress may be a better indicator than T_{max} for pollen germination. However, understanding the physiological or biochemical reasons for relatively greater tolerance of pearl millet pollen to HT can help breed for tolerance to HT stress.

Seed-set percent, defined as number of seeds panicle^{-1}, mainly depends on the functionality of male and female gametes. Growing conditions during floral development and anthesis can negatively influence the performance of gametes, resulting in decreased seed-set percentage. The study showed that during reproductive stages the most sensitive periods to HT based on pollen germination and number of seeds panicle^{-1} were between 10 and 8 days before anthesis (gametogenesis) and between 2 and 0 days before anthesis (anthesis, fertilization and embryo formation). Similar results on cowpea (*Vigna unguiculata* L. Walp., Ahmed et al. 1992), common bean (*Phaseolus vulgaris* L.; Gross and Kigel 1994), groundnut (Prasad et al. 2001), wheat (Prasad and Djanaguiraman 2014), sorghum (Prasad et al. 2008, 2015), cowpea (Ahmed et al. 1992) and common bean (Gross and Kigel 1994) showed that gametogenesis and anthesis stages were the two most sensitive stages to HT stress causing maximum decreases in seed-set. It is known that the export rate of sucrose from source (leaves) to sink (seeds) organs correlates linearly with the current photosynthetic rate (Huber et al. 1984). In the present study HT stress affected seed-set percentage, but not photosynthetic rate (Fig. 3) leading to development of new tillers, leaves, more panicles and enhanced biomass (Fig. 3d,e). Similar results were observed in sorghum (Prasad et al. 2006) and maize (*Zea mays* L., Quattar et al. 1987) where under stress, the assimilate reserves from vegetative organs might have been re-mobilized and used for production of new tillers and panicles.

High temperature stress during pre-anthesis stage decreases pollen or ovule viability and/or stigma receptivity (Prasad et al. 2008; Nguyen et al. 2013; Djanaguiraman et al. 2014; Prasad and Djanaguiraman 2014), reproductive structures abnormalities (Prasad and Djanaguiraman 2014) and oxidative damage, resulting in pollen sterility and decreased seed-set (Djanaguiraman et al. 2014). High temperature stress at the time of anthesis can decrease floret fertility even when the pollen is viable (Prasad and Djanaguiraman 2014). The decreased floret fertility at this stage is commonly due to poor stigma receptivity, dryness of the stigma, poor pollen germination on the stigmatic surface and decreased

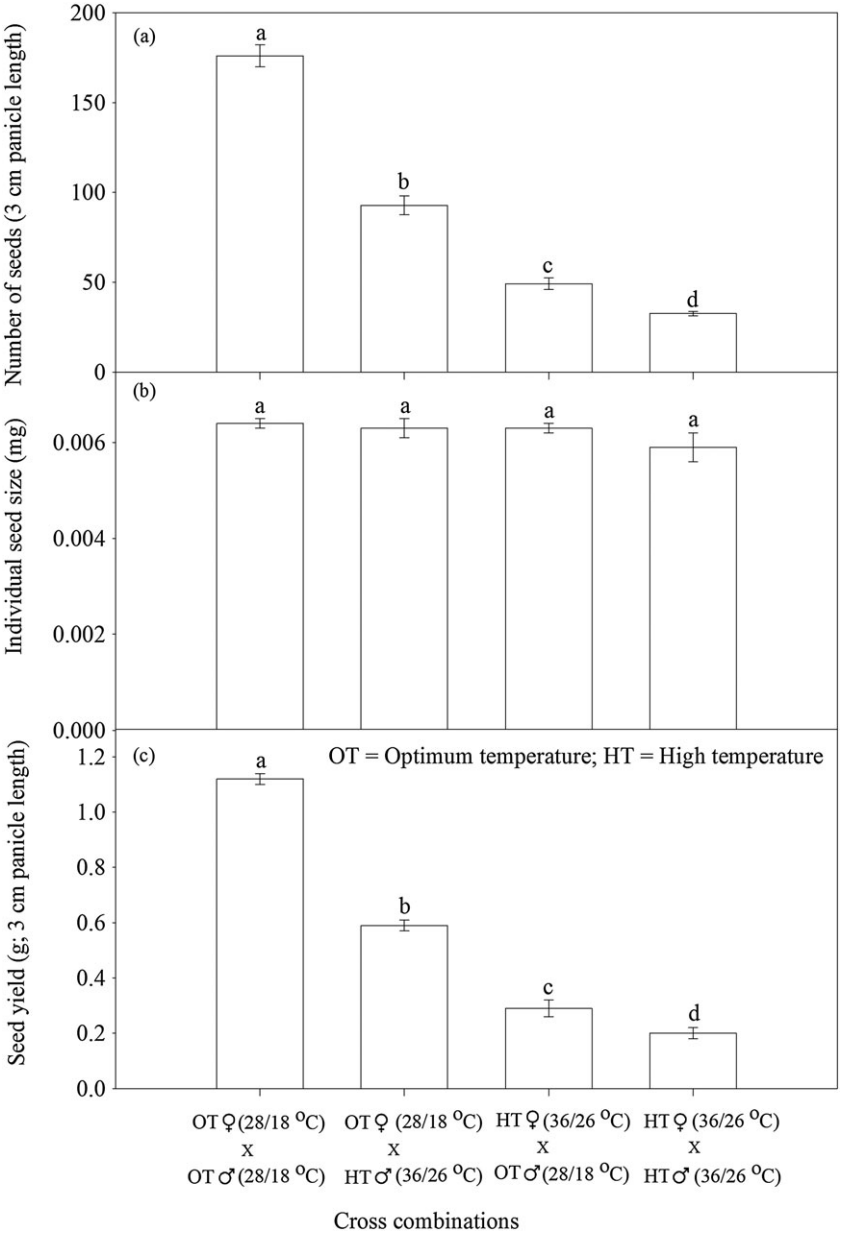

Figure 7. Impact of high temperature (36/26°C) and control (28/18°C) pollen crosses with high temperature (36/26°C) or control (28/18°C) pistils on (a) number of seeds (from 3 cm marked portion of panicle), (b) individual seed weight (mg from 3 cm marked portion of panicle) and (c) seed yield (g from 3 cm marked portion of panicle) to study the effect of high temperature stress on male and female reproductive organs. Vertical bars denote ±S.E. of mean. The symbols '♂' and '♀' represent male and female lines, respectively. Means with different letters are significantly different according to the least significant difference test at $P < 0.05$.

rate of pollen tube growth, leading to unsuccessful fertilization and lower seed-set (Kakani et al. 2002; Prasad and Djanaguiraman 2014).

Pollination of panicles grown at OT (28/18°C) with pollen collected from plants grown under OT resulted in the highest numbers of seeds and seed yield. Whereas, when OT plants were crossed with pollen collected from plants grown at HT (36/26°C) caused significant decreases in seed numbers (about 47%) and seed yield. Similarly, when plants grown under HT were crossed with pollen from OT, decreases in seed numbers (about 72%) and seed yield were greater. This implies that the pistil rather than pollen grains are relatively more vulnerable to HT stress. This is in accordance with the finding of Gupta et al. (2015) who observed similar responses. In contrast, the level of HT stress that caused male sterility in wheat had no damaging influence on the functions of the pistil indicating that the female reproductive tissues had the greater HT stress tolerance (Saini and Aspinall 1982). Similarly, Devasirvatham et al. (2012) reported that in chickpea (*Cicer arietinum* L.), pollen is the most sensitive to HT compared with pistil tissues. Likewise, in maize, the megagametophytes appear to be unaffected under HT stress, and it is desiccation and loss of

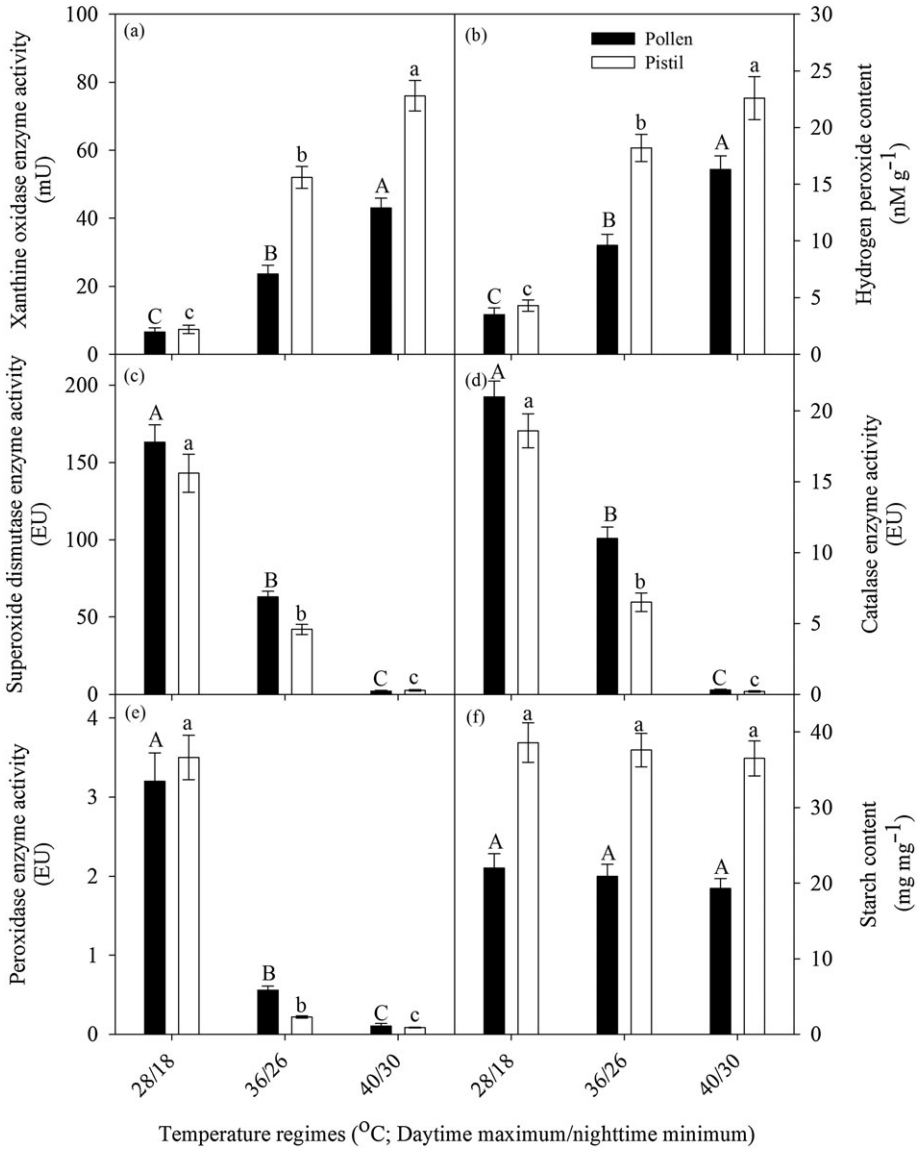

Figure 8. Impact of high temperature stress (36/26°C or 40/30°C daytime maximum/nighttime minimum temperature) from booting to 3 days after anthesis (15 days) on (a) xanthine oxidase enzyme activity (mU), (b) hydrogen peroxide contents (nM g^{-1}), (c) superoxide dismutase enzyme activity (EU), (d) catalase enzyme activity (EU), (e) peroxidase enzyme activity (EU) and (f) starch concentration (μg g^{-1}) in pollen grains and pistils. Vertical bars denote ±S.E. of mean. Means with different letters (capital letter for pollen and small letter for pistil) are significantly different according to the least significant difference test at $P < 0.05$.

pollen viability that are thought to be the primary cause of HT induced yield reductions (Herrero and Johnson 1980; Dupuis and Dumas 1990). This clearly suggests differential response and sensitivities of male and female reproductive organs to HT stress (Herrero 2003; Hedhly et al. 2008).

High temperature stress induced higher concentrations of ROS (O_2^- and H_2O_2) in both pistils and pollen grains. However, pistils had higher ROS than pollen grains (Fig. 8a,b). Greater concentrations of ROS causes oxidative damage through the imbalance in the generation and metabolism of ROS, with more ROS produced than are metabolized (Apel and Hirt 2004; Djanaguiraman et al. 2010; Prasad and Djanaguiraman 2011). Greater ROS production under HT stress in pistils could cause increased membrane damage and susceptibility compared with pollen grains (Djanaguiraman et al. 2014). Plants have an enzymatic antioxidant defense system to protect against ROS-induced membrane damage. CAT and POX coordinate with SOD to protect the plant in the scavenging processes. We found that the SOD activity decreased by HT in both pollen grains and pistils (Fig. 8c). However, maximum decrease was observed in pistil, indicating less efficient scavenging of the superoxide radical by SOD to form H_2O_2 in the pistil than pollen grains. The increase in H_2O_2 level in pistil compared with pollen grains may be due to higher superoxide radical dismutation in the presence of a reductant or elevated synthesis and/or decreased activity of POX and/or CAT. The greater inhibition of CAT and POX in HT stressed pistil relative to pollen grains indicates greater inactivation of

these enzymes by HT stress. This might be due to the toxic effects and high turnover rate of ROS, which impair enzyme activities (Noctor and Foyer 1998). However, it needs to consider that pearl millet is protogynous and cross pollinated crop, where stigma protrudes out of florets and exposed to outside environmental conditions, compared with other cereals crops such as sorghum, rice or wheat and other legumes (groundnut and soybean), which are all self-pollinated and have cleistogamy and stigma and pollen is protected from outside environment. This exposure to outside environment may explain greater sensitivity of pistil and greater concentrations of ROS. Further research is required to understand these mechanisms and responses.

In summary, our research demonstrated that (1) increases in growth temperature from emergence to maturity from 32/22°C to 44/34°C decreased the seed yield and seed size; (2) HT of 40/30°C caused close to 95% sterility and resulted in no seed formation when HT stress was imposed from booting to maturity or booting to start of grain filling; (3) there were two periods (10–8 days and 2–0 days before anthesis) that were most sensitive to short episodes (2 days) of HT stress resulted in decreased seed-set; (4) genotypic variability for HT stress in terms of cardinal temperatures for pollen germination (mainly explained by differences in T_{max} for pollen germination) was found; and (5) there was negative impact of HT stress on function (fertility) of pollen grains and pistil; however, pistil was relatively more sensitive to HT than pollen, which was explained by greater oxidative damage in pistils than pollen grains. Compared with other cereals, pearl millet has greater ceiling temperatures for grain yield, thus an important climate resilient crop suitable for cropping systems in the semi-arid regions of the world. Further research should focus on determining mechanisms for relatively greater tolerance of pearl millet pollen grains to HT stress, which can potentially guide breeding for HT tolerance in other cereals crops where pollen is more sensitive to HT stress. The results of this research can help with developing efficient methods to screen germplasm collection of pearl millet to identify lines that are tolerant to HT stress and use them in breeding programme to develop parental lines or hybrids with HT tolerance. Deployment and adoption of HT tolerant genotypes or hybrids will increase resilience of millet based cropping systems in current and future climates.

ACKNOWLEDGMENTS

We thank the USAID for supporting CGIAR and US University linkage grant from International Crop Research Institute for Semi-Arid Tropics; and Feed the Future Innovation Lab for Collaborative Research on Sustainable Intensification (Grant no. AID-OAA-L-14-00006) for financial support. We thank Tamil Nadu Agricultural University, India, for permitting Dr M. Djanaguiraman to conduct post-doctoral research at Kansas State University, USA. This is contribution no. 17-197-J from the Kansas Agricultural Experiment Station. We declare that we have no conflict of interest.

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Received 30 November 2016; received in revised form 9 January 2017; accepted for publication 16 January 2017