

Various temperature effects on spikelet growth in hulless oat during grain-filling stage

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Temperature conditions affect growth and grain development during the grain-filling stage, but a comprehensive analysis of oat subjected to different temperatures during grain development has not been studied. In this study, an integrated physiological and proteomic examination of oat spikelets was performed to analyze the influence of five different day-time temperatures on stress-relative parameters and grain development. Physiological analysis showed decrease of total chlorophyll, shoot dry weight and spikelet shape development and increased activation of MDA, soluble sugar and antioxidant enzymes, with increase of temperatures. However, considering major grain yield components and storage materials, there should be an optimum temperature during ripening period. The result of proteomic analysis showed significantly high expressions of stress-related gene in high temperature treatment and grain storage materials in optimum temperature. Our findings indicate that temperature conditions during the grain-filling period exert a major influence on yield potential.

Key words: *Avena nuda*, temperature stress, physiological measurement, proteome analysis, stress tolerance, grain development

Introduction

Oat (*Avena sativa* L.), a crop that grows well under poor soil and adverse environmental conditions, is primarily cultivated in countries such as Russia, Canada, and Europe (Schnitzenbaumer and Arendt 2014). Compared to the nutritional value of other cereals, oat has a higher nutritional value because it naturally contains many important nutrients such as soluble fibers, proteins, unsaturated fatty acids, vitamins, minerals, and antioxidants (Lásztity 1998). In addition, the grains contain large quantities of compounds such as β -glucan, oil, and protein, because of which oat is a valuable resource for industrial applications (Gorash et al. 2017). The protein composition of oat grains is also dominated by globulins rather than prolamins that are found in barley or wheat (Schnitzenbaumer et al. 2012). Oat florets are branched cluster of loose lemma and palea. The composition and level of multiple proteins accumulated in *Avena* husks were significantly changed under multiple stresses (Graf and Singiri 2022). Because of the high nutrient density in the grains, oat has become extremely popular in recent years.

Oat can be classified into two types according to the presence or absence of hull, namely, hulled oat (*A. sativa* L.) and hulless oat (*A. nuda* L.). The nutritional value of hulled oat is lower than that of other cereals because of its high fiber content, and it needs to be dehulled before being rolled or processed into flour, which is used for human consumption or as feed for non-ruminant animals (Zarkadas et al. 1995). After the initial release and commercial success of hulless oat cultivars, many early maturing genotypes showing increased grain yield, superior agronomic traits, increased disease resistance, improved seed quality, and higher energy and protein content were subsequently released. Because of these beneficial traits, the economic importance of hulless oat has recently risen. However, abiotic stresses limit the growth of hulless oat, thereby reducing the crop yield.

Oat has been shown to respond to a variety of environmental conditions, including drought, salt, and infectious diseases. It undergoes morphological and physiological changes in response to abiotic stresses (Wu et al. 2017, Jinqiu et al. 2021). Temperature stress, one of the key elements influencing oat growth conditions and grain production, causes serious damage during the grain-filling stage in oat. High temperatures during the grain development and filling phases reduced the period from spikelet emergence to spikelet maturity and adversely affected the grain-filling process (Jaiswal et al. 2017). Nevertheless, the optimal temperature required during the grain development stage in oat is not known till date.

Although this nutritious cereal crop is grown worldwide, the extent of genetic research on this crop has fallen behind the volume of research on other cereal grains. Currently, there is no comprehensive and widely annotated oat reference genome, and only a few transcriptome analyses have been reported so far. Proteins are involved in all biological processes and plant signaling networks (Wang et al. 2016). As transcriptome data do not always align

with protein expression levels, it is important to analyze protein expression as well (Jiang et al. 2017). Proteomic analysis is a powerful method for studying total protein expression that helps in revealing the specific proteins associated with any biological process (Li et al. 2015).

In this study, various analyses were performed during the grain-filling stage to examine the mechanism of response to temperature stress in oat. An integrated physiological, and proteomic examination of oat spikelets was performed during the grain-filling stage to analyze the effect of variation in temperature on grain physiology. The spikelets were exposed to five different temperatures to investigate the stress response mechanism and the effect on grain yield distribution along the spikelet. The results of the stress measurement assays indicated that grain growth was affected by the variation in temperature. Identification of the optimal temperature needed during the grain-filling stage to maximize crop yield will also accelerate research on other oat seed quality parameters in the future.

Materials and methods

Plant growth conditions

The Korean hulless oat cultivar ‘Choyang’ (accession no. IT276394), which was developed in 2007 by the Department of Rice and Winter Cereal Crop, National Institute of Crop Science, Rural Development Administration, Republic of Korea, was used in this study. Seeds of oats were vernalized at 4 °C for 4 weeks. After the vernalization, each plant was transferred into a pot (12 × 10 × 12 cm, top × height × bottom diameter) filled with soil (Baroker; Seoul Bio, Korea). The experiment was conducted under well-controlled conditions in a greenhouse. The temperature was set at 22–25 °C/15–18 °C (maximum day-time temperature/minimum night-time temperature) with a photoperiod of 16 h/8 h. Temperatures were monitored every 15 min using data recorders during all stages of plant development (HOBO UX100-003, Onset Computer Corporation).

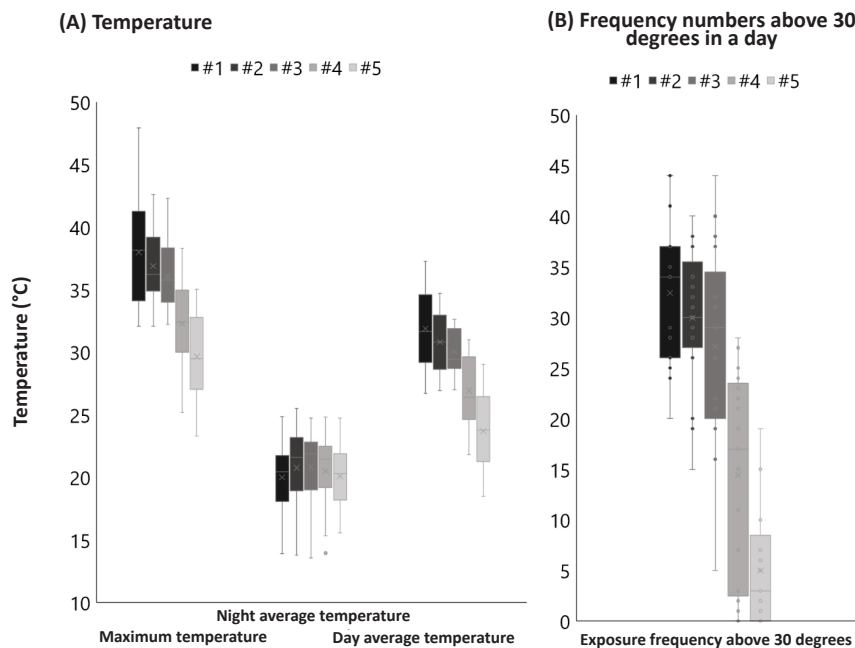


Fig. 1. Temperatures observed during the oat grain-filling period. Temperatures were automatically recorded every 15 min using a thermometer (HOBO Temp/RH Logger, USA) throughout the treatment period. (A) Maximum and daily average temperature. (B) Number of times the temperature rose above 30 °C per day. #1: 33 °C, #2: 31 °C, #3: 29 °C, #4: 27 °C, and #5: 25 °C.

The number of days taken by the main tillers of each plant to transition from panicle initiation to heading of spikelets was noted, and each plant was duly labeled with their respective heading dates. After 10 days of heading, fifteen plants at similar stages of growth were planted in separate greenhouses set at five different day-time temperatures (33 °C [#1], 31 °C [#2], 29 °C [#3], 27 °C [#4], and 25 °C [#5]), while the night-time temperatures were maintained at 20 °C until grain maturation. The average day-time temperatures, night-time temperatures

and maximum day-time temperatures were recorded (Fig. 1A). The average number of times (or the average frequencies) that the temperatures in the greenhouses were observed to rise above 30 °C in a single day (Fig. 1B).

Physiological measurement of plant growth

The total chlorophyll content was determined using a chlorophyll meter (SPAD-502 Plus, FieldScout® meters, USA). The middle portions of flag leaves on the main tillers (which were tagged earlier) of the ten plants that were subjected to high temperature stress were measured in triplicates on 0, 5, 12, 16, 20, 24, 26, 29, and 33 days after treatment (DAT) at 09:00. Yield and yield components were measured after harvesting the grains. All the plants were collected and grouped based on the number of tillers (6, 7, and 8). Plants without spikelets were dried at 60 °C for 10 days to estimate dry shoot weight. Spikelets were counted for assessment of spikelet development (single, double, triple, small multiflorous, and large multiflorous kernels) following the method used by (Doehlert et al. 2006) and the spikelet shapes were noted. Ten spikelet samples were dried for three days by incubating at 35 °C before being threshed to extract grains. Grain size, 100-grain weight, and seed numbers were measured for each plant and analyzed. All physiological measurements were performed in ten replicates.

Measurement of different parameters

Dehulled spikelets were collected on DAT 14 and DAT 20 for enzyme assays. Spikelets were harvested from three individual plants with tagged tillers and immediately frozen in liquid nitrogen before being stored at –80 °C. The amount of malondialdehyde (MDA) present in the spikelet tissue was measured using thiobarbituric acid (TBA) assay with some modifications (Senthilkumar et al. 2021). The soluble sugar content was measured using the anthrone method with some modifications (Hansen and Møller 1975). Antioxidant enzyme assays were performed as previously described (Ko et al. 2018). All enzyme assays were performed in three replicates.

Measurement of components of the harvested grain

After recording the physiological grain yield components, the grains were ground using a flour mill grinder (Krupps Garantie-Karte, KM75, Mexico) to measure the grain components. Amounts of β -glucan and total starch present in the sample were determined using the Megazyme assay kit (K-YBGL and K-TSTA-100A, respectively; Megazyme, Wicklow, Ireland) following the manufacturer's instructions. Total globulin content was measured using a general globulin assay kit (MBS8309619; MyBioSource, San Diego, CA, USA). To examine the composition and molecular weight of globulins and albumins present in the sample, the total storage proteins were subjected to one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Total oat storage protein was extracted according to the protocol (Nałęcz et al. 2017). Protein concentration was examined using the Bradford method (Bradford 1976). SDS-PAGE analysis was performed following a previously published protocol with some modifications (Laemmli 1970). All measurements of grain components were performed in three replicates.

Determining the association between measured characteristics

The XLSTAT software (<https://www.xlstat.com>) was used to conduct a linear regression analysis to determine the probable causal effect of variation in temperature on grain development and stress tolerance, with a significance threshold of $p = 0.05$. MDA level, soluble sugar content, ascorbate peroxidase (APX) activity, catalase (CAT) activity, peroxidase (POD) activity, superoxide dismutase (SOD) activity, seed weight, seed number, β -glucan content, total starch content, and globulin content were assigned as the dependent variables (X), and the different temperatures used to create stress in the plants and the duration of stress treatment were allocated as independent factors (Y). All the variables assessed were subjected to correlation analysis (CA). Through graphical representation, a symmetric plot was utilized to demonstrate the relationship between the combined parameters and their effects on the plants that were subjected to stress.

Proteome analysis

Two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF) analyses were performed on total protein extracted using GENOMINE (Pohang, Korea). Isoelectric focusing (IEF) was performed using a Multiphor II electrophoresis device connected to an EPS 3500 XL power supply (Amersham Biosciences), following the manufacturer's instructions. Before being subjected to the second dimensional electrophoresis, the strips were incubated for 10 min in the equilibration buffer (50 mM Tris-HCl containing 6 M urea, 2% sodium dodecyl sulfate [SDS], and 30% glycerol; pH 6.8)

containing 1% DTT, and then with equilibration buffer containing 2.5% iodoacetamide. The equilibrated strips were inserted into SDS-PAGE gels (20 × 24 cm, 10–16%). SDS-PAGE was performed using a Hoefer DALT 2D system (Amersham Biosciences), following the manufacturer's instructions. 2-DE gels were run at 20 °C for 1700 Vh. The 2-DE gels were stained with Coomassie G250, using the method (Anderson and Anderson 1991). For MALDI-TOF/TOF analysis, gel pieces were washed with 50% acetonitrile. After being washed and dehydrated, the spots were vacuum dried to remove the solvent before being rehydrated with trypsin (8–10 ng μl^{-1}) solution in 50 mM ammonium bicarbonate, pH 8.7, and incubated for 8–10 h at 37 °C. A BRUKER Autoflex maX with LIFT ion optics was used to evaluate the samples. MS and MS/MS data were collected using a SMARTBEAM LASER with a 2 kHz repetition rate, and each spectrum received up to 4000 shots. Both MS and MS/MS data were acquired using default settings of the instrument, without applying any internal standard or external calibration. MS/MS ion searches were performed using the Mascot database for in-house use.

Statistical analysis

All experiments were performed in triplicates, making sure that there was no variation in the temperatures (to which the replicates were exposed). SPSS11.0 (SAS Institute Inc., Cary, NC, USA) was used to perform a two-way analysis of variance for all assays and parameters.

Results

Analysis of phenotype and related parameters in different temperature conditions

Heat stress has a considerable impact on the grain-filling stage in oat. In general, the plants completed the grain-filling stage in approximately 35 days. Heat stress, on the other hand, accelerated maturation. Total chlorophyll content can be used to investigate plant senescence and stress tolerance. The total chlorophyll content measured in flag leaves of plants #1, #2, and #3 was considerably lower than that in flag leaves of the other treated plants (Fig. 2A). The influence of temperature on the treated plants was observed to be more after DAT 29 and DAT 33 than its effect on any other day. The total chlorophyll content decreased as temperature increased. However, in the case of spikelets, the days to maturity did not change in response to high temperatures.

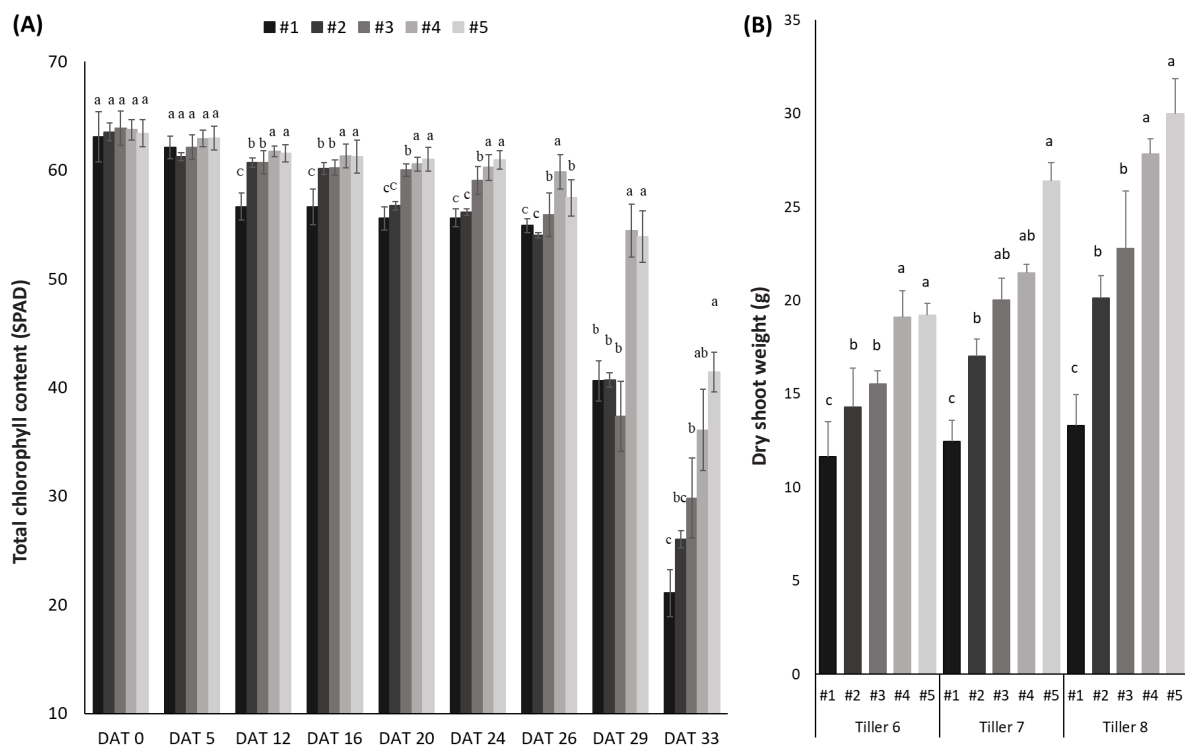
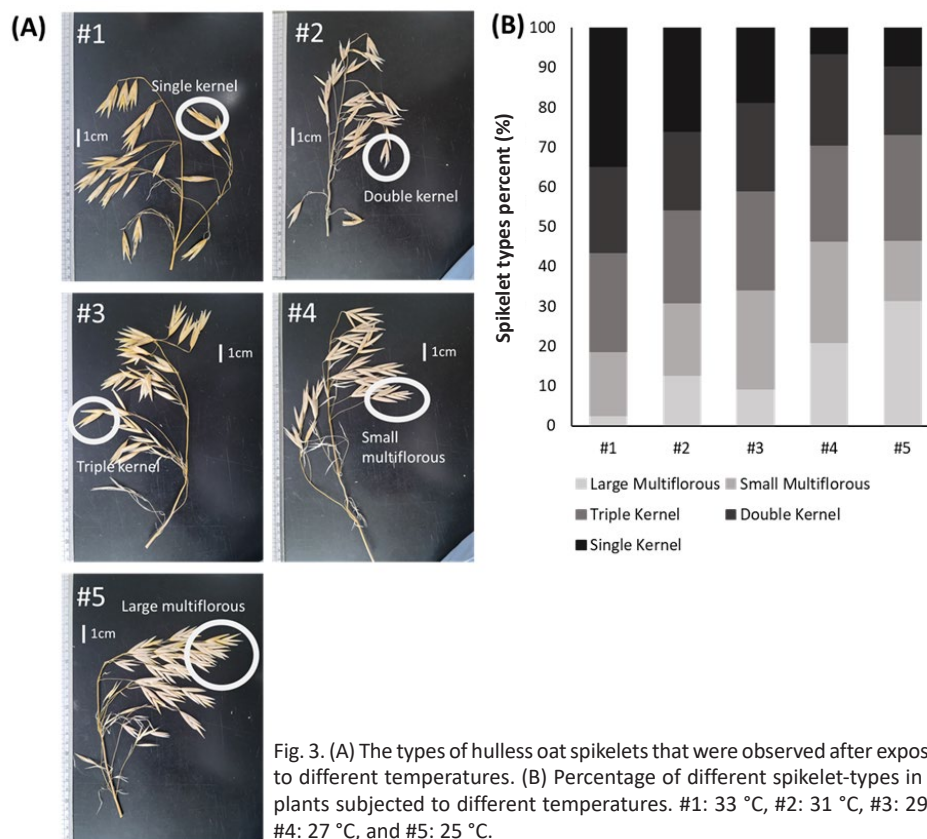


Fig. 2. (A) Total chlorophyll content observed from DAT (days after treatment) 0 to DAT 33. X-axis shows time lapse. Y-axis indicates total chlorophyll content. (B) Comparison of shoot dry weight of plants having same number of tillers. The five different temperatures to which the plants were exposed were 33 °C (#1), 31 °C (#2), 29 °C (#3), 27 °C (#4), and 25 °C (#5). The letters written on top of the vertical bars represent the levels of significance of differences in the shoot dry weight of the treated plants ($p \leq 0.05$).

Analysis of dry shoot weight in plants revealed variation among samples kept in different temperature conditions. Temperature stress significantly reduced the dry shoot weight in plants #1, but the change was not so noticeable in plants #5 (Fig. 2B). A lower shoot weight was observed in plants #1, independent of the number of tillers (11.65 [6 tillers], 12.457 [7 tillers], and 13.312 g [8 tillers]). In contrast, the shoot weight of plants #5 plants increased significantly (19.225 [6 tillers], 26.398 [7 tillers], and 29.99 [8 tillers]). Reduction in shoot dry weight was considerably higher for treated plants with 8 tillers than the reduction of shoot dry weight of plants with 6 or 7 tillers.

Hulless oat spikelets were composed of five different types of florets containing single, double, triple, small multiflorous, and large multiflorous kernels (Fig. 3A). In general, differences in the type of spikelet observed was influenced by the plant genotype and environmental factors. In this study, however, the spikelet form and features were attributable to varying magnitudes of response to environmental variables, especially temperature. We examined the changes in spikelet-type and the variation in temperature (Fig. 3B). Single kernels were abundant in plants #1 (34.71%), whereas large multiflorous kernels were abundant in plants #5 (31.42%). Triple kernels were present in all the spikelets regardless of the temperature (24.87% [#1], 23.24% [#2], 24.87% [#3], 24% [#4], and 26.67% [#5]). The proportion of single and large multiflorous kernels found on the spikelets was significantly affected by the variation in temperature.



Overall, total chlorophyll content, shoot dry weight, and spikelet-type were significantly influenced by high temperatures. Thus, variation in temperature affected the morphological characteristics of the plants. Further, under heat stress, there was a reduction in total chlorophyll content and shoot dry weight.

Effect on malondialdehyde and soluble sugar levels and antioxidant enzyme activities because of the variation in temperature

Plants generate MDA, soluble sugars, and antioxidant enzymes such as APX, CAT, POD, and SOD to defend themselves from environmental stress. Variation in temperature and duration of exposure to high-temperature stress had a substantial effect on all measured physicochemical characteristics.

Soluble sugars and MDA are synthesized by plants for osmotic adjustment of the cells that are exposed to high-temperature stress. Therefore, we conducted this experiment to investigate whether the variation in temperature affected the levels of soluble sugars and MDA in the stressed plants. There was a slight difference in the MDA and soluble sugar levels of plants #1 and #5 on DAT 14 (Fig. 4). There was no significant change in the soluble sugar levels on DAT 14 (8.9 [#1], 8.41 [#2], 8.39 [#3], 8.26 [#4] and 7.98 mg l⁻¹ [#5]). However, there was a marked difference in the soluble sugar levels on DAT 20 (8.09 [#1], 7.68 [#2], 5.98 [#3], 6.26 [#4] and 5.68 mg l⁻¹ [#5]). Moreover, the levels of MDA had significantly increased by the end of the experiment (DAT 20). The results revealed that cellular damage was reduced in plants #4 and #5 that were subjected to low-temperature stress conditions.

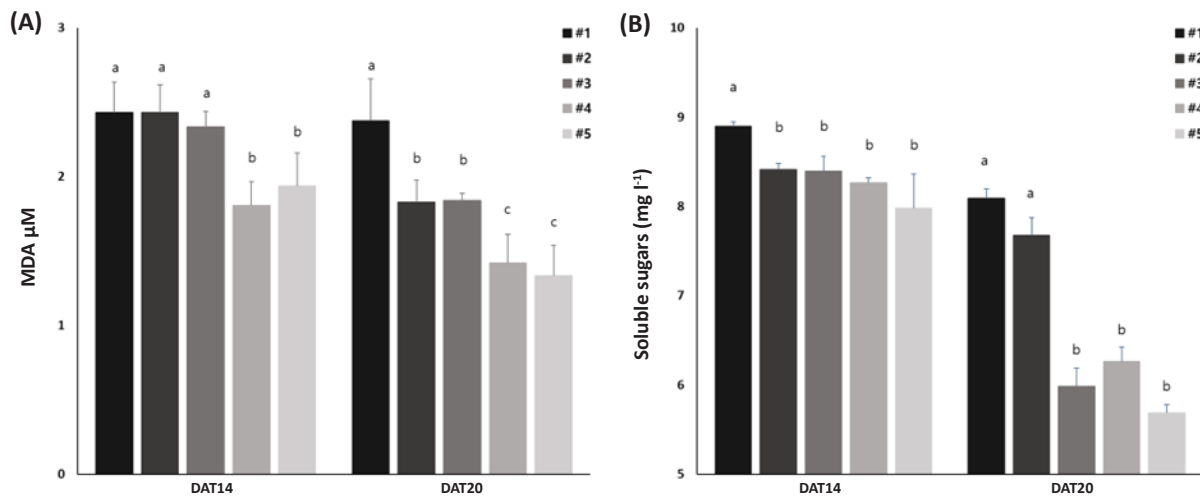


Fig. 4. Levels of (A) MDA and (B) soluble sugars on DAT 14 and DAT 20 in plants that were exposed to different temperature conditions. #1: 33 °C, #2: 31 °C, #3: 29 °C, #4: 27 °C, and #5: 25 °C. The letters written on top of the vertical bars represent levels of significance of the differences in the (A) MDA and (B) soluble sugar contents ($p \leq 0.05$).

The activities of antioxidant enzymes such as APX, CAT, POD, and SOD were measured (Fig. 5). Antioxidant defense mechanisms help in maintenance of redox homeostasis under high-temperature stress. APX, CAT, and POD showed similar levels of activity when the plants were subjected to different temperature conditions. The enzyme activities decreased under low-temperature conditions. On DAT 20, in all the plants that were exposed to the different temperature conditions, level of activity of each of the enzymes (except SOD) had decreased (9.75 [#1], 10.3 [#2], 9.12 [#3], 6.89 [#4] and 7.53 units mg⁻¹ [#5]) from the level of activity that had been observed on DAT 14. However, in the early phases of stress (DAT 14), the level of activity of each of the enzymes had increased. In plants #1, antioxidant enzyme activities were significantly elevated on DAT 14 as well as on DAT 20. These findings indicate that the activities of antioxidant enzymes increased significantly under high-temperature conditions.

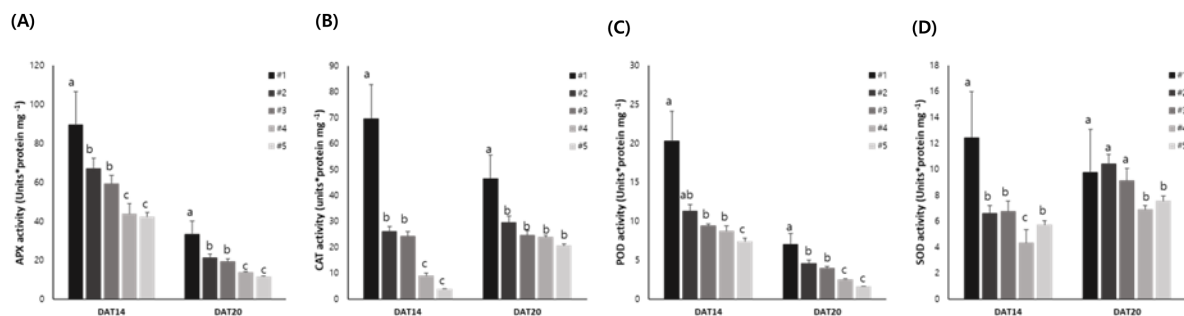


Fig. 5. Activities of antioxidant enzymes (A) APX (ascorbate peroxidase), (B) CAT (catalase), (C) POD (peroxidase), and (D) SOD (superoxide dismutase) on DAT 14 and DAT 20 in plants that were exposed to different temperatures during the grain-filling period. #1: 33 °C, #2: 31 °C, #3: 29 °C, #4: 27 °C, and #5: 25 °C. The letters on the vertical bars represent the levels of significance of the difference between enzyme activities ($p \leq 0.05$).

Analysis of grain width, weight, and yield under different temperature conditions

Oat grains were harvested at full maturity. When the plants reached complete physiological maturity, the yield components, such as grain dry weight, and number of grains per plant, were measured. For all these parameters, the results obtained from three independent replicates were averaged to minimize experimental error.

Out of all the plants subjected to different temperature conditions, the grain width and grain dry weight per plant was the highest in plants #3 and the lowest in plants #1 and #5 (Fig. 6A and 6B). The seed number per plant was greatly reduced in plants #1, but the number of seeds had not substantially decreased in plants #4 (Fig. 6C). The number of seeds in all the plants ranged from 52.6 (#1) to 144.75 (#4). The results of analysis of variance showed that there was no significant variation in seed numbers of plants #3, #4, and #5. However, the width and dry weight of the grains decreased under high-temperature stress, and there were significant differences in the seed numbers of plants that were exposed to different temperature conditions. There was a considerable difference in the 100-seed weight of plants #3 (3.22 g) and #1 (1.62 g). Plants #3 and #4 exhibited significant increase in grain width and grain dry weight, whereas plants #1 and #5 showed significantly lower values for these parameters. Optimal temperatures for grain-filling were deduced to be 27 °C (#4) and 29 °C (#3). High-temperature (33 °C, #1) and low-temperature (25 °C, #5) conditions significantly reduced grain production.

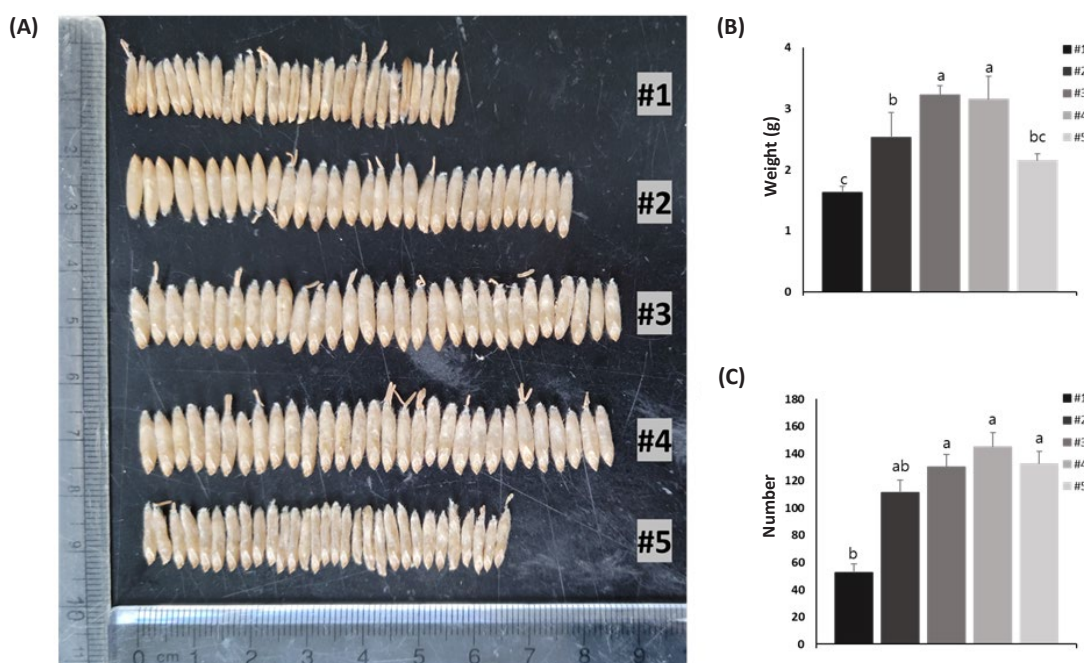


Fig. 6. (A) Morphology of mature grains harvested from the plants that were subjected to the different temperature conditions. (B) 100-seed weight and (C) seed number per plant observed in the plants that were subjected to the different temperature conditions. #1: 33 °C, #2: 31 °C, #3: 29 °C, #4: 27 °C, and #5: 25 °C. The letters written on top of the vertical bars represent the levels of significance of the differences in (B) 100-seed weight and (C) seed number per plant ($p \leq 0.05$).

Analysis of grain components in different temperature conditions

Differences in grain storage components could be observed in oat grains subjected to temperature stress. Analysis of grain components (β -glucan, total starch, and total globulin content) was performed on grains that were harvested and milled at maturity.

The β -glucan, total starch, and globulin contents (Fig. 7) were significantly higher in plants #3, #4 and lower in plants #1, #5. On average, the highest quantities of these seed components were reported from plants that were subjected to optimal temperature conditions (29 °C, #3) during the grain-filling stage (β -glucan: 734.85 mg, total starch: 787.569 mg, and total globulin: 40.634 mg), whereas lower quantities of these components were found in plants subjected to temperatures of 25 °C (354.6, 392.616, and 31.051 mg, respectively) or 33 °C (322.65, 352.888, and 33.086 mg, respectively). Overall, exposure to optimal temperature conditions (27 °C [#4] and 29 °C [#3])

resulted in a significant increase in the analyzed grain components compared with the exposure to temperature stress (25 °C [#5] and 33 °C [#1]) that led to a decrease in the quantities of these components. Although the plants exposed to 25 °C temperature produced many large multiflorous spikelets (about 28%) with variable number of florets within the spikelets, the quantities of the analyzed grain components were reduced. Significant reduction in quantities of the analyzed seed components was recorded in plants exposed to non-optimal temperatures.

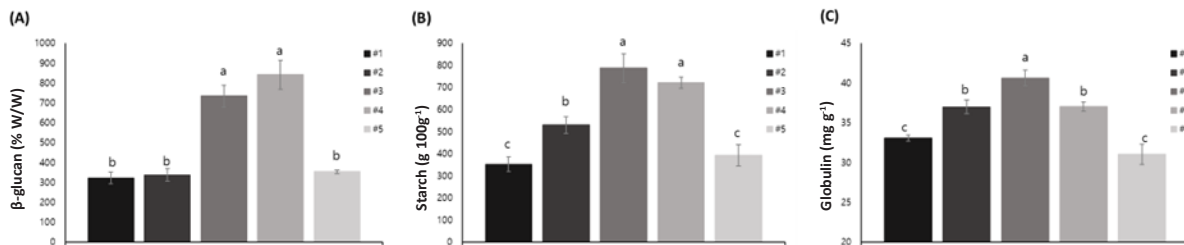


Fig. 7. (A) β -glucan, (B) total starch, and (C) total globulin contents of seeds from plants exposed to different temperature conditions. #1: 33 °C, #2: 31 °C, #3: 29 °C, #4: 27 °C, and #5: 25 °C. The letters on top of the vertical bars represent the levels of significance of differences in the quantities of the analyzed seed components ($p \leq 0.05$).

The storage proteins present in the seeds were separated by SDS-PAGE. All the samples exhibited distinct bands ranging in size from 30 kDa to 65 kDa. On the basis of analysis of these separated protein bands, storage proteins could be classified into two categories: albumins and globulins (65 kDa-7S globulins, 45 kDa-albumins, 40 kDa-albumins, and 30 kDa- β -globulins) (Sánchez-Velázquez et al. 2021). Compared to the expression of albumins and globulins in the seeds of plants #3 and #4, the expression of these proteins was much lower in the seeds of plants #1 and #5 (e.g., the expression of 30 kDa- β -globulins in plants #5; Fig. 8). The composition and quantity of storage proteins was distinctly dependent on the temperature conditions present during the grain-filling stage.

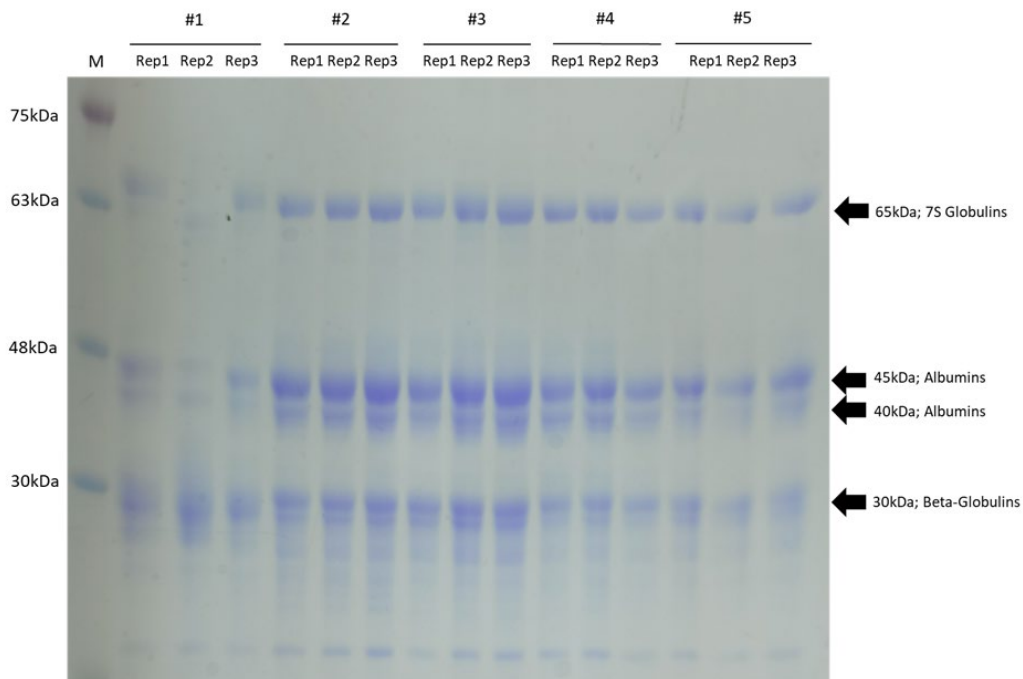


Fig. 8. Analysis of globulin and albumin profile by SDS-PAGE. These storage proteins were obtained from the grains harvested from plants that were exposed to different temperature conditions. Lane M – molecular weight marker. #1: 33 °C, #2: 31 °C, #3: 29 °C, #4: 27 °C, and #5: 25 °C.

Association between stress tolerance and seed development in different temperature conditions

Correlation analysis (CA) was used to analyze the relationships between the parameters measured and the combined factors. Based on the results of CA (Fig. 9), the parameters measured were categorized into two distinct types: seed development parameters (left region) and stress tolerance parameters (right region). These findings revealed that enhanced antioxidant enzyme activity, especially that of POD and CAT, seems to be a crucial element associated with oat stress tolerance. The seed number and seed weight parameters were placed near plants #5, while other grain development parameters were placed near plants #2, #3, and #4 (Fig. 9A). DAT 14-plants #2 were positioned on the borderline between stress tolerance and seed development parameters. Except for plants #1, the difference between data obtained on DAT 14 and DAT 20 for the analyzed parameters from all the other plants was insignificant (Fig. 9B). These results indicated that the results of the relative stress measurement assays and grain development parameters in the temperature-stressed plants were influenced by the variation in temperature during the grain-filling stage.

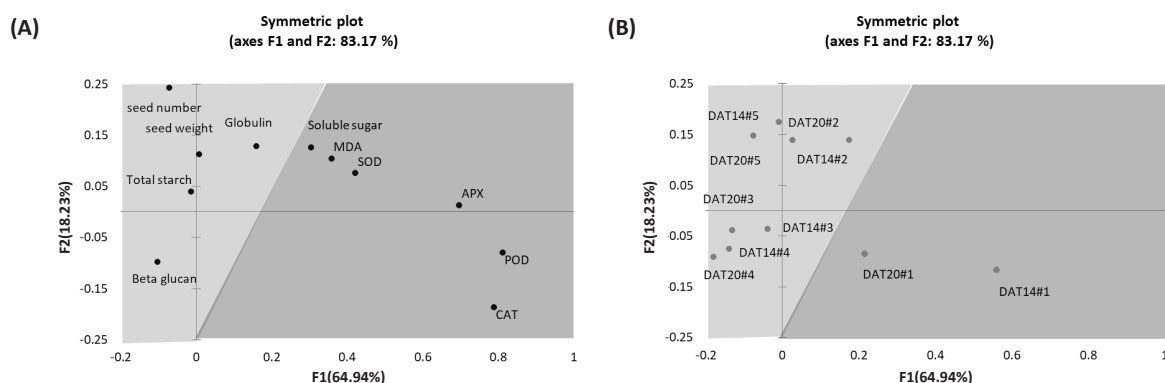


Fig. 9. Association analysis of (A) seed number, seed weight, and seed content parameters and (B) different temperatures to which the plants were exposed during the grain-filling stage, and changes in the grain development parameters that were observed on different days after treatment (DAT; or the number of days after exposure to temperature stress). The left region corresponds to combined grain development parameters and the right region corresponds to combined stress tolerance parameters. F1: First principal component, F2: Second principal component.

Comparative analysis of changes in protein expression during the grain-filling stage in different temperature conditions using 2-DE maps

2-DE analysis of total proteins extracted from oat grains subjected to different temperature conditions was performed to study changes in the oat grain proteome in response to the variation in temperatures. The 2-DE electrophoretic maps obtained from the mature seeds are shown (Fig. 10). The protein spots were widely distributed in the pI range from 4.0 to 10.0 representing proteins with molecular masses of 10–100 kDa. On Coomassie B-250 stained gels, approximately 700 spots were observed, with 138 spots exhibiting considerably higher expression levels. Analysis of trait-specific protein expression revealed that 15 proteins of plants #1 and 28 proteins of plants #3 showed significantly upregulated spots in comparison with the protein spots obtained from plants #2, #4, and #5 (Fig. 10). The 43 significantly distinct proteins were examined using MALDI-TOF/TOF. When spot identification on the basis of peptide mass fingerprinting of tryptic peptides proved to be unsuccessful, *de novo* sequencing was performed, allowing database searches for homologous sequences from related plant species. Fifteen of the protein spots of plants #1 revealed several unique proteins that were expressed in response to high-temperature stress, including E3 ligase, disease resistance protein, peroxidase, phosphatase, F-box protein, transcription-repair coupling factor, kinase, and leucine-rich repeat-containing surface protein (Table 1). 23 of the protein spots of plants #3 contained several proteins associated with grain development, such as GDSL-lipase, fatty acyl-CoA reductase, glutelin precursor, pentatricopeptide repeat, maturase, UDP-glycosyltransferase, DNA helicase, α -amylase trypsin inhibitor, avenin, gliadin, kinase, globulin, and plexin (Table 2). For the proteomic analysis, the upregulated spots were divided into two groups on the basis of the temperature conditions that were present during the grain-filling stage.

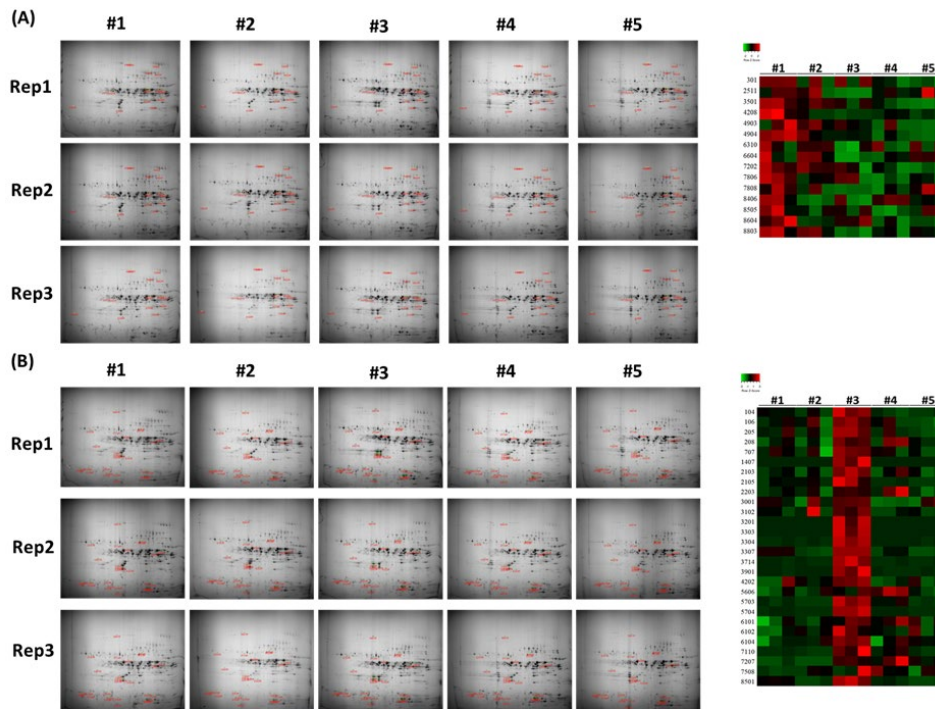


Fig. 10. Identification of upregulated protein spots for plants exposed to (A) high temperatures and (B) optimal temperatures. Spot intensity was visualized using a heatmap. The numbers on 2-DE gels and heatmaps indicate spot numbers. #1: 33 °C, #2: 31 °C, #3: 29 °C, #4: 27 °C, and #5: 25 °C.

Table 1. Identification of proteins extracted from seeds of plants subjected to high-temperature stress using MALDI-TOF/TOF mass spectrometry

Spot no.	Amino acid sequence showing high identity on similarity search	Name of protein	Accession number	e-value	Percent identity	Molecular weight (kDa)	pI
301	GAPQRSYGVVER	E3 ubiquitin-protein ligase SINA-like 2	XP_037411032.1	0.16	100.00%	23.00	4.36
2511	TTLAKSIYHDDK	putative disease resistance protein	XP_024187134.1	0.009	100.00%	33.15	5.21
3501	SHSQSINIKPPGPR	P1 (Rice black streaked dwarf virus)	AOS58258.1	1.00E-04	100.00%	32.78	5.41
4208	SAIDSAISSEARMGASLIR	cationic peroxidase 1	POF26732.1	7.00E-09	100.00%	21.06	5.61
4903	RDCVFEDSFHR	HECT domain-containing ubiquitin-protein ligase	QDZ19375.1	0.11	100.00%	65.67	6.09
4904	EMWEQDKAQMR	IQ motif, EF-hand binding site	XP_003078633.2	9.00E-04	100.00%	65.42	6.23
6310	HAGIPIRSK	P5 (Barley yellow dwarf virus PAV)	AUX14000.1	20	100.00%	26.73	7.34
6604	MRGSVPFEEIDLTR	Phosphoserine phosphatase	PKA60320.1	3.00E-05	100.00%	33.87	7.33
7202	LSFLYPACTGR	1-cysteine peroxiredoxin 1	EOY06870.1	0.058	100.00%	22.31	7.71
7806	ILPLPPSNFDPR	F-box protein	GER56839.1	0.003	100.00%	59.46	7.86
7808	GHPGHGSR	transcription-repair coupling factor	MAG32526.1	256	100.00%	48.14	7.37
8406	ADLLAKLAITR	UvrD-helicase domain-containing protein	MCD8307001.1	0.65	100.00%	27.59	8.21
8505	TGLVVLVGVGI AVLGLLVIVGIFVLWR	probable LRR receptor-like serine/threonine-protein kinase At1g561	XP_020103081.1	3.00E-17	100.00%	32.90	8.26
8604	TESRSPDNTSANDMAFSIGGLQGSR	protein Suppressor of quenching 1	XP_028061681.1	1.00E-16	100.00%	33.91	8.03
8803	TTMMSAF	BspA family leucine-rich repeat surface protein	EGF4233558.1	289	100.00%	46.36	8.01

Table 2. Identification of proteins extracted from seeds of plants subjected to optimal temperature conditions using MALDI-TOF/TOF mass spectrometry

Spot no.	Amino acid sequence showing high identity on similarity search	Name of protein	Accession number	e-value	Percent identity	Molecular weight (kDa)	pI
104	ESSDSSMVVKGNLR	GDSL-like lipase/acylhydrolase	YP_009273344.1	53	100.00%	15.32	4.77
106	RPVTEERDLALR	B3 domain-containing protein	XP_021316377.1	7.00E-04	100.00%	15.69	4.51
205	ALGEMLIQSR	fatty acyl-CoA reductase 3-like	XP_043697197.1	1.3	100.00%	16.25	4.54
208	QKEFLLAGNNQR	glutelin C precursor	AAR06951.1	0.009	100.00%	16.42	4.73
707	MPEKNVVTWTGIITGYVK	pentatricopeptide repeat-containing protein	XP_022736550.1	4.00E-10	100.00%	39.47	4.75
1407	RWLIQAMGAAGPSGPGAGPR	sodium/calcium exchanger NCL-like	XP_013687843.1	1.00E-10	100.00%	29.51	4.93
2103	RLGSALFEFFMSEEEVLSLTFPLPR	maturase K	ABO68908.1	8.00E-18	100.00%	14.27	5.20
2105	MLEPWLNR	UDP-glycosyltransferase	RVW94960.1	4	100.00%	14.89	5.28
2203	NDASGSGPARGAVELR	DNA helicase	GEU44823.1	2.00E-05	100.00%	16.64	5.19
3001	SRPDQIGGLIDLPQCPR	avena alpha amylase trypsin inhibitor-2	AOA33790.1	5.00E-08	100.00%	12.43	5.41
3102	MGPEPELGGR	predicted protein	XP_003058410.1	0.76	100.00%	14.43	5.50
3201	SQILQQSSCQVMR	avenin-3	P80356.1	2.00E-04	100.00%	22.38	5.50
3303	QFLVQQCSPVAVVPFLR	gliadin-like avenin	AGB56859.1	2.00E-08	100.00%	23.18	5.50
3304	QFLVQQCSPVAEVPFLR	avenin (<i>Avena sativa</i>)	CCH22495.1	2.00E-08	100.00%	24.56	5.51
3307	QFLVQQCSPVAVVPFLR	gliadin-like avenin	AGB56859.1	2.00E-08	100.00%	23.08	5.59
3714	VGSLPVKYLGLPLGAR	protein reticulata-related 1	RVW54319.1	7.00E-06	100.00%	38.80	5.45
3901	SCTPNGLGDMVMVTLK	hypothetical protein SOVF	KNA25450.1	2.00E-06	100.00%	67.02	5.45
4202	QSALSSFINSFTISKTASK	coat protein ORF3	BCP56395.1	1.00E-08	100.00%	22.09	5.98
5606	DAGVKDSGSLIPGHGSR	phosphatidate cytidyltransferase 4	CAN65330.1	2.00E-05	100.00%	35.93	5.83
5703	HHHSSGGPNNGHWR	serine-threonine/tyrosine-protein kinase catalytic domain	KAG7551173.1	9.00E-06	100.00%	40.29	6.70
5704	DMLVGKETFDQINK	hypothetical protein	MBA0771169.1	2.00E-05	100.00%	41.37	6.74
6101	LQAFEPLR	11S globulin (<i>A. sativa</i>)	CAA52763.1	90	100.00%	13.78	6.95
6102	NPLALVLEIPR	protein trigalactosyldiacylglycerol 2	XP_008802738.1	0.12	100.00%	13.52	7.01
6104	LQAFEPLR	plexin B2	KAF6121087.1	90	100.00%	13.80	7.17
7110	SQAGITEYFDEQNEQFR	11S globulin (<i>A. sativa</i>)	CAA52763.1	8.00E-09	100.00%	15.23	7.35
7207	SQAGITEYFDEQNEQFR	11S globulin (<i>A. sativa</i>)	CAA52763.1	8.00E-09	100.00%	15.80	7.04
7508	SQAGITEYFDEQNEQFR	11S globulin (<i>A. sativa</i>)	CAA52763.1	8.00E-09	100.00%	31.92	7.48
8501	AKDVTEFTLGISGADK	pentatricopeptide repeat-containing protein At3g180	XP_020253832.1	7.00E-06	100.00%	32.91	7.91

Discussion

Plants are exposed to diverse environmental conditions, which affect their growth and development. Changes in temperature have a significant impact on plant growth, development, reproduction, and yield (Sadras and Dreccer 2015). Each plant species has a preferred temperature range, which can be characterized by optimum, maximum, and minimum temperatures. The optimum temperature varies for each stage of plant growth and development. It is the temperature or the temperature range, which is most effective in accelerating plant growth (Izaurre et al. 2011). During the grain-filling stage in rice, temperatures ranging from 21.7 to 26.7 °C are best for grain development, whereas temperatures over 27 °C result in a reduction in grain production because of a decrease in grain weight (Tashiro and Wardlaw 1991). Wheat is frequently exposed to temperatures above the optimal temperature (24 °C) during the reproductive and grain-filling stages (Tashiro and Wardlaw 1990). Temperatures above 30 °C during grain-filling are known to reduce kernel weight in wheat (Wardlaw 1994). However, very few studies have

been conducted to examine the impact of temperature on grain development in oat. Identification of the optimal temperature needed during the grain-filling stage will help in increasing the oat crop production.

High temperatures can induce morphological, physiological, biochemical, and molecular changes in plants (Hasanuz-zaman et al. 2013). Moreover, high temperatures lead to chlorophyll degradation and decrease the photosynthetic efficiency (Momčilović et al. 2016). Tolerance towards heat stress can be predicted by examining the chlorophyll content of leaves during the maturation phase (Reynolds et al. 1994). In the present study, we found a significant decline in the chlorophyll content at high temperatures. The reduction in soluble protein content resulted in a decrease in the photosynthetic rate and plant shoot weight. Low temperatures delayed leaf senescence (Fig. 2).

Plants respond to heat stress by maintaining cellular homeostasis, which reduces cellular damage (Sairam et al. 2000). They synthesize specific proteins that assist cells in tolerating heat stress by maintaining the structural integrity of enzymes in the cell, which prevents disruption of enzyme function (Ahmad et al. 2009). Malondialdehyde is produced by the peroxidation of unsaturated fatty acids that are present in phospholipids, and is frequently used as an indicator of lipid peroxidation in cells (Halliwell and Gutteridge 2015). Presence of soluble sugars in the cell is essential for adjustment of the osmotic potential under stress conditions (El-Bassiouny and Sadak 2015). High temperatures can alter antioxidant enzyme activity and increase the production of reactive oxygen species, which in turn can lead to peroxidation of membrane lipids, resulting in membrane damage (Levitt 1972). Our results showed that high temperatures enhanced MDA content and increased concentration of soluble sugars in oat cells, both of which were associated with an increase in cell membrane permeability. In high-temperature conditions, antioxidant enzyme activities also increased transiently, which led to the production of active oxygen species (Figs. 4 and 5). In this study, the proteins whose expression levels were elevated upon exposure to high temperatures, were associated with the process of stress tolerance. Cationic peroxidase 1 (Li et al. 2020), phosphoserine phosphatase (Ho and Saito 2001), and peroxiredoxins (Wang et al. 2020), were associated with responses to biotic and abiotic stresses that entailed the decomposition of reactive oxygen species and prevention of changes to cell membrane permeability. Expression levels of E3 ligase (Stone 2019), Hul5 HECT ubiquitin ligase (Fang et al. 2011), and F-box protein (Li et al. 2018) showed an increase in some temperatures, and a decrease in other temperatures. Both the scenarios resulted in modification in the cellular pattern of protein degradation or protein stabilization, changes in gene expression patterns, and alteration of essential physiological responses. Several genes, including plant-specific IQ67 domain (Wang et al. 2022), genes associated with MMR complexes (Tuteja and Tuteja 2013), leucine-rich repeats receptor-like kinases (Ma et al. 2022), and suppressor of quenching 1 (Brooks et al. 2013), are associated with the response to several environmental stresses, and play essential roles in the repair of damaged DNA. Furthermore, in this study, high temperatures hastened senescence in oat. Overall, plants use several enzymes, transcription factors, and translation factors to counter environmental stresses.

Temperatures during the grain-forming and grain-filling stages affect the time taken by the plant to mature after emergence of the ear, and are responsible for changes that occur during the grain-filling stage. High temperatures induced rapid senescence and resulted in a significant loss of oat grain productivity. However, this did not imply that low temperatures were favorable for the production of oat grains. The grain weight in plants exposed to low temperatures was more susceptible to change in case there was a variation in temperature conditions, but the grain weight of plants subjected to high temperatures was maintained even when there was a change in the temperature. The yield components and weight, number, and length of dry seeds tended to decrease with any increase or decrease in the temperature (Fig. 6). The globulin content mostly influences the physical and chemical characteristics of oat proteins (He et al. 2021). Oat β -glucan, a mixed linkage β -(1,3;1,4)-D-glucan polymer, is the most abundant non-starch polysaccharide, accounting for 3–7% of oat grain composition (Zhao et al. 2014). Total starch accounts for 0.4–12.8% of oat grain composition (Drzikova et al. 2005). In plants exposed to high or low temperatures, oat grain components such as globulin, β -glucan, and starch showed significantly decreased levels in assays and SDS-PAGE (Figs. 7 and 8). Decrease in quantity of the main components that make up a majority of the grain storage protein reveals the temperature sensitivity of oat seeds. The proteins whose expression levels were upregulated at 27–29 °C, were associated with the process of synthesis of grain storage compounds, grain development, or related enzymes. Enzymes such as glutelin (Anderson 2014), GDSL lipase (Zhao et al. 2020), UDP-glycosyltransferase (Zhou et al. 2019), phosphatidate cytidyltransferase (Sparrow and Raetz 1985), and digalactosyldiacylglycerol (Basnet et al. 2019), are major seed components. B3 domain transcription factors (Yang et al. 2021), fatty acyl-CoA synthetase (Watkins 2013), maturase K (Liere and Link 1995), α -amylase/trypsin inhibitor (Zhou et al. 2017), and plexin-B2 (Yu et al. 2017), were expressed specifically in the developing seed, and are involved in the regulation of grain maturation and influence the factors affecting grain quality. Similarly, high- or low-temperature stress induced a decrease in the translation levels of grain-synthesizing factors and limited accumulation of storage compounds in developing grains.

Analysis of variation in spikelet morphology with change in temperature, which is a distinctive feature of hullless oat, helped in deciphering the optimal temperature conditions required during the grain-filling stage. Oat spikelet shapes were not similar in individuals of the same genotype and showed variation between plants and panicles, and within panicles as well (Pellizzaro et al. 2016). Environmental factors have been found to have a significant effect on the development of multiflorous spikelets (Zimmer et al. 2019). In this study, variation in spikelet shape was associated with variation in temperature. High-temperature conditions reduced multiflorous spikelets. Hullless oats responded to the environmental stimuli. Environmental signals, such as temperature, may explain the variable expressivity observed in oat panicles. Although the genetic mechanisms controlling multiflorous spikelet formation in oats remain unclear, an environmental influence on its expression can be assumed. The optimal temperature range for the grain-filling stage was 27–29 °C. At low temperature (25 °C), numerous multiflorous spikelets were formed, but the number of seeds did not increase, and small-sized seeds were produced. The spikelets comprising a larger number of the triple-kernels exhibited higher seed number and seed weight. As the temperature decreased, the proportion of multiflorous spikelets increased, which is not favorable for oat seed production.

The ideal temperature for highest yield should be differed by chosen varieties and other associated environmental factors. The oat variety ('Choyang') used in this experiment showed highest yield at 27 °C and showed similar 100 seeds weight with 29 °C. Furthermore, as depicted in Fig. 1, it is difficult to fix the exact treatment temperature during the treatment period in the controlled glass house. Repeated trials as well as mass experiment or field study with appropriate lines numbers may needed to provide optimum temperature for chosen varieties in that region.

Conclusion

Long-term high temperature stress in oat influences several stress-related parameters and adversely affects seed development. Low temperatures were considered favorable for spikelet growth; however, grain development was strongly reduced. Numerous abiotic stress-related parameters were measured at different grain developmental stages, as well as at different locations of inflorescences on each plant. The reproductive organs of oat responded differently to diverse temperatures, which suggests that optimal temperature conditions are necessary to maximize grain yield. Although there are many genetic or environmental reasons, one of the reasons for poor grain yield under low-temperature stress could be the shape of the hullless oat spikelet. As the temperature during the grain-filling period is considered a major factor affecting the yield potential, we expect that the obtained results will allow us to deduce the optimal temperature needed to maximize oat grain yield.

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