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Improving Photosynthetic Responses during Recovery from Heat Treatments with Brassinosteroid and Calcium Chloride in Indian Bread Wheat Cultivars

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Abstract

Climate change is expected to unleash severe and frequent heat waves in future, adversely affecting crop productivity. The aim of this study was to examine the effect of two separate episodes of heat stress, mimicking heat wave conditions on the physiology of four Indian bread wheat cultivars and to study the ameliorating effects of epibrassinolide (BR) and calcium chloride on the recovery of these cultivars. The two thermo-tolerant cultivars C306 and K7903 suffered less inhibition of photosystem II efficiency as compared to the two thermo-susceptible cultivars HD2329 and PBW343. Application of BR and calcium chloride resulted in faster recovery in all the four cultivars. Measurement of the minimum fluorescence (Fo) versus temperature curves revealed a higher inflection temperature of Fo (Ti) for the two tolerant cultivars as compared to the susceptible cultivars, emphasizing greater thermo stability of the photosynthetic apparatus. The two thermo-tolerant cultivars showed higher photochemistry (φPSII) relative to the two susceptible cultivars. An increase in the steady state fluorescence was observed in both the susceptible cultivars as compared to the tolerant cultivars. Expression analysis revealed faster recovery of the transcripts involved in photosynthesis in tolerant cultivars as compared to susceptible cultivars. Exogenous application of the ameliorating compounds resulted in faster recovery of transcripts in all the cultivars. The result suggested that under severe stress conditions tolerant cultivars showed faster recovery and a better thermo-stability of its photosynthetic apparatus as compared to susceptible cultivars and application of epibrassinolide and calcium chloride could ameliorate the damaging effect of severe temperature stress to a considerable level in all the four cultivars under study.

Keywords

Non Photochemical Quenching, Photosynthetic Efficiency

1. Introduction

Previous century has seen an increase of 0.74˚C in global mean temperature which currently continuing to increase at ~0.1˚C per decade [1]. Global mean temperature increase will be associated with extreme heat events including severe heat waves along with hot days with more severity and frequencies over most land areas [1] [2]. Few studies have examined the responses of crop plants, to heat waves [3]-[6]. The highly susceptible state of photosynthesis will adversely affect photosynthesis at elevated temperature [7], photosynthetic capacity and photochemical efficiency [8]. Susceptibility of PSII and OEC to thermal stress has been shown [9].

Brassinosteroids (BR) are a group of steroids regulating plant growth and development by possible involvement in signaling pathways [10]-[12]. Important insights were made of the BR-insensitive transduction pathways due to discovery of BR-insensitive mutants in *Arabidopsis* (*Arabidopsis thaliana*), Pea (*Pisum sativum*), tomato (*Solanum lycopersicum*), and rice (*Oryza sativa*) leading to the isolation and characterization of BRI1 and its homolog [13]-[16]. Role of BR in imparting tolerance to different abiotic stresses including heat and cold stress, drought and salinity stress has been demonstrated [17]-[20]. Although the exact mechanism of the BR induced tolerance is not known, various reports have suggested increased expression of heat shock proteins via influence on some components of the translational machinery [21] [22].

Apart from its role as a nutrient, calcium also acts as a secondary messenger and is known to ameliorate water stress induced effects in higher plants [23]. Calcium is known to be involved in H_2O_2 perception, induction of antioxidant genes [24], cellular signaling processes [25], most notably by deciphering calcium signatures [26]. Influence of calcium in ameliorating the negative effects of different abiotic stresses is reported in a variety of plants [27]-[30]. A known calcium induced protective mechanism against abiotic stresses in plants is via the induction of anti-oxidative responses [28].

2. Materials and Methods

2.1. Temperature Treatment

For simulation of two consecutive heat wave effect on the four Indian bread wheat cultivars namely *C*306, *K*7903, *HD*2329 and *PBW*343, potted plants at anthesis stage were taken for this study. The schematic diagram of the experiment designed is presented in **Figure 1**. The experiment which was carried out over a span of five days with exposure to thermal stress of 43˚C for 8 h with prior acclimatization at 37˚C for 1 h was given on day one (D-1) and day three (D-3), day two (D-2), day four (D-4), day five (D-5) were the recovery period, when plants were kept at 22˚C without stress. All analysis were carried out with the flag leaf. The 5 days of experiment starting from zero time were depicted as D-0, D-1, D-2, D-3, D-4 and D-5. For foliar application, a solution of BR (0.05 µM) was mechanically sprayed on leaves for five days 12 h prior to exposure to high temperature stress during the span of experiment. Calcium chloride (10 mM, 1 L) was added to pot 12 h prior to the start of experiment.

2.2. Temperature Induction Response (TIR)

For Temperature Induction Response (TIR), 20 seeds of uniform size were imbibed for 16 h in petri plates with approximately 15 ml distilled water along with different chemicals including *calcium chloride* ($CaCl_2$) (1 mM, 5 mM and 10 mM), *Brassinosteroid* (BR) (0.01 µM, 0.05 µM and 0.1 µM), *salicylic acid* (SA) (100 µM, 500 µM and 1000 µM), *abcissic acid* (ABA) (5 µM) and 1-aminocyclopropane-1-carboxylic acid (ACC) (100 µM). Seeds showing germination were subjected to lethal temperature stress of 51˚C for 3 h with prior induction at sublethal temperature of 37˚C for 1.5 h. Immediately after the treatment, seedlings were allowed to recover at 22˚C ± 1˚C. On the 10th day, from the date of imbibition of seeds, the percentage survival of seeds were scored [31].

2.3. Chlorophyll Fluorescence and Photosynthesis

Chlorophyll fluorescence parameters were measured continually *in situ* from D-0 to D-5 with Licor 6400
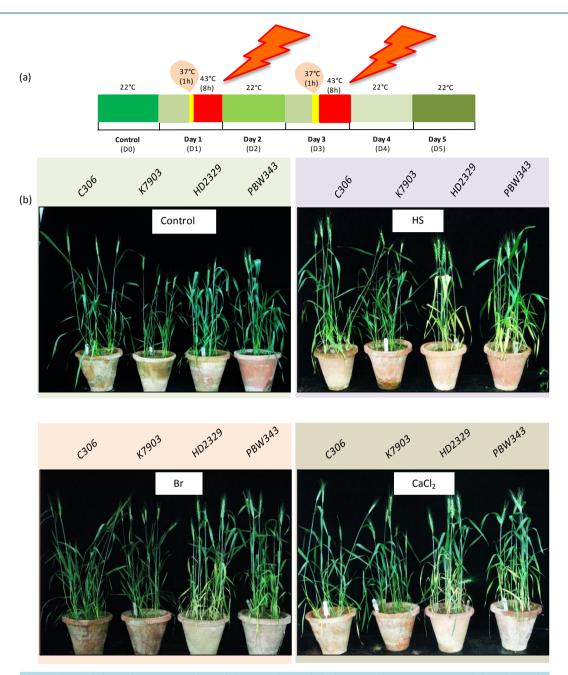
Figure 1. (a) Schematic representation of the experimental design spanning over a span of five days with temperature stress given on day 1 (D-1) and day 3 (D-3); (b) Influence of exogenously applied compounds including CaCl₂ (Calcium chloride) (10 mm) and Br (Brassinosteroid) (0.05 μM) followed by heat treatment in different wheat cultivars on Day 5.

(Portable Photosynthesis System, Li-Cor, Lincoln, NE, USA). For measuring Fm, dark adapted leaves were given a 0.8 s saturating pulse at 8000 μmol m^{-2} s^{-1} after 30 min of dark adaptation. After recording steady state fluorescence, a second 0.8 s saturating light of 8000 μmol photons m^{-2}s^{-1} was provided to determine the maximum fluorescence in the light-adapted state (Fm'). After turning off the actinic light, 3 s of far red light was followed by noting the minimal fluorescence in the light-adapted state (Fo'). The following parameters were then calculated: Fv/Fm = Fm − Fo/Fm; ϕPSII = Fm' − Fo'/Fm'; ETR = ϕPSII × PPFD × A × 0.5; qP = Fm' − Fs/Fm' − Fo'; ϕNPQ = (Fs/Fm') − (Fs/Fm); ϕf, d = Fs/Fm. The inflexion temperature and the peak temperature were determined by using Junior PAM-250 (WALZ, Germany). Dark adapted leaf disc were kept in water bath of

25˚C - 60˚C and readings were taken after every degree rise in temperature.

2.4. Total Chlorophyll Estimation

Total chlorophyll was estimated fluorometrically by taking 100 mg leaf tissue in 20 ml of dimethylsulphoxide (DMSO) at 65˚C for 4 h in dark and total chlorophyll content was calculated [32].

2.5. Membrane Injury Index

Membrane Injury Index was measured with 100mg fresh leaf tissue in 20 mL milli-Q water per tube. Electrical conductivity (EC) was measured with an EC-meter (Eutech, Singapore). Test tubes were autoclaved for 10 min at 0.10 MPa pressure to release all the electrolytes. Final EC was measured after bringing down the temperature to 25˚C. Percentage Relative Injury Index (RII %), was than calculated [33].

2.6. Real Time Expression Analysis

Total RNA was isolated from control and treated samples using the RNeasy Plant mini kit (Qiagen, Germany) according to the manufacturer's instructions, followed by on-column DNase I treatment to remove genomic DNA contamination. 1.5 µg of the total RNA was used as template for cDNA synthesis employing the High Capacity cDNA Archive kit (Applied Biosystems, USA) and mixed with 200 nM of forward and reverse primers (**Table 1**) and SYBR Green PCR Master Mix (Agilent) for real time PCR analysis. Normalization of PCR was done by using internal control, actin. Three independent RNA isolations (biological replicates) were used for expression analysis.

2.7. Seed Weight

The mature spikes having fully developed grains were harvested from experimental and control plants and 100 seed weight was measured.

2.8. Heat Susceptible Index (HSI)

Heat Susceptible Index was calculated [34] using the following equation HSI = $(1 − Xh/X)/(1 − Yh/Y)$, where Xh and X are the mean of physiological trait for each cultivar under heat stressed and control conditions, respectively. Whereas, Yh and Y are the physiological mean of all genotypes under heat stress and control conditions. The values for heat susceptibility index was used for generation of a heat map.

2.9. Statistical Analysis

All experiments were carried out with at least 3 biological replicates. Results were analyzed by one-way ANOVA to identify significant differences between the groups and their significance levels ($p < 0.05$) were determined.

3. Results

Terminal heat stress can substantially reduce grain yield depending upon the cultivar in wheat. Previous reports have shown the positive impact on grain yield upon application of various compounds. Hence in this study, the role of different compounds in ameliorating the negative effect of high temperature stress was investigated.

3.1. Germinating Stage

A tolerant (*CPAN*1676) and susceptible (*HD*2428) cultivars were selected based on their thermotolerance performance studied earlier in the lab. Different compounds were checked for their ameliorating effect, including *Calcium Chloride* (*CaCl₂*), *Abscisic acid* (*ABA*), *Salicylic acid* (*SA*), *Brassinosteroid* (*BR*), and 1-*aminocyclopropane*-1-*carboxylic acid* (*ACC*) which were previously reported to be providing thermotolerance to plants. Hence we have used different concentrations for optimization of ameliorating effect, if any, on heat stress in wheat cultivars.

Seeds were imbibed for 16 h with or without ameliorating compounds. Application of calcium chloride at

Table 1. List of qRT-PCR primers used in the experiment.

Gene	Primer	Sequence (5'-3')
Ascorbate peroxidase	F	ATGCGCCCCCATCATG
	R	CACCAGTTCTTGTGTTCACATCATAG
Rubisco Large subunit	F	AACGAAGGGCGCGATCTT
	R	CATTTGCAAGCTGCTCGGATA
Rubisco small subunit	F	CCTTCTCCTTGTGTTAGCATCGA
	R	TTGCACGGATGACCATTAGG
Oxygen evolving complex	F	AGCCGCTCATCGACAAGAA
	R	CCTGAGGCGGAGGTCGTT
PsbO	F	AGGCCGAGGGCATCCA
	R	ACGGGTCATGAGCTTGGTTT
PsbP	F	AAGGCGCAACGAGACAAGAG
	R	CGGCGTTCTCGACGAACTT
Actin	F	CCTTGTTTGCGACAATGGAA
	R	AGCCCTTGGTGCATCATCTC

various concentration (1 mM, 5 mM and 10 mM) showed an increase in percentage survivability of imbibed seeds as compared to imbibed heat treated seeds with temperature stress at 37˚C/1.5 h followed by 51˚C for 3 h (**Figure S1**). Over the period of time, the lethal temperature stressed untreated plants progressively became more stunted as compared to CaCl₂ treated plants. Heat treated *CPAN*1676 and *HD*2428 showed a decrease in percentage survivability of 42.5% and 70% whereas CaCl₂ treated germinating seeds showed an increased percentage survivability at all concentrations (**Figure S1**), with maximum increase in survivability observed at 1 mM and 5 mM CaCl₂. At a concentration of 1 mM CaCl₂, *CPAN*1676 and *HD*2428 showed a decrease in percentage survivability of 22.5% and 50%, respectively (**Figure S1**).

Treatment with different concentrations of Brassinosteroid (BR) (0.01 µM, 0.05 µM and 0.1 µM) showed a concentration of 0.01 µM to be most effective in imparting thermotolerance to seedlings following lethal temperature stress (**Figure S1**).

Heat treated plants showed a percentage decrease in survival of 27.5% and 75% for *CPAN*1676 and *HD*2428 whereas BR (0.01 µM) treated seeds showed a decrease in percentage survivability of 7.5% and 55% for *CPAN*1676 and *HD*2428, respectively (**Figure S1**).

Out of the different concentrations (100 µM, 500 µM and 1000 µM) of salicylic acid, a concentration of 100 µm showed a slightly better performance as compared to heat treated germinating seeds (**Figure S1**). Heat treated germinating seeds showed a decrease in percentage survival of 40% and 77.5% for *CPAN*1676 and *HD*2428, whereas salicylic acid (SA) (100 µM) treated germinating seeds showed a percentage decrease in survival of 12.5% and 67.5% for *CPAN*1676 and *HD*2428, respectively (**Figure S1**).

Abscisic acid (*ABA*) at a concentration of 5 µM showed dramatic increase in percentage survivability in *CPAN*1676 reaching upto 97.5% whereas *HD*2428 showed a lower survivability percentage of 50% in ABA treated germinating seeds, whereas heat treated germinating seeds showed a percentage survival of 27.7% and 77.5% for *CPAN*1676 and *HD*2428, respectively (**Figure S1**).

1-aminocyclopropane-1-carboxylic acid (*ACC*) at a concentration of 100 µM was used and the treated plants showed better recovery in terms of percentage decrease in survivability with heat treated plants showing a percentage decrease in survival of 27.5% and 77.5% in *CPAN*1676 and *HD*2428, respectively, whereas ACC treated germinating seeds showed a percentage decrease in survival of 12.5% and 52.5% in *CPAN*1676 and *HD*2428, respectively (**Figure S1**).

3.2. Mature Plant Stage

Since treatment with CaCl₂ and BR increased the percentage survival of germinating seeds, both these com-

pounds were used approximately a day before anthesis to check their ameliorating effect after heat treatment. Hence we have used $CaCl_2$ at a concentration of 10 mM and Brassinosteroid (BR) at a concentration of 0.05 µM, since BR at a concentration of 0.01 µM did not showed optimal results in mature plants. This was probably due to the limitation in BR entry inside the plant through the surface upon foliar spray. The experiment was designed over a span of 5 days, with moderate temperature stress of 37°C/1 h followed by high temperature stress of 43°C for 8 h were given on D-1 and D-3. D-2, D-4 and D-5 are the recovery period at 22°C ± 1°C. Schematic representation of the experimental design along with the recovery plants on day 5 are shown in **Figure 1**.

3.3. Photosystem II Efficiency

In mature plants, high temperature has profound effect on photosynthetic yield measured as fluorescence efficiency (Fv/Fm) (**Figure 2**). All cultivars showed a decrease in Fv/Fm after heat treatment on D-1 and D-3, whereas by D-5, wheat cultivars *C*306 and *K*7903 showed complete recovery while *HD*2329 showed a continuous decrease in Fv/Fm which kept decreasing till D-5, pointing towards irreversible damage to the photosynthetic apparatus.

3.4. Minimum Fluorescence

The minimum fluorescence (Fo) showed a small increase after thermal stress on D-1 and D-3 in all cultivars, although the increase was slightly higher in *HD*2329 and *PBW*343 as compared to *C*306 and *K*7903 (**Figure 3**).

A steep increase in Fo was observed on D-4, in all cultivars, with sensitive cultivars *HD*2329 and *PBW*343 showing higher Fo values as compared to the tolerant cultivars *C*306 and *K*7903. All except *HD*2329 showed recovery by D-5, with treated plants showing faster recovery, *HD*2329 showed an increasing trend in Fo which reached to maximum level by D-5 with the treated plants too showing a similar trend. A slight decrease in Fo was observed in treated plants as compared to HS, suggesting irreversible damage to chloroplast membrane (**Figure 3**).

Thermal stress induced injury to PSII have been earlier shown [35]. Our results are consistent with earlier report in *Vitis* [6], where high temperature episodes caused inhibition of PSII activity. By D-5 of HS, both *C*306 and *K*7903 showed higher ϕPSII and ETR as compared to *PBW*343 and *HD*2329.

3.5. Electron Transport

Additionally, both ϕPSII and ETR showed a consistent decrease in efficiency after high temperature treatment (**Figure 4**). Highest decrease in both these parameters were observed on D-1 and D-3, whereas by D-5 all except *HD*2329 showed recovery to various levels with treated plants showing faster recovery. *HD*2329 showed a steep decrease in ϕPSII and ETR starting from D-3 which kept decreasing till D-5.

The BR and $CaCl_2$ treated plants showed stability by D-5 as compared to HS plants in *HD*2329. This suggests that treated plants had undergone relatively lesser injury as compared to non-treated plants. The faster recovery in treated plants results in better energy utilization, which over the span of the growing season adds to a significant increase in yield. Excess energy dissipated thermally is a photo protective mechanism in plants.

3.6. Energy Distribution

Energy partitioning model have earlier been proposed by giving insight into the energy assimilation and dissipation mechanism for protecting photosynthetic apparatus [36]. ϕf, d (Steady state fluorescence) which depicts the total absorbed energy lost as heat and as chlorophyll fluorescence showed complete recovery by D-5 with treated plants showing similar trend (**Figure 5**).

High temperature induced injury results in an imbalance between energy absorption and utilization. The energy not utilized in carbon assimilation can be easily directed in formation of Reactive Oxygen Species (ROS) which further degrades the photosynthetic apparatus [37]. Nonphotochemical quenching is one mechanism to prevent ROS formation.

Non photochemical quenching regulated by thylakoid lumen pH and PsbS showed a significant increase in all the four cultivars with *HD*2329 showing maximum increase in NPQ by D-5 (**Figure 5**). The amount of constitutive thermal dissipation (ϕf, d) thus showed an increase after D-1 and D-3 in all the four cultivars. Since ϕf, d points to the inevitable energy loss, *C*306 and *K*7903 seems to be better performer than *PBW*343 and *HD*2329.
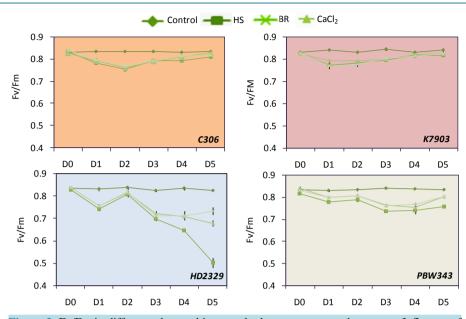
Figure 2. Fv/Fm in different wheat cultivars under heat treatment and recovery. Influence of exogenously applied compounds on Fv/Fm in different wheat cultivars at control temperature 22˚C, during heat and recovery.
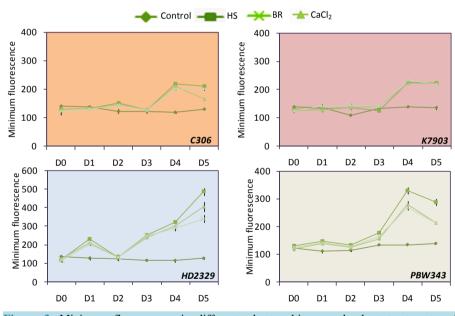

Figure 3. Minimum fluorescence in different wheat cultivars under heat treatment and recovery. Influence of exogenously applied compounds on minimum fluorescence in different wheat cultivars at control temperature 22˚C, during heat and recovery.

In fact *HD*2329 showed a maximum increase in ϕf, d by D-5 suggesting damage to photosynthetic apparatus and chlorophyll, hence affecting energy absorption and utilization. Further, ϕNPQ which depicts the fraction of the regulated ΔpH and xanthophyll dependent thermal dissipation process (ϕNPQ), showed an increase after D-1 and D-3 and by D-5 showed different levels of increase in *C*306, *K*7903, *HD*2329 and *PBW*343 (**Figure 5**).

These results indicate that *C*306 and *K*7903 are more efficient in regulating energy partitioning in PSII complexes to minimize damaging potential simultaneously retaining the efficiency for carbon assimilation. The effective energy partitioning could possibly be contributing to acclimation mechanism by withstanding higher temperatures in *C*306 and *K*7903.
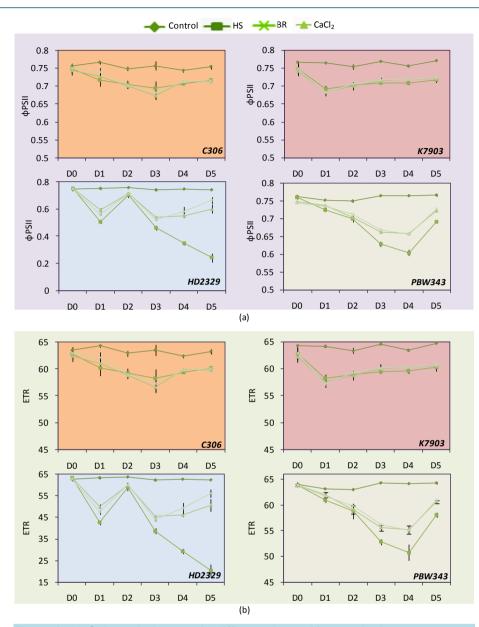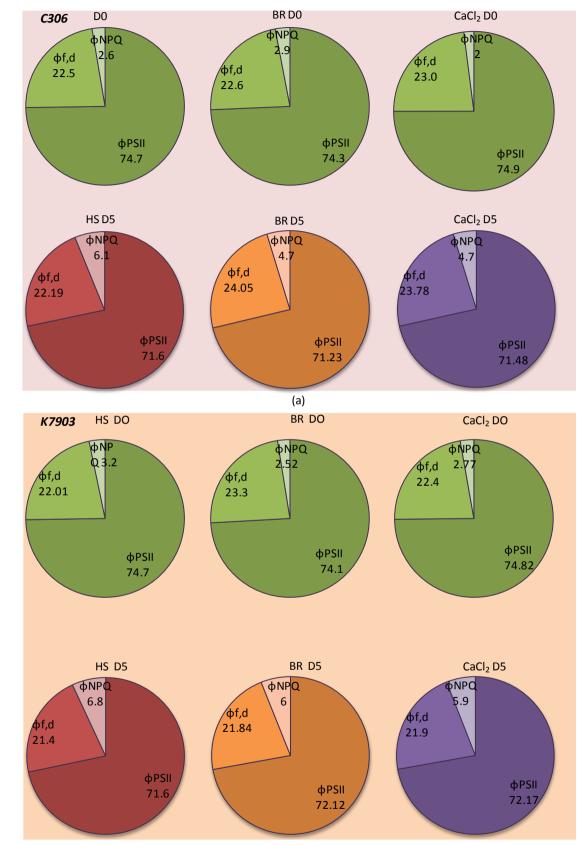
Figure 4. (a) φPSII and, (b) ETR, in different wheat cultivars under heat treatment and recovery. Influence of exogenously applied compounds on ETR in different wheat cultivars at control temperature 22°C, during heat treatment and recovery.

3.7. Light Quenching

Decrease in qP (Photochemical quenching by PSII) was observed after D-1 and D-3 of heat stress, by D-5 all cultivars except *HD*2329 showed stability in qP. A consistent decrease in qP was observed in *HD*2329, although BR and calcium chloride heat stress treated plants showed comparatively stable qP by D-5 in *HD*2329 (**Figure 6**).

Following treatment, all cultivars showed faster recovery as compared to heat stressed plants. Estimation of redox state of QA, the first molecule that accept electron from PSII in z-scheme, using lake model presuming all photosynthetic units are connected [38], showed tolerant cultivars along with treatment given plants recovering faster as compared to the susceptible cultivars. qP indicates the proportion of reaction centres that are open. Earlier it has been used to indicate the onset of photo inhibition [39] and for determining the level of photo protective quenching of fluorescence [6].
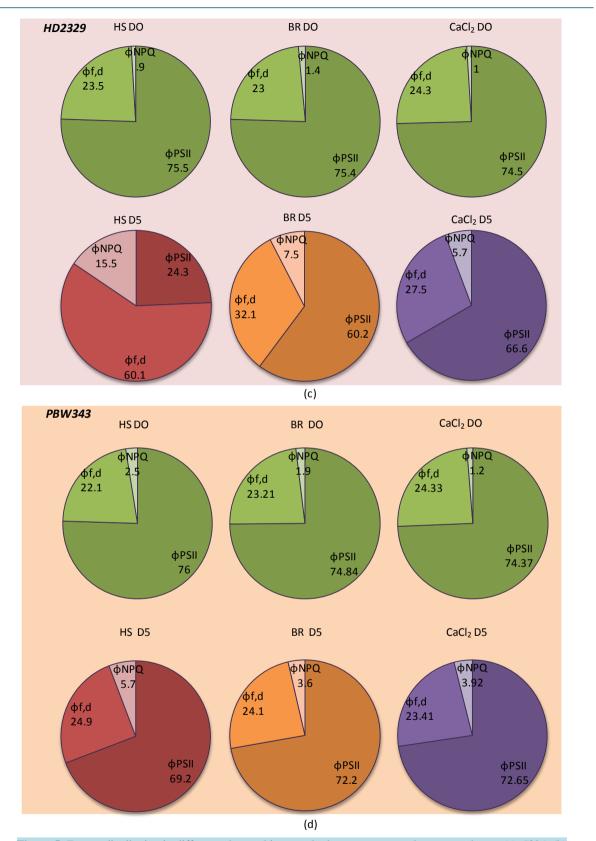
(a)

(b)

Figure 5. Energy distribution in different wheat cultivars under heat treatment and recovery phases. (a) *C*306; (b) *K*7903; (c) *HD*2329; (d) *PBW*343.
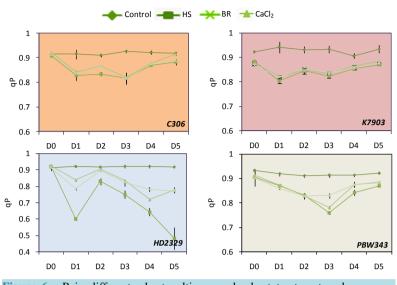
Figure 6. qP in different wheat cultivars under heat treatment and recovery. Influence of exogenously applied compounds on qP in different wheat cultivars at control temperature 22˚C, during heat and recovery.

3.8. Membrane Thermo-Stability

To measure the thermo-stability of the photosynthetic membranes, Fo versus T curves in the leaves of all four cultivars were measured (**Figure 7**). Fo versus T curve showed a parabola, with a higher inflection temperature (Ti) at which a steep rise in Fo is observed in both the tolerant cultivars and showed Ti of 46˚C and 47˚C in *C*306 and *K*7903 as compared to the susceptible cultivars *HD*2329 and *PBW*343, which showed a Ti of 40˚C and 44˚C. No significant change in inflexion temperature was observed in any of the BR and CaCl₂ treated plants. A corresponding increase in the peak temperature for Fo (Tp) was observed with *K*7903 showing higher peak temperature followed by *C*306 and *PBW*343 and *HD*2329 showing minimum peak temperature of 52˚C, 52˚C, 45˚C and 51˚C, respectively (**Figure 7**).

For better efficiency of the photosynthetic apparatus, better membrane stability is advantageous. Increase in Fo in the dark under increasing temperature conditions (Fo versus T curve) has been frequently used to determine the thermo-stability of the photosynthetic apparatus [40]. Irreversible damage to photosynthetic apparatus can be predicted by noting the sharp increase in Fo (Ti) highlighting the critical point. In our study, we have observed tolerant cultivars *C*306 and *K*7903 having higher Ti as compared to susceptible cultivars *HD*2329 and *PBW*343, suggesting higher thermo-stability of photosynthetic apparatus in both tolerant cultivars.
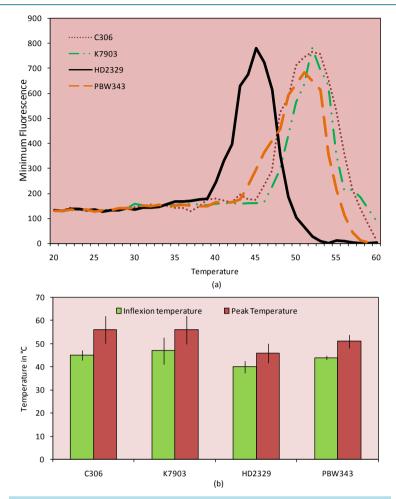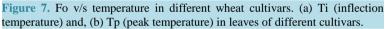
3.9. Light Avoidance

The total chlorophyll content in both *C*306 and *K*7903 showed a slight dip on D-1 and D-3 but showed complete recovery by D-5. *K*7903 showed higher chlorophyll accumulation by D-5 as compared to control. In contrast, both *HD*2329 and *PBW*343 showed a continuous decrease in total chlorophyll. Although in all four cultivars, higher levels of chlorophyll was observed in BR and calcium chloride treated plants (**Figure 8**).

3.10. Membrane Injury

Membrane Injury Index has been widely used in identification of tolerant members of specie, highlighting the degree of damage to membrane. High temperature induced increase in membrane fluidity resulted in an increase in membrane leakage rate of all the four wheat cultivars after D-1 and D-3 of HS. Although by D-4, both *C*306 and *K*7903 started showing lower membrane injury and by D-5 showed nearly complete recovery (**Figure 9**).

Similar trend in MII was observed with BR and calcium chloride treated plants. *HD*2329 showed an increase in MII on D-4, which further increased on D-5, *HD*2329 treatment given plants with both the ameliorating agents too showed similar trend as of HS plants, with increase in membrane injury of 303 fold, 229 fold and 281 fold in HS, BR and CaCl₂ treated plants, respectively. In *PBW*343 HS, BR and calcium chloride treated plants a

(a)

(b)

Figure 7. Fo v/s temperature in different wheat cultivars. (a) Ti (inflection temperature) and, (b) Tp (peak temperature) in leaves of different cultivars.
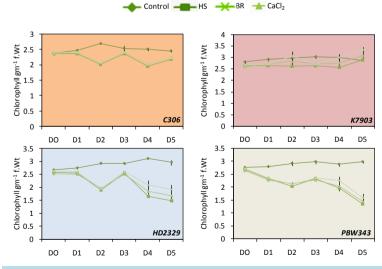

Figure 8. Total chlorophyll in different wheat cultivars under heat treatment and recovery. Influence of exogenously applied compounds on total chlorophyll in different wheat cultivars at control temperature 22°C and during heat treatment and recovery.
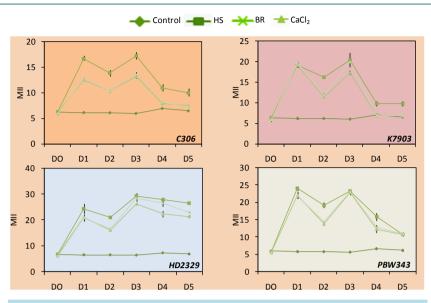
Figure 9. MII in different wheat cultivars under heat treatment and recovery. Influence of exogenously applied compounds on MII in different wheat cultivars at control temperature 22˚C and during heat treatment and recovery.

similar increase in MII as *HD*2329 on D-4 was observed but started showing stability by D-5, with BR and CaCl$_2$ treated plants showing slight decrease in injury as compared to HS (**Figure 9**). Better membrane stability under stress conditions will invariably be advantageous for the plant, since a part of light reaction of photosynthesis is membrane localized; a better membrane stability will help in efficient electron transport and an overall better performance of photosynthesis.

3.11. Temperature Induced Seed Weight Variation

The 100-seed-weight showed a higher percentage decrease in seed weight in susceptible cultivars *HD*2329 and *PBW*343 as compared to the two tolerant cultivars *C*306 and *K*7903 (**Figure 10(b)**). Upon treatment with *BR* and calcium chloride, all cultivars showed a significant increase in 100-seed-weight along with an increased size (**Figure 10(a)**). No difference was observed in seed weight in control plants treated with *BR* and calcium chloride (**Figure S2**).

3.12. Gene Expression Analysis

A substantial decline in the expression of all photosynthetic genes were observed in all cultivars immediately after heat stress, although the decrease in tolerant cultivars was slightly lower as compared to the two susceptible cultivars and faster recovery in the expression of the transcripts of all genes was observed in the tolerant cultivars along with treated plants of the four cultivars (**Figure 11**).

Expression analysis by real time PCR revealed a decrease in the expression of all photosynthetic genes in all cultivars after temperature stress on D-1 and D-3. For all genes under study namely rubisco large and small subunit, PsbO, PsbP, oxygen evolving complex and ascorbate peroxidase, both the tolerant cultivars showed faster recovery in expression of these genes as compared to the two susceptible cultivars. Faster recovery was also observed in all cultivars after treatment with BR and calcium chloride (**Figure 11**).

3.13. Heat Injury Index

Further to determine the thermos-tolerance level of all the four cultivars, Heat Susceptibility Index (HSI) was estimated (**Figure 12**). HSI depict the high temperature susceptibility of plant under the parameter in consideration. A higher index generally depicts high susceptibility towards particular stress. Thermo-tolerant cultivars showed a decrease in Heat Susceptibility Index (HSI) as compared to the two thermo-susceptible cultivars. Treatment with the ameliorating agents decreased the HSI in all cultivars to a considerable level (**Figure 13**).
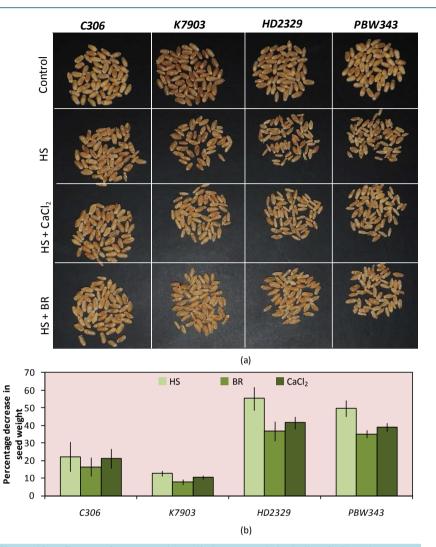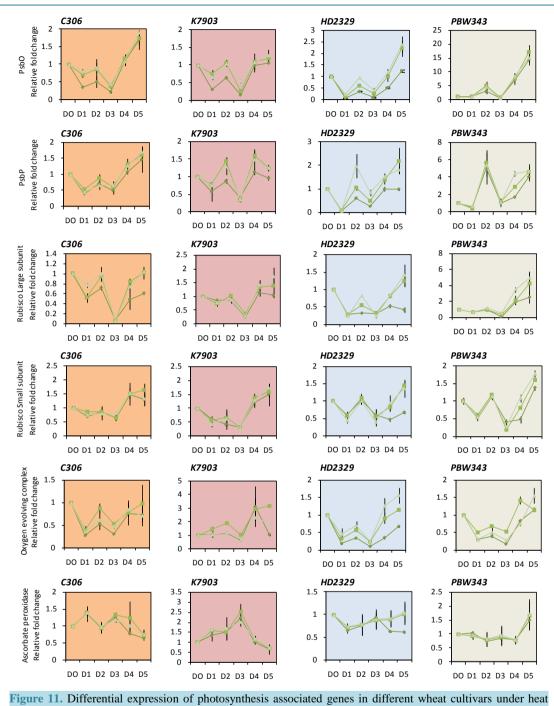
(a)

(b)

Figure 10. Influence of exogenously applied compounds including $CaCl_2$ (10 mM) and BR (0.05 µM) on (a) seed seed size; (b) seed weight in different wheat cultivars at control temperature 22˚C and two episodes of high temperature stress of 37˚C for 1 h followed by 43˚C for 8 h.

Heat Susceptibility Index for heat treated *HD*2329 was maximum, although with BR and $CaCl_2$ treatment *HD*2329 showed a decrease in HSI. Minimum HSI was shown by treated plants of *K*7903. Different cultivars responded differently to the ameliorating agents. Although *C*306 and *HD*2329 showed lower HSI with $CaCl_2$, whereas *K*7903 showed low HSI with BR (**Figure 13**). These results suggest towards the possible cultivar dependent response of different ameliorating agents. Both the tolerant cultivars showed a comparatively low HSI as compared to the two susceptible cultivars.

4. Discussion

Earlier reports [31] [41] [42] have suggested the identification of tolerance level in different genotypes following exposure to a sub-lethal induction stress. This can chiefly be attributed to the changing transcriptome upon exposure to thermal stress [42] [43]. The prior treatment of germinating seeds and mature plants with moderately high temperature of 37˚C in general can act as an inducer of the stress responsive pathways. Under natural conditions, the plants are exposed to a gradual increase in temperature and not directly to heat shock thereby suggesting that genetic variability for thermotolerance between cultivars can be observed only after exposure to an induction temperature.

Figure 11. Differential expression of photosynthesis associated genes in different wheat cultivars under heat treatment and recovery.

The adverse effect of thermal stress on PSII had earlier been reported [35]. High temperature treatment resulted in a significant decrease in PSII function in all cultivars after D-1 and D-3 of heat stress. The observable changes in PSII and Fo are dependable diagnostic indicators of photosystem efficiency [44].

Estimation of reduced state of QA based on Puddle model reflect the reaction centres that are open and had been commonly used to indicate the onset of photo inhibition [39] and for determining the level of photo protective quenching of fluorescence [45].

The minimum fluorescence has been extensively used in thermo-stability of the photosynthetic apparatus [40] [46]-[48]. A sharp increase in Fo (Ti) indicates severe damage to photosynthetic apparatus [9]. In our study, we
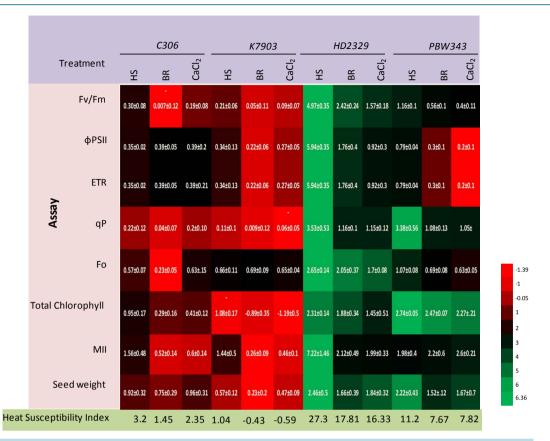
Treatment	C306			K7903			HD2329			PBW343		
Assay	HS	BR	CaCl₂	HS	BR	CaCl₂	HS	BR	CaCl₂	HS	BR	CaCl₂
Fv/Fm	0.30±0.08	0.007±0.12	0.19±0.08	0.21±0.06	0.05±0.11	0.09±0.07	4.97±0.35	2.42±0.24	1.57±0.18	1.16±0.1	0.56±0.1	0.4±0.11
ΦPSII	0.35±0.02	0.39±0.05	0.39±0.2	0.34±0.13	0.22±0.06	0.27±0.05	5.94±0.35	1.76±0.4	0.92±0.3	0.79±0.04	0.3±0.1	0.2±0.1
ETR	0.35±0.02	0.39±0.05	0.39±0.21	0.34±0.13	0.22±0.06	0.27±0.05	5.94±0.35	1.76±0.4	0.92±0.3	0.79±0.04	0.3±0.1	0.2±0.1
qP	0.22±0.12	0.04±0.07	0.2±0.10	0.11±0.1	0.009±0.12	0.06±0.05	3.53±0.53	1.16±0.1	1.15±0.12	3.38±0.56	1.08±0.13	1.05±
Fo	0.57±0.07	0.23±0.05	0.63±.15	0.66±0.11	0.69±0.09	0.65±0.04	2.65±0.14	2.05±0.37	1.7±0.08	1.07±0.08	0.69±0.08	0.63±0.05
Total Chlorophyll	0.95±0.17	0.29±0.16	0.41±0.12	1.08±0.17	-0.89±0.35	-1.19±0.5	2.31±0.14	1.88±0.34	1.45±0.51	2.74±0.05	2.47±0.07	2.27±.21
MII	1.56±0.48	0.52±0.14	0.6±0.14	1.44±0.5	0.26±0.09	0.46±0.1	7.22±1.46	2.12±0.49	1.99±0.33	1.98±0.4	2.2±0.6	2.6±0.21
Seed weight	0.92±0.32	0.75±0.29	0.96±0.31	0.57±0.12	0.23±0.2	0.47±0.09	2.46±0.5	1.66±0.39	1.84±0.32	2.22±0.43	1.52±.12	1.67±0.7
Heat Susceptibility Index	3.2	1.45	2.35	1.04	-0.43	-0.59	27.3	17.81	16.33	11.2	7.67	7.82

Figure 12. The Heat Susceptibility Index (HSI) for different parameters for HS and treatment given plants was calculated. Heat map depicting heat susceptibility of different parameters. Red colour depict minimum HSI and green colour depict maximum HSI.
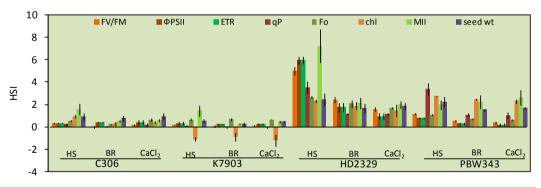

Figure 13. A comparative graph of heat stress and ameliorating agent treated cultivars. Vertical lines on top of bars indicate standard error of means.

found *K*7903 and *C*306 exhibited a higher Ti than the two thermo-susceptible cultivars *HD*2329 and *PBW*343 suggesting that K7903 and *C*306 have better thermo-stability of its photosynthetic apparatus as compared to the two thermo-susceptible cultivars *HD*2329 and *PBW*343.

High temperature induced photo inhibition result in an imbalance in light energy absorption and utilization. The energy not utilized can result in the formation of free radicals, which can damage the photosynthetic apparatus [37] [49]. The consecutive heat treatment showed a significant decrease in pigment content in the two susceptible cultivars *HD*2329 and *PBW*343, which is considered as a protective mechanism to decrease light absorption and hence energy imbalance. In fact a slight increase in total chlorophyll was observed in *K*7903 at the end of recovery.

Cellular membrane are the chief sites for physiological injury by high temperature [50]. Hence membrane injury can be used as an indicator of the physiological state of the plant. Better membrane stability of $C306$ and $K7903$ might correlate with membrane composition. Membrane fluidity is generally dependent on membrane composition including unsaturation level of phospholipids and glycolipids [51]. Earlier reports have shown a correlation between membrane composition and PSII efficiency [52], since components involved in light reaction in plants are membrane embedded, hence a change in membrane fluidity will directly affect PSI and PSII complexes. The consistent increase in membrane injury index in $HD2329$ even till D-5 shows its inability to recover after treatment with high temperature.

Lower decrease in 100-seed-weight was observed in thermo-tolerant cultivars as compared to thermo-susceptible cultivars after heat treatment. Upon application of ameliorating agents, considerable increase in 100-seed-weight was observed in all cultivars. Increased yield in treated plants can be correlated with the better photosynthetic activity, since addition of ameliorating agents invariably resulted in an increase in PSII efficiency and ultimately photosynthetic yield. Any increase in photosynthetic activity will contribute to the assimilatory efficiency of plant, and hence will improve yield in terms of grain weight.

Light energy distribution showed different level of energy utilization between different cultivars. Whereas both the tolerant cultivars $C306$ and $K7903$ showed only a slight increase in ϕf, d (Constitutive thermal dissipation), and ϕNPQ (non photochemical quenching) by D-5, $HD2329$ showed a significant increase in both ϕf, d and ϕNPQ, although $PBW343$ showed stability in ϕf, d and ϕNPQ by D-5. The increase in ϕf, d in $HD2329$ highlight the damaging effect of high temperature stress on the thylakoid membrane and thus resulting in a decrease in efficiency in energy utilization via PSII complexes and hence an increase in damage to carbon assimilation pathway.

A decrease in the transcripts level of genes involved in the process of photosynthesis was observed. A decrease in photosynthesis can possibly be an adaptive mechanism for high temperature tolerance in plants. Shut down of the carbon assimilation can possibly aid in imparting thermotolerance to plants by keeping a strong proton motive force, thus preventing membrane collapse. In addition, rubisco deactivation can avert photorespiration under conditions of high temperature and thus preventing the formation of phosphoglycolate and omit the carbon that could be fixed.

Environmental stress inducing ROS production triggers enhancement in APX expression [53] [54]. Under conditions of damaged PSII, higher expression of APX can scavenge the H_2O_2 as part of the ascorbate-glutathione or Asada-Halliwell-Foyer pathway [53].

4.1. Role of BR in Stress Tolerance

Various studies have highlighted BR induced abiotic stress tolerance in plants [19]-[21] [55] [56], however the precise mechanism of action remained enigmatic, primarily due to the strong and pleiotropic phenotypes of BR biosynthesis and signaling mutants, including extreme dwarfism, associated with dark green and epinastic leaves [55] [57]-[59]. Microarray analysis revealed BR induced expression of stress associated genes including heat shock protein and oxidative stress related genes in *Arabidopsi* [58] [60].

4.2. Role of Calcium Chloride in Stress Tolerance

Transient Ca^{2+} peaks have been observed across the plasma membrane during heat stress, corroborating the role of heat stress sensing by plasma membrane [61]-[64]. Rapid influx of Ca^{2+} was observed in plants treated with benzyl alcohol, a known membrane fluidizer, inducing a rapid influx of Ca^{2+} and a progressive expression of HSP's resulting in developing thermotolerance [63].

Increased expression of Cam3 and Cam7 is observed during thermal stress resulting in expression of HSPs [62] [65]. Cams further interact with calcium dependant kinases such as AtCBK3, resulting in the activation of HSFs, which further regulate HSP and thus impart thermotolerance to plants [63]. The HSF expression/regulation seems to be governed by membrane physiological state and perhaps triggered by increased fluidity of membrane, thus resulting in expression of different HSPs and also the change in proportion of saturated and unsaturated fatty acid. During recovery period, calcium treatment resulted in higher thermo-stability possibly due to the higher expression of stress associated genes especially anti-oxidative response.

In our study, we have observed that application of BR and $CaCl_2$ on plants before heat stress can decrease the injury level and increase the recovery after heat stress. Faster recovery after heat stress in all the cultivars based

on the florescence observation along with membrane injury index, total chlorophyll content and faster recovery of genes involved in photosynthesis points towards the ameliorating effect of BR. Further, higher seed weight observed in treated plants points towards the significance of these compounds in ameliorating the negative effect of heat treatment and an overall increase in grain yield.

5. Conclusion

In conclusion, the two thermo-tolerant cultivars, *C*306 and *K*7903 performed better under high temperature treatment as compared to the two thermosensitive cultivars *HD*2329 and *PBW*343. Exogenous application of Brassinosteroid and calcium chloride resulted in faster recovery in all the four cultivars.

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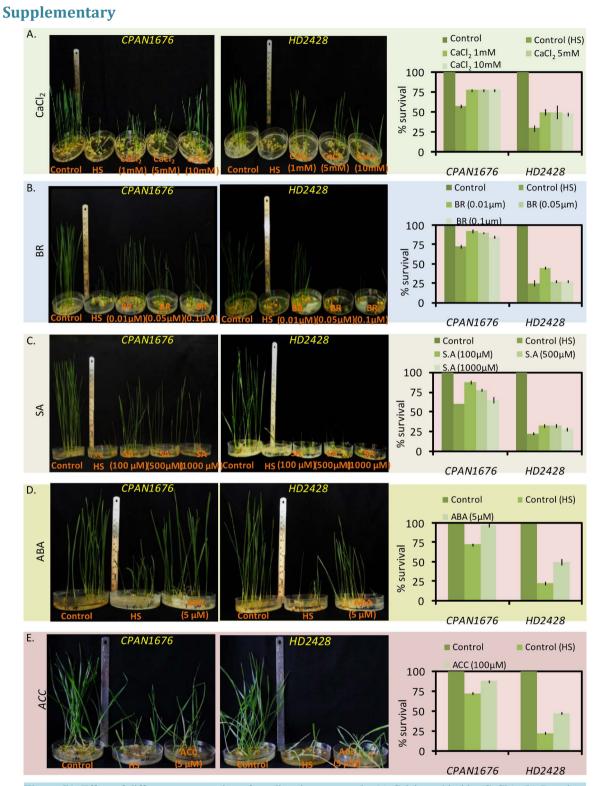
Supplementary

Figure S1. Effect of different concentration of ameliorating compunds. (a) Calcium chloride (CaCl$_2$); (b) Brassino-steroid (BR); (c) Salicylic acid (SA); (d) Abcissic acid (ABA); (e) 1-aminocyclopropane-1-carboxylic acid (ACC), on percentage survival in response to heating in different wheat cultivars (*CPAN*1676 and *HD*2428). Plants were imbibed for 12 h in different concentrations of of these compounds prior to lethal temperature stress of 51˚C for 3 h. Graphs indicate percentage survival 10 days after the temperature treatment.

A.

C306   K7903   HD2329   PBW343

Control (C)
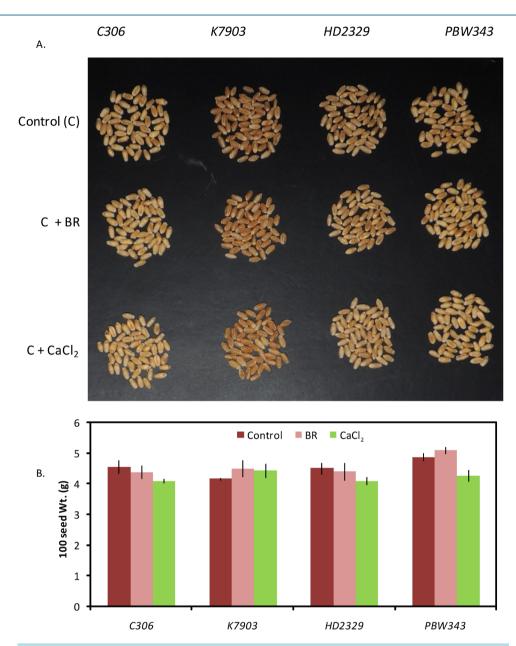
C + BR

C + CaCl$_2$

B.

Figure S2. A comparative graph of control and ameliorating agent treated cultivars. Vertical lines on top of bars indicate standard error of means.