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RESEARCH ARTICLE

Interactive effect of temperature and water stress on physiological and biochemical processes in soybean

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Abstract Drought and heat stress are important abiotic stress restricting plant growth, while the two stresses often occur at the same time in nature and little is known about when these stresses occur in combination. Therefore, attempts were made to understand the impact of water stress imposed under different temperature conditions on photosynthesis, chlorophyll fluorescence, antioxidant enzymes, lipid peroxidation, chlorophyll, proline, free amino acid, epicuticular wax content and seed yield. Soybean genotype EC 538828 was grown under greenhouse conditions at day/night temperatures of 30/22, 34/24, 38/26 and 42/28 °C. At each temperature, pots were divided into two sets, one set was unstressed while second was subjected to water stress at reproductive stage (beginning of seed fill). High temperature significantly declined the rate of photosynthesis, stomatal conductance, water use efficiency, Fv/Fm ratio, photochemical quenching, PhiPSII, electron transport rate, ascorbic acid, total free amino acids, chlorophyll content and seed yield. As against this with increase in temperature from 30/22 to 42/28 °C intercellular CO_2, transpiration rate, vapor pressure deficit, non photochemical quenching, proline content, SOD, POD, APX, GR, MDA and epicuticular wax content were increased. Water stress when imposed at different temperature further aggravated the effects of temperature, and the combination of water stress and high temperature had more detrimental effect.

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Introduction

Soybean is the main rainfed crop of India; currently it is grown in 10.6 million ha with an annual production of 11.0 million tons (FAO 2017). However, the productivity of soybean has remained low (about 1 t/ha) due to the occurrence of drought and high temperature conditions at one or the other stage of crop growth (Bhatia et al. 2014a, b; Bhatia and Jumrani 2016; Jumrani et al. 2017; Jumrani and Bhatia 2018). Water deficit and high temperature are important abiotic stress factors limiting plant growth, while the two stresses often occur at same time in nature (Shah and Paulsen 2003), little is known about their combined effect (Rizhsky et al. 2004). Soybean is often affected by environmental stresses, particularly the drought and high temperature stress during various stages of development. Knowledge of how soybean is affected by environmental stresses is important in determining overall plant survival strategies, which is expected to occur simultaneously in the future.

Photosynthesis in plants has been considered as main indicator of growth because of its relationship with productivity (Piao et al. 2008). High temperatures result in a decline in leaf photosynthesis (Taub et al. 2000), and change in water status affect plant chlorophyll, growth rate of photosynthesis and seed yield (Guenni et al. 2004). The interactive effect of drought and high temperature on photosynthetic parameters is more complex (Hamerlynck et al. 2000) and could be caused by stomatal or non-stomatal factors (Yamori et al. 2009; Djanaguiraman and

Prasad 2010). Thus, drought and heat stress can affect the light harvesting photosystems, electron transport rate, NADPH and ATP synthesis, carbon cycle, and utilization of photosynthates. In recent years, chlorophyll fluorescence techniques have been used as a powerful tool to quantify the non-stomatal inhibition to photosynthetic efficiency and for screening of tolerant genotypes under abiotic stress conditions (Oukarroum et al. 2007). Efficiency of photosystem II decreases under abiotic stresses, due to imperfect energy dissipation (Centritto et al. 2003; Monneveux et al. 2003; Jumrani et al. 2017) which ultimately decline the photosynthetic efficiency under combined stress.

Both water stress and temperature stress are known to trigger wide variety of plant responses, and are known to produce reactive oxygen species (ROS) that can produce serious damage to biological system which can inactivate metabolic enzymes, attack membrane lipids and nucleic acids ultimately leading to cell death (Gill and Tuteja 2010). In various crops, oxidative stress has been observed in response to drought (Sairam et al. 1998) and high temperature (Sairam et al. 2000). To protect them against these reactive oxygen species, plants have antioxidant defense systems, which avoid deleterious effects produced by toxic intermediates. The antioxidant enzymatic system includes catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX), superoxide dismutase (SOD), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and non-enzymatic antioxidants are Glutathione (GSH), Ascorbate (AsA), and tocopherols (Miller et al. 2010). Under conditions of severe stress in plants, the production of ROS may exceed the scavenging capacity of the antioxidant defense system as a result of it ROS can accumulate and cause lipid peroxidation which can be calculated by measurement of malondialdehyde (MDA) content (Girotti 1990). Chlorophyll pigment breakdown can be caused due to intracellular membrane damage by ROS (Scandalios 1993). Chlorophyll concentration has been known as an indicator for evaluation of photosynthates (Zobayed et al. 2005); therefore, decline in chlorophyll content has been considered as a non-stomatal limiting factor under abiotic stress.

Increase in free amino acids content has been reported in plants under enviornmental stresses (Lugan et al. 2010). Free amino acids are always present in tissues for protein synthesis and they can accumulate in response to abiotic stresses (Lea and Azevedo 2007). Proline, an amino acid, plays a key role in plants exposed to various stresses (Hayat et al. 2012). One of the ways to prevent water stress and heat tolerance is by reducing loss of water from leaf surfaces. Epicuticular wax and hairs help in reducing the water loss by increasing the albedo and lower transpiration without decreasing stomatal conductance or photosynthesis (Premachandra et al. 1994; Jenks et al. 1996).

The harvested yield is in a way a manifestation of the impact of temperature or water stress on physiological and biochemical processes. Understanding the physiological and biochemical responses to drought and temperatures stresses is therefore essential for a proper observation of plant defense mechanisms to these stresses. Moreover, there are a number of reports illustrating the impact of individual temperature/water stress on the physiological and biochemical processes but there are very few reports on the combined effect of these two stresses on physiological and biochemical processes. Therefore, attempts were made in this study to understand the effect of water stress occurring at different temperatures on key physiological and biochemical processes of soybean.

Materials and methods

Green houses/temperature treatments

The experiment was conducted under four green house conditions at ICAR-Indian Institute of Soybean Research, Indore (22.72°N, 75.83°E). Soybean genotype EC 538828 was grown in cement pots (45 cm height and 18 cm diameter) maintained at day/night temperatures of 30/22, 34/24, 38/26 and 42/28 °C with an average temperature of 26, 29, 32 and 35 °C, respectively. Before sowing, a recommended dose of fertilizers was applied and the seeds were treated with recommended fungicides bavistin and dithane M and inoculated with slurry of *Rhizobium japonicum*. Ten seeds of uniform size were sown at 4 cm depth in each pot. One week after sowing, thinning was done to three plants per pot which were maintained until maturity.

Imposition of soil moisture stress

In each greenhouse, pots were divided into two sets. One set was kept as control (unstressed), second set was subjected to water stress at initiation of seed fill stage (R5 stage). In stress treatment, the supply of water in the pots was stopped till the leaf water potential was reduced to − 2.0 MPa after which the stress was released by regular watering of plants till maturity. In order to monitor the water status of the plants during water stress treatment, leaf water potential (Ψ_L) was measured daily in water stressed and control plants using Psychrometers (Wescor Inc, USA). All the analysis was carried out when the plants were subjected to water stress at R5 stage in the fully expanded leaves of both the control (unstressed) and water stressed plants grown at different temperature conditions.

Gas exchange measurements

Gas exchange parameters measured at R5 stage in intact fully expanded leaf from top in five plants selected from different pots in control and water stress plants. In water stressed plants the parameters were recorded when water potential was − 2.0 MPa along with their respective control plants at four different temperatures using portable photosynthesis system (LI-6400 XT, LI-COR, Inc., Lincoln, NE, USA). A leaf was fitted into 6 cm^2 leaf chamber and gas exchange measurements were made at PAR of 1000 µmol m^{-2} s^{-1} and ambient CO_2 concentration (380–400 µmol CO_2 mol^{-1} air) between 10.00 to 12.00 h. Net photosynthetic rate, stomatal conductance and vapor pressure deficit in terms of leaf temperature were measured. Chlorophyll fluorescence parameters Fv/Fm, PhiPSII, ETR, photochemical and non-photochemical quenching were recorded in the dark adapted leaf for ~ 30 min (fully expanded third leaf from the top) using LI-6400 XT portable photosynthesis system combined with 6400-40 leaf chamber fluorometer (Jumrani et al. 2017). Summary of parameters and their description using data extracted from leaf chamber fluorometer (IRGA) are given below:

Fo	Minimal fluorescence of dark-adapted leaves
Fm	Maximal fluorescence of dark-adapted leaves
Fv/Fm	Variable to maximal fluorescence of dark-adapted leaves (maximum PSII efficiency)
Fo′	Minimal fluorescence of light adapted leaves
Fm′	Maximal fluorescence of light adapted leaves
Fv′/Fm′	Variable to maximal fluorescence of light adapted leaves
Fs	Steady state fluorescence
PhiPSII	PSII efficiency
ETR	Electron transport rate
qP	Photochemical quenching
qN	Non-photochemical quenching.

Chlorophyll fluorescence

Chlorophyll a (Chl a) fluorescence induction kinetics of dark-adapted (30 min) was measured between 10.00 to 12.00 h from five plants on an intact fully expanded leaf from top in plants at R5 stage grown at four different temperatures in control and water stressed plants using a Handy PEA Fluorimeter (Plant Efficiency Analyzer, Hansatech Instruments, King's Lynn, Norfolk, UK). Excitation light of 650 nm (peak wavelength) from an array of six light-emitting diodes focused on a 4 mm diameter spot on the leaf surface to provide a homogenous illumination. The light intensity reaching the leaf was 600 Wm^{-2} (3.2 mmol m^{-2} s^{-1}) which was sufficient to generate maximal fluorescence for all treatments. The energy pipeline model was prepared using a chlorophyll fluorescence-analyzing program (Biolyzer HP 3 software, Bioenergetics Laboratory, University of Geneva, Switzerland). In the phenomenological leaf model each relative value is represented by the size of the proper parameters (arrow), the width of each arrow denotes the relative size of the fluxes or the antenna, empty circles represent reducing QA reaction centers (active), full black circles represent non-reducing QA reaction center (inactive or silent).

Chlorophyll content

The total chlorophyll was determined using dimethyl sulfoxide (DMSO) according to the method of Hiscox and Israelstam (1979). For the extraction of chlorophyll, 50 mg well cleaned fresh leaf was chopped and transferred to a test tube containing 10 ml of DMSO. The contents were incubated at 65 °C for 3 h and volume was made up to 10 ml with DMSO. The content was allowed to settle down and the absorbance was recorded at 645 and 663 nm. Equations of Wellburn and Lichtenthaler (1984) were used to calculate the total chlorophyll concentrations.

Estimation of L-ascorbic acid

Ascorbic acid was measured based on reduction of Fe^{3+} to Fe^{2+} with ascorbic acid in acid solution followed by the formation of a red chelate between ferrous ion and bipyridyl (Arakawa et al. 1981): 100 mg tissue sample was homogenized in 2 ml ice cold 5% TCA. The homogenate was filtered through four layers of muslin and centrifuged at 15,000 rpm for 15 min at 4 °C. The supernatant was used for total ASA assay. Total ASA was expressed as µmol ASA/g leaf fresh weight.

Extraction and estimation of the antioxidants enzymes

All operations were performed at 4 °C. The enzyme extract was prepared by homogenizing 100 mg leaves with 10% (w/v) polyvinyl polypyrrolidone and 5 ml of 0.1 mol/L phosphate buffer (pH 7.0) for SOD, APX, GR and POD. The homogenate was filtered through three layers of cheesecloth, centrifuged at 15,000 rpm for 30 min and the supernatant obtained was used to determine the activity of antioxidant enzymes. Protein was estimated by the method of Lowry et al. (1951).

Superoxide dismutase (SOD)

[EC 1.15.1.1] activity was assayed by the method of Beauchamp and Fridovich (1971). The reaction mixture

contained 0.24 mM riboflavin, 2.1 mM methionine, 1% Triton-X 100, 1.72 mM nitroblue tetrazolium chloride (NBT) in 50 mM sodium phosphate buffer (pH 7.8) and 200 µl of enzyme extract in a final volume of 3 ml. The activity was expressed as Units/mg protein. One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition in the rate of NBT photo reduction.

Ascorbic acid peroxidase (APX)

[EC 1.11.1.11] activity was measured by the method of Nakano and Asada (1987). The 3 ml reaction mixture contained 2.5 ml sodium phosphate buffer (pH 7.4, 50 mM), 0.3 mM ascorbate and 0.06 mM EDTA, 300 µl enzyme extract and 200 µl of 2 mM H_2O_2. The decrease in absorbance at 290 nm was recorded at 25 °C for 1 min. The activity was expressed as µM ascorbic acid oxidized/min/mg protein.

Glutathione reductase (GR)

[EC 1.6.4.2] activity was determined at 25 °C as described by Rao et al. (1996). The 3 ml assay mixture contained 300 µl enzyme extract, 10 µl NADPH (1 mM), 100 µl oxidized glutathione (15 mM), and 2590 µl potassium phosphate buffer (50 mM; pH 7.8). The decrease in absorbance was recorded at 340 nm for 10 min. The enzyme activity was expressed as µM NADPH oxidized/min/mg protein.

Guaiacol peroxidase (POD)

[EC 1.11.1.7] was assayed as described by Chance and Maehly (1955). The reaction mixture contained 0.5 ml enzyme extract, 1 ml 20 mM guaiacol and 3 ml 0.02 M phosphate buffer. The reaction was started by the addition of 0.03 ml of H_2O_2 (88.2 mM). The initial and final absorbance was recorded at 475 nm for 2 min. The activity was calculated as the change in OD/min/mg protein.

Lipid peroxidation (MDA)

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) content according to the method of Heath and Parker (1968). Leaf tissue (100 mg) was homogenized in 5 ml of 50 mM Phosphate buffer (pH 7.2) containing 10% (w/v) polyvinyl polypyrrolidone and centrifuged at 10,000 rpm for 10 min. An aliquot of 0.5 ml supernatant was added to 4 ml TBA-TCA reagent (0.25% Thiobarbituric acid in 10% Trichloroacetic acid) and incubated at 95 °C for 30 min and again centrifuged at 10,000 rpm for 15 min at 4 °C. Absorbance was read at 532 nm and value for the non- specific absorption was read at 600 nm. The amount of malondialdehyde (A_{532}–A_{600}) present was calculated using Malondialdehyde as a standard.

Total free amino acids

For the extraction of total amino acids, the method of Noorudeen and Kulandaivelu (1982) was followed. Preweighed (100 mg) tissue was extracted twice with 80% chilled acetone (5 ml). The homogenate was centrifuged (10,000 rpm, 20 min) and the supernatant was collected. Amino acids were estimated following the method of Troll and Cannan (1953). The reaction mixture consisted of 1 ml of extract + 1 ml of KCN-Pyridine-phenol reagent which was kept in boiling water bath for 10 min. To this mixture 0.2 ml of 0.5% ninhydrin made in absolute alcohol was added and the tubes were kept in boiling water bath for another 15 min. The tubes were cooled to room temperature; the volume was adjusted to 10 ml with 60% ethanol and absorbance was read at 575 nm.

Proline content

Proline content in leaves was estimated by the method of Bates et al. (1973). A known quantity of fresh leaf sample (500 mg) was homogenized with 10 ml of 3% sulphosalycylic acid. The homogenate was filtered through Whatman filter paper No. 1 and the filtrate was used for the estimation of proline. An aliquot of 2 ml from each sample was taken and to each test tube 2 ml of glacial acetic acid and 2 ml of ninhydrin reagent was added and boiled on a hot water bath for 1 h. The test tubes were then transferred to ice bath for cooling. To this 4 ml of toluene was added, shaken thoroughly, and allowed to form separate layer. The upper toluene layer containing the color complex due to proline ninhydrin reaction was taken into a separate test tube and color was read at 520 nm against toluene blank.

Epicuticular wax content

Leaf surface waxes were extracted and quantified using a colorimetric method as described by Ebercon et al. (1976) at R5 stage. Freshly harvested leaf was immersed for 15 s in 30 ml redistilled chloroform by holding the petiole. The extract was filtered and the chloroform was evaporated to dryness at 70 °C in water bath. To the extracted waxes, 5 ml of acidic potassium dichromate reagent was added (prepared by dissolving 20 g of potassium dichromate in 1 l of H_2SO_4 and boiled for half an hour) and the samples were heated for 30 min at 100 °C. After cooling, 12 ml of deionized water was added to each sample and allowed for color development. The optical density of the samples was measured at 590 nm. Total leaf surface wax amount was expressed as mg/dm^2 by recording the leaf area.

Seed yield

At maturity, plants from 5 pots (3 plants/pot) in each treatment were sampled and data on seed weight was recorded.

Statistical analysis

Analysis of variance was carried out for all the data sets using SAS statistical software (ver. 9.2; SAS Institute, Cary, NC). The treatment mean was compared based on least significant differences (LSD) at $P \leq 0.05$ using Duncan multiple range test (DMRT).

Results

Rate of photosynthesis

Increasing temperatures as well as water stress had a significant negative effect on the rate of photosynthesis. The rate of photosynthesis in control plants at 30/22 °C was 28.4 µmol CO_2 m^{-2} s^{-1}, which significantly declined to 26.6 (6%), 24.3 (15%) and 22.9 µmol CO_2 m^{-2} s^{-1} (19%) at 34/24, 38/26 and 42/28 °C, respectively. The rate of photosynthesis in water stressed plants at 30/22 °C was 12.3 µmol CO_2 m^{-2} s^{-1}, which significantly declined to 10.7 (13%), 9.4 (23%) and 8.2 µmol CO_2 m^{-2} s^{-1} (33%) in plants grown at 34/24, 38/26 and 42/28 °C, respectively. The non-significant interaction between temperature and water stress indicated a similar change in rate of photosynthesis in control and water stressed plants grown at different temperatures in soybean (Table 1). The rate of photosynthesis was significantly higher in control plants by 57, 60, 61 and 64% as compared to water stressed plants grown at 30/22, 34/24, 38/26 and 42/28 °C, respectively (Fig. 1a).

Vapor pressure deficit

The average vapor pressure deficit with respect to leaf temperature (VPDL) increased significantly with increasing temperatures. The average VPDL value in control plants was 1.47 kPa at 30/22 °C which increased to 1.59, 1.76 and 2.12 kPa indicating a 8, 20 and 44% increase in plants grown at 34/24, 38/26 and 42/28 °C, respectively. The average VPDL in water stressed plants was 2.33 kPa at 30/22 °C which increased to 2.60, 3.14 and 3.56 kPa indicating a 12, 35 and 53% increase in plants grown at 34/24, 38/26 and 42/28 °C, respectively. The interaction of temperature and water stress treatment interaction was not significant indicating a similar response of VPDL in control and water stressed plants to increasing temperatures (Table 1). The VPDL was significantly high in water stress plants as compared to control plants at all the temperatures and the magnitude of difference increased by 58, 63, 79 and 68% at 30/22, 34/24, 38/26 and 42/28 °C, respectively (Fig. 1b).

Stomatal conductance

The increasing temperatures had a negative influence on the average stomatal conductance. In leaves of control plants grown at 30/22 °C the stomatal conductance was 0.66 mol H_2O m^{-2} s^{-1} which was reduced to 0.61 (7%), 0.57(14%) and 0.55 mol H_2O m^{-2} s^{-1} (16%) in plants grown at 34/24, 38/26 and 42/28 °C, respectively. The water stress resulted a severe reduction in stomatal conductance which was 0.31 mol H_2O m^{-2} s^{-1} at 30/22 °C and declined to 0.27 (12%), 0.24 (23%) and 0.20 mol H_2O m^{-2} s^{-1} (36%) as the temperature increased to 34/24, 38/26 and 42/28 °C, respectively. The non-significant interaction between temperature and water stress indicated that the stomatal conductance was influenced in the similar manner in control and water stressed plants grown at different temperature (Table 1). The stomatal conductance was significantly higher in control plants by 53, 55, 58 and 64% as compared to water stressed plants grown at 30/22, 34/24, 38/26 and 42/28 °C, respectively (Fig. 1c).

Fv/Fm ratio

The average Fv/Fm ratio declined significantly with increasing temperatures. It declined from 0.822 at 30/22 °C to 0.778 at 42/28 °C. There was slight reduction in average Fv/Fm ratio in water stressed plant (0.714) at 42/28 °C as compared to plants grown at 30/22 °C (0.764). The interaction of temperature with water stress treatment was not significant for Fv/Fm ratio indicating similar change in it in control and water stressed plants in response to increasing temperatures (Table 1) (Fig. 1d).

Photochemical quenching

Photochemical quenching in soybean leaves was significantly affected by temperature and water stress. The average photochemical quenching declined progressively as the growing temperatures increased. In control plants compared to average photochemical quenching value of 0.593 at 30/22 °C, it declined by 6% at 34/24 °C (0.560) and by 14 and 16% at 38/26 (0.513) and 42/28 °C (0.501), respectively. In water stress plants average photochemical quenching value was 0.481 at 30/22 °C which was reduced to 0.437 (9%), 0.368 (24%) and 0.332 (31%) as the growing temperature increased to 30/22, 34/24, 38/26 and 42/28 °C, respectively. Significant interaction of

Table 1 ANOVA table for effect of temperature and water stress on physiological and biochemical parameters

Parameters	Temperature	Water stress	Temperature × water stress
Photosynthesis	< 0.0001	< 0.0001	0.0845
VPDL	< 0.0001	< 0.0001	0.1482
Stomatal conductance	< 0.0001	< 0.0001	0.7439
Fv/Fm ratio	< 0.0001	< 0.0001	0.1434
Photochemical quenching	< 0.0001	< 0.0001	< 0.0001
Non photochemical quenching	< 0.0001	< 0.0001	< 0.0001
PhiPSII	< 0.0001	< 0.0001	< 0.0001
Electron transport rate	< 0.0001	< 0.0001	< 0.0001
Total chlorophyll content	< 0.0001	< 0.0001	0.0052
Wax content	< 0.0001	< 0.0001	< 0.0001
Superoxide dismutase	< 0.0001	< 0.0001	0.0036
Guaicol peroxidase	< 0.0001	< 0.0001	< 0.0001
Ascorbate peroxidase	< 0.0001	< 0.0001	< 0.0001
Glutathione reductase	< 0.0001	< 0.0001	0.0192
Ascorbic acid content	< 0.0001	0.0414	< 0.0001
Malondialdehyde	< 0.0001	< 0.0001	< 0.0001
Total free amino acids	< 0.0001	< 0.0001	< 0.0001
Proline content	< 0.0001	< 0.0001	< 0.0001
Seed yield	< 0.0001	< 0.0001	< 0.0001

Values under ANOVA are the probabilities (P values) of the sources of variation ($P \leq 0.05$)

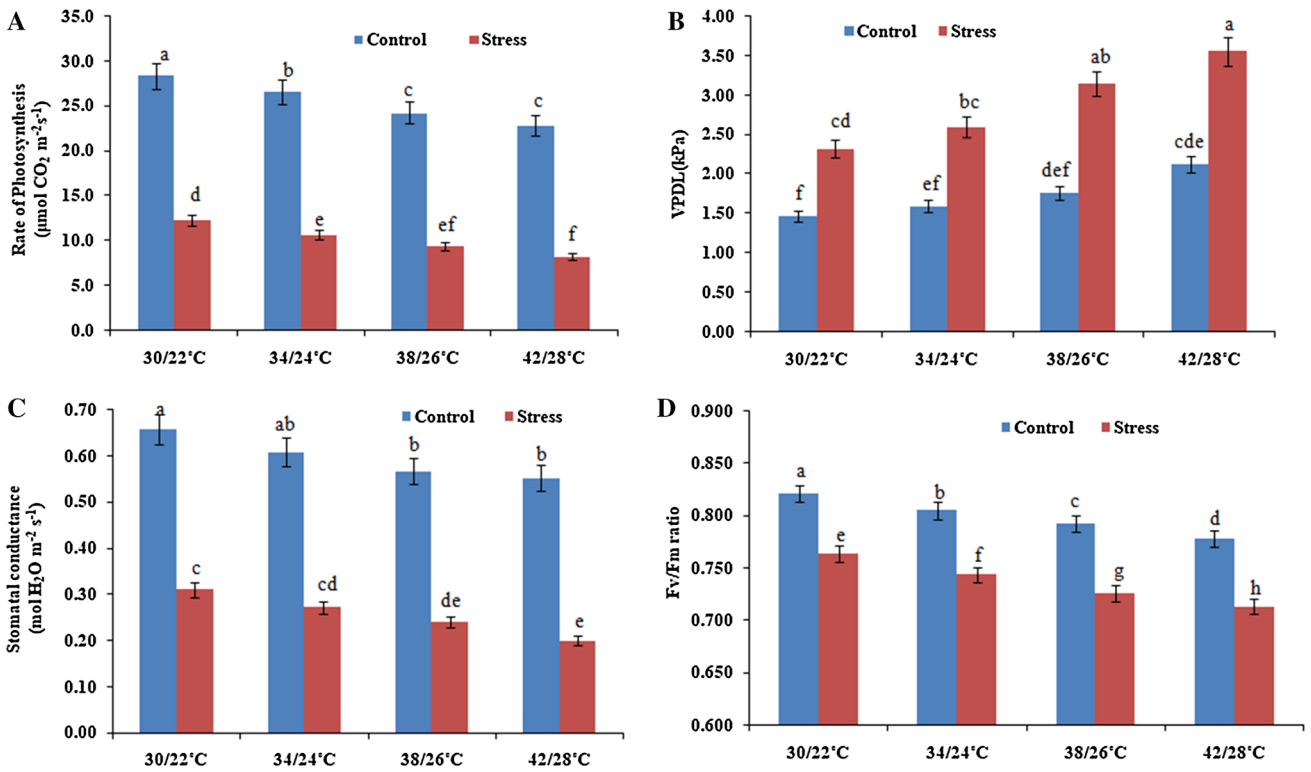

Fig. 1 Interactive effect of temperature and water stress on rate of **a** photosynthesis, **b** VPDL, **c** stomatal conductance and **d** Fv/Fm ratio. The vertical bar indicates ± SE for mean. The vertical bar indicates ± SE for mean. Values followed by the same letter are not significantly different at $P \leq 0.05$

temperature with water stress treatments revealed that the reduction in photochemical quenching due to increasing temperatures was of higher magnitude in water stressed plants as compared to control plants (Table 1). The photochemical quenching was significantly higher in control plants by 19, 22, 28 and 34% as compared to water stressed plants grown at 30/22, 34/24, 38/26 and 42/28 °C, respectively (Fig. 2a).

Non-photochemical quenching

The increasing temperatures resulted in slight increase in average non-photochemical quenching in soybean. In control plants, it increased from 0.764 in plants grown at 30/22 °C to 0.806 (5%) at 42/28 °C whereas, in water stressed plants the average non-photochemical quenching value was 0.782 at 30/22 °C and increased to 0.867 (11%) at 42/28 °C. The interaction of temperature with water stress treatment was significant indicating that non-photochemical quenching was differentially influenced due to water stress treatments at increasing temperatures (Table 1). The non-photochemical quenching was significantly higher in water stressed plants by 2, 4, 5 and 8% as compared to control plants grown at 30/22, 34/24, 38/26 and 42/28 °C, respectively (Fig. 2b).

PhiPSII

The PhiPSII was significantly affected by temperature and water stress. The average PhiPSII was reduced as the plants were grown at increasing temperatures. Maximum average PhiPSII value was 0.343 at 30/22 °C, and declined by 9 (0.311), 14(0.296) and 21% (0.271) as the growing temperature increased to 34/24, 38/26 and 42/28 °C. The average PhiPSII value in water stressed plants was 0.290 at 30/22 °C and reduced to 0.255 (12%), 0.229 (21%) and 0.185 (36%) at 30/22, 34/24, 38/26 and 42/28 °C, respectively. Interaction of temperature with water stress treatments was significant, indicating a differential change in PhiPSII in control and water stressed plants grown at different temperatures (Table 1). The reduction in PhiPSII values in response to increasing temperatures was of relatively higher magnitude in water stressed plants as compared to control plants. The average PhiPSII values was significantly higher in control plants by 16, 18, 23 and 32% as compared to water stressed plants grown at 30/22, 34/24, 38/26 and 42/28 °C, respectively (Fig. 2c).

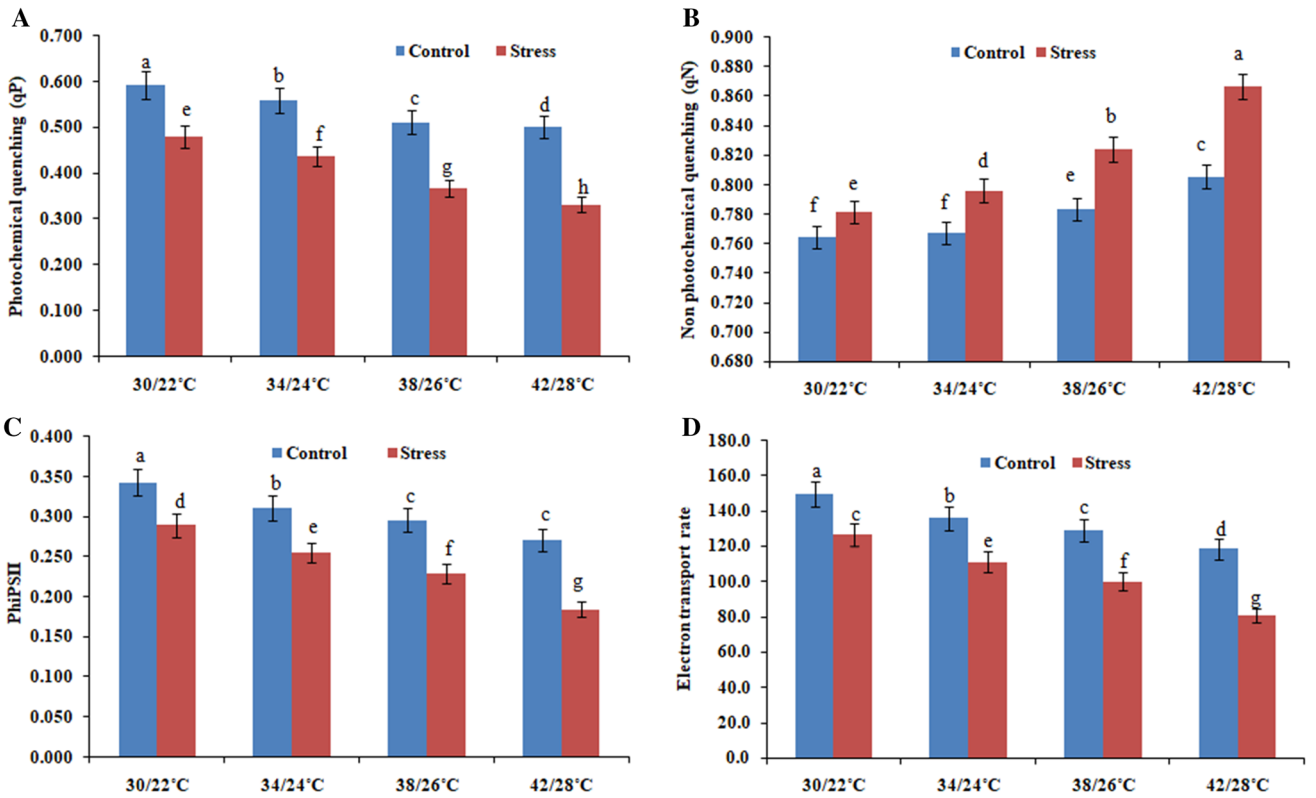

Fig. 2 Interactive effect of temperature and water stress on **a** photochemical quenching (qP), **b** non photochemical quenching (qN), **c** PhiPSII and **d** electron transport rate The vertical bar indicates ± SE for mean. Values followed by the same letter are not significantly different at $P \leq 0.05$

Electron transport rate

The electron transport rate in soybean was significantly affected by temperature and water stress. The increasing temperatures had a negative influence on average electron transport rate values. The average electron transport rate value was 149.7 in plants grown at 30/22 °C, which declined by 9, 14 and 21% as the growing temperature increased to 34/24 (136.0), 38/26 (129.2) and 42/28 °C (118.0). The average electron transport rate value in water stressed plants was 126.6 at 30/22 °C and reduced by 12, 21 and 36% at 34/24 (111.3), 38/26 (100.0) and 42/28 °C (81.0), respectively. In response to temperature, the reduction in electron transport rate was differentially influenced in control and water stressed plants as indicated by a significant interaction of temperature with water stress treatments (Table 1). The average electron transport rate value was significantly higher in control plants by 16, 18, 23 and 32% as compared to water stressed plants grown at 30/22, 34/24, 38/26 and 42/28 °C, respectively (Fig. 2d).

Total chlorophyll content

The total chlorophyll (Chl) content in soybean was though significantly affected by temperature and water stress, the impact was moderate. Compared to average chlorophyll content in control plants grown at 30/22 °C (2.06 mg/g fw), a reduction to 1.94 (6%), 1.93 (6%) and 1.83 mg/g fw (11%) was observed in plants grown at 34/24, 38/26 and 42/28 °C, respectively. Total chlorophyll content in water stressed plants was maximum at 30/22 °C (1.78 mg/g fw) which was declined to 1.70 (4%), 1.60 (10%) and 1.41 (21%) at 34/24, 38/26 and 42/28 °C, respectively. The interaction of temperature with water stress was not significant which indicated that there was a similar response of control and water stressed plants when grown at different temperatures (Table 1). The chlorophyll content was significantly higher in control plants by 14, 12, 17 and 23% as compared to water stressed plants grown at 30/22, 34/24, 38/26 and 42/28 °C, respectively (Fig. 3a).

Epicuticular wax content

A significant increase in epicuticular wax (EW) content in soybean leaves due to temperature and water stress treatments was observed. The average wax content in control plants at highest temperature (42/28 °C) was 2.53 mg/dm^2, which was significantly higher by 17% (2.10 mg/dm^2), 18% (2.07 mg/dm^2) and 23% (1.96 mg/dm^2) as compared to plants grown at 38/26, 34/24 and 30/22 °C, respectively. The average wax content was very high in water stressed plants as compared to control plants. Maximum EW was observed in plants water stressed at 42/28 °C (4.10 mg/dm^2) which was significantly higher by 31% (2.82 mg/dm^2), 36% (2.61 mg/dm^2) and 43% (2.34 mg/dm^2) as compared to plants grown at 38/26, 34/24 and 30/22 °C, respectively. The significant interaction of temperature and water stress treatment indicated a variable response of EW content with the imposition of water stress at different temperatures (Table 1). The EW content was significantly high in water stressed plants as compared to control plants at all the temperature. However, the magnitude of increase in EW content in water stressed plants as compared to control plants increased with increasing temperatures. The EW content was significantly very high in water stressed plants by 19, 26, 35 and 62% as compared to control plants grown at 30/22, 34/24, 38/26 and 42/28 °C, respectively (Fig. 3b).

Phenomenological leaf model

A phenomenological leaf model generated by Biolyzer HP 3 software depicts more active reaction centers per unit area of the leaf of plants grown at low temperatures (30/22 and 34/24 °C) as compared to high temperatures (38/26 and 42/28 °C). In this model open circles represent the active reaction center and control and water stressed plants grown at lower temperatures had more active reaction centers combined with higher efficiency of electron transport indicated by broader width of the arrow in the leaf models (Fig. 4a–d) as compared to plants grown at higher temperatures (Fig. 4e–h). The phenomenological fluxes were significantly affected by the high temperature and water stress conditions.

Superoxide dismutase

The Superoxide dismutase (SOD) activity in soybean was significantly affected by temperature and water stress. The SOD activity was similar at lower temperature both under control and water stress condition as compared to high temperature. The average SOD activity significantly increased as the growing temperatures increased. In control plants the average activity was 0.088 Units/mg protein at 30/22 °C, which was increased by 3, 43 and 96% in plants grown at 34/24, 38/26 and 42/28 °C, respectively. The SOD activity in water stressed plants was minimum at 30/22 °C (0.092 Units/mg protein) which was increased to 0.095 (4%), 0.138 (50%) and 0.197 Units/mg protein (114%) at 34/24, 38/26 and 42/28 °C, respectively. The significant interaction of temperature and water stress treatment indicated a variable response of SOD activity with the imposition of water stress at different temperatures (Table 1). The SOD activity was higher in water stressed plants by 5, 5, 10 and 14% as compared to control plants

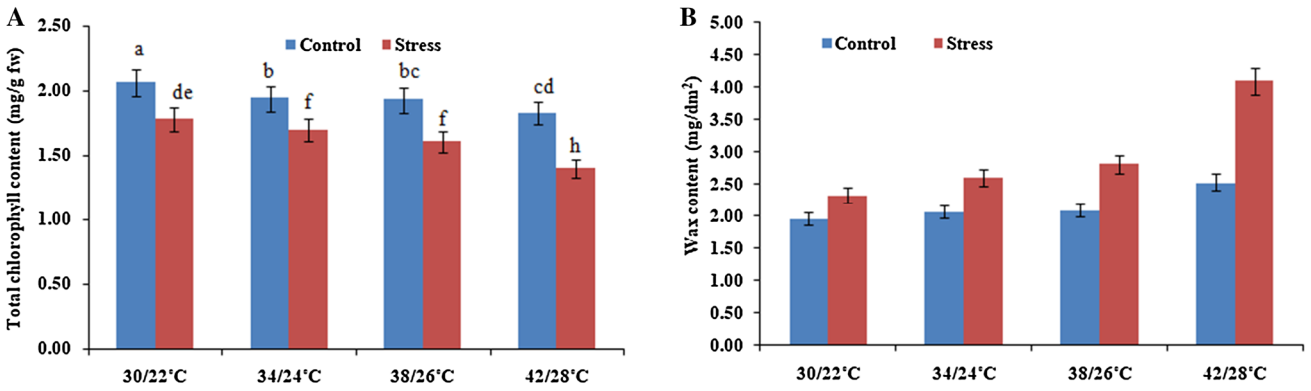

Fig. 3 Interactive effect of temperature and water stress on **a** total chlorophyll content and **b** wax content. The vertical bar indicates ± SE for mean. Values followed by the same letter are not significantly different at $P \leq 0.05$

grown at 30/22, 34/24, 38/26 and 42/28 °C, respectively (Fig. 5a).

Guaicol peroxidase

The average activity of Guaicol peroxidase (POD) also significantly increased with increasing temperatures. Compared to 30/22 °C (2.07 OD/min/mg protein), the POD activity increased to 3.25, 4.00 and 5.98 OD/min/mg protein indicating a 57, 93 and 189% increase at temperatures 34/24, 38/26 and 42/28 °C, respectively in control plants. In water stressed plants the POD activity was minimum at 30/22 °C (0.53 OD/min/mg protein) which was increased to 0.65 (24%), 2.80 (433%) and 3.91 OD/min/mg protein (643%) at 34/24, 38/26 and 42/28 °C, respectively. The interaction of temperature and water stress treatment was significant indicating a differential change in POD activity when water stress was imposed at different temperatures in soybean (Table 1). Astonishingly, between the control and water stress plants, the average POD activity was significantly high in control plants as compared to water stressed plants unlike to other antioxidants enzymes. However, the differences were much higher (75 and 80%) at lower temperatures (30/22 and 34/24 °C) as compared to higher temperatures (30 and 35%) at 38/26 and 42/28 °C, respectively (Fig. 5b).

Ascorbate peroxidase

The average activity of Ascorbic acid peroxidase (APX) which was 0.003 mM ascorbic acid oxidized/min/mg protein at 30/22 °C progressively increased with increasing temperatures to 0.005 mM ascorbic acid oxidized/min/mg protein (67%) at 42/28 °C in control plants. The APX activity in water stressed plants was 0.004 mM ascorbic acid oxidized/min/mg protein which was increased to 0.008 mM ascorbic acid oxidized/min/mg protein (122%) at 42/28 °C. The interaction of temperature and water stress treatment was significant indicating a differential change in APX activity when water stress was imposed at different temperatures (Table 1). The APX activity was higher in water stressed plants by 17, 20, 46 and 56% as compared to control plants grown at 30/22, 34/24, 38/26 and 42/28 °C, respectively (Fig. 5c).

Glutathione reductase

The average activity of Glutathione reductase (GR) also significantly increased with increasing temperatures. Compared to 30/22 °C (0.018 µM NADPH oxidized/min/mg protein), the GR activity increased to 0.019, 0.020 and 0.040 µM NADPH oxidized/min/mg protein indicating a 6, 10 and 119% increase at temperatures 34/24, 38/26 and 42/28 °C, respectively in control plants. In water stressed plants the GR activity was minimum at 30/22 °C (0.023 µM NADPH oxidized/min/mg protein) which was increased to 0.026 (12%), 0.029 (23%) and 0.058 µM NADPH oxidized/min/mg protein (149%) at 34/24, 38/26 and 42/28 °C, respectively. The interaction of temperature with water stress was not significant which indicated that there was a similar response of control and water stressed plants when grown at different temperatures (Table 1). The GR activity was higher in water stressed plants by 28, 35, 44 and 46% as compared to control plants grown at 30/22, 34/24, 38/26 and 42/28 °C, respectively (Fig. 5d).

Ascorbic acid content

A significant reduction in average ascorbic acid content was observed with elevated temperatures. The average ascorbic acid content in soybean plants grown at 30/22 °C was 8.82 µmoles/g fw, which was decreased by 3, 8 and 16% in control plants grown at 34/24, 38/26 and 42/28 °C, respectively. As far as the water stress treatments is concerned, the average ascorbic acid content at 30/32 °C was 9.07 µmol/g fw and declined in similar fashion as in

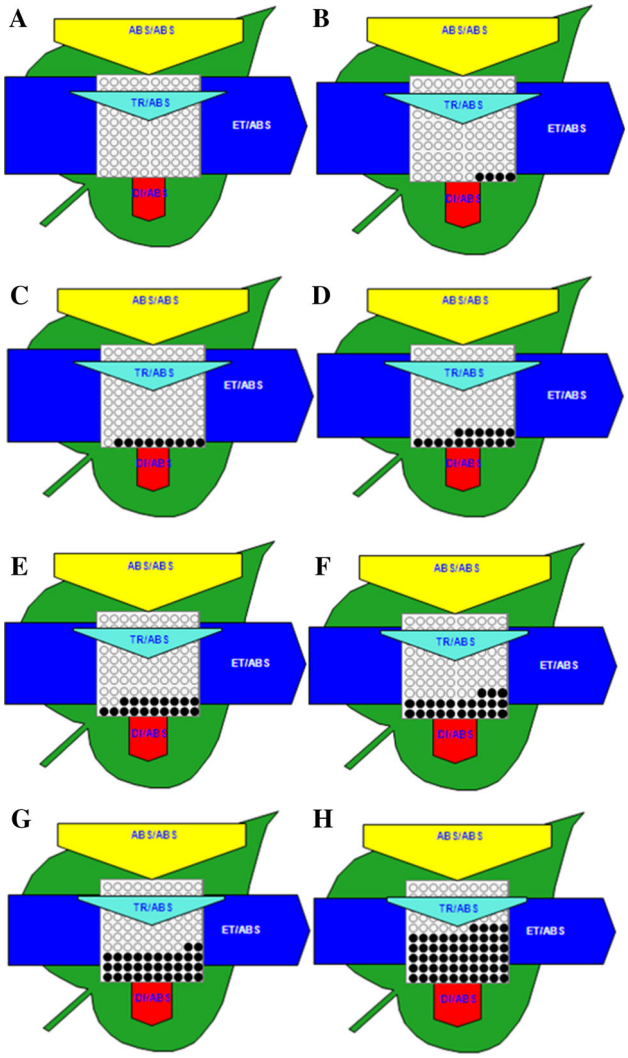

Fig. 4 Phenomenological leaf model of soybean leaves showing the combined effect of temperature and water stress. Each relative value is represented by the size of the proper parameters (arrow), the width of each arrow denotes the relative size of the fluxes or the antenna, empty circles represent reducing QA reaction centers (active), full black circles represent non-reducing QA reaction center (inactive or silent). **a**, **c**, **e**, **g** Represents unstressed leaves at 30/22, 34/24, 38/26 and 42/28 °C. **b**, **d**, **f**, **h** Represents water stressed leaves at 30/22, 34/24, 38/26 and 42/28 °C, respectively

control plants by 2, 13 and 19% in plants grown at 34/24, 38/26 and 42/28 °C, respectively. The interaction of temperature and water stress indicated that the water stress treatments differently influenced the ascorbic acid content when imposed at various temperatures. There was no significant difference in ascorbic acid content in control and water stressed plants at all the temperatures (Fig. 6a).

Malondialdehyde

The average Malondialdehyde (MDA) content in control plants was 0.066 mg/g fw in plants grown at 30/22 °C, which remained same at 34/24 °C. However, there was significant increase of 7 and 22% in plants grown at 38/26 and 42/28 °C, respectively. The MDA content in water stressed plants at 30/22 °C was 0.090 mg/g fw which was increased by 5, 27 and 60% as the temperature increased to 34/24, 38/26 and 42/28 °C, respectively. Thus, higher amount of MDA was observed at high temperatures and the magnitude of increase was greater in water stressed plants indicating the higher degree of lipid peroxidation under these conditions. The interaction of temperature and water stress indicated that the water stress treatments differently influenced the MDA content when imposed at various temperatures. The MDA content was higher in water stressed plants by 38, 42, 63 and 82% as compared to control plants grown at 30/22, 34/24, 38/26 and 42/28 °C, respectively (Fig. 6b).

Total free amino acids

Average total free amino acid (FAA) content in soybean genotypes significantly declined with an increasing magnitude as the growing temperatures increased. In control plants the FAA content was 0.702 mg/g of leaf tissue when grown at 30/22 °C declined by 3, 29 and 33% in plants grown at 34/24, 38/26 and 42/28 °C, respectively. The FAA content in water stressed plants was 1.102 mg/g of leaf tissue when grown at 30/22 °C which declined by 5, 28 and 68% in plants grown at 34/24, 38/26 and 42/28 °C, respectively. In response to temperature, the reduction in FAA was differentially influenced in control and water stressed plants as indicated by a significant interaction of temperature with water stress treatments (Table 1). The average total FAA content was significantly high (54–60%) in water stressed plants at all the temperatures except at highest temperature (42/28 °C) as compared to control plants. Compared to control plants there was 26% less FAA content in water stressed plants at 42/28 °C (Fig. 6c).

Proline content

Average proline content in soybean genotypes significantly increased with increase in growing temperatures under water stress condition. However, it did not show much difference under control conditions. The proline content in control plants was 0.741 µmol/g fw of leaf tissue when grown at 30/22 °C, which increased by 1, 7 and 9% as the temperature increased to 34/24, 38/26 and 42/28 °C, respectively. However, magnitude of increase in average proline content with rise in temperature was more in water stressed plants. The increase was 7, 15 and 22% as the temperature increased to 34/24, 38/26 and 42/28 °C, as compared to plants grown at 30/22 °C (1.842 µmol/g fw), respectively. The interaction of temperature and water

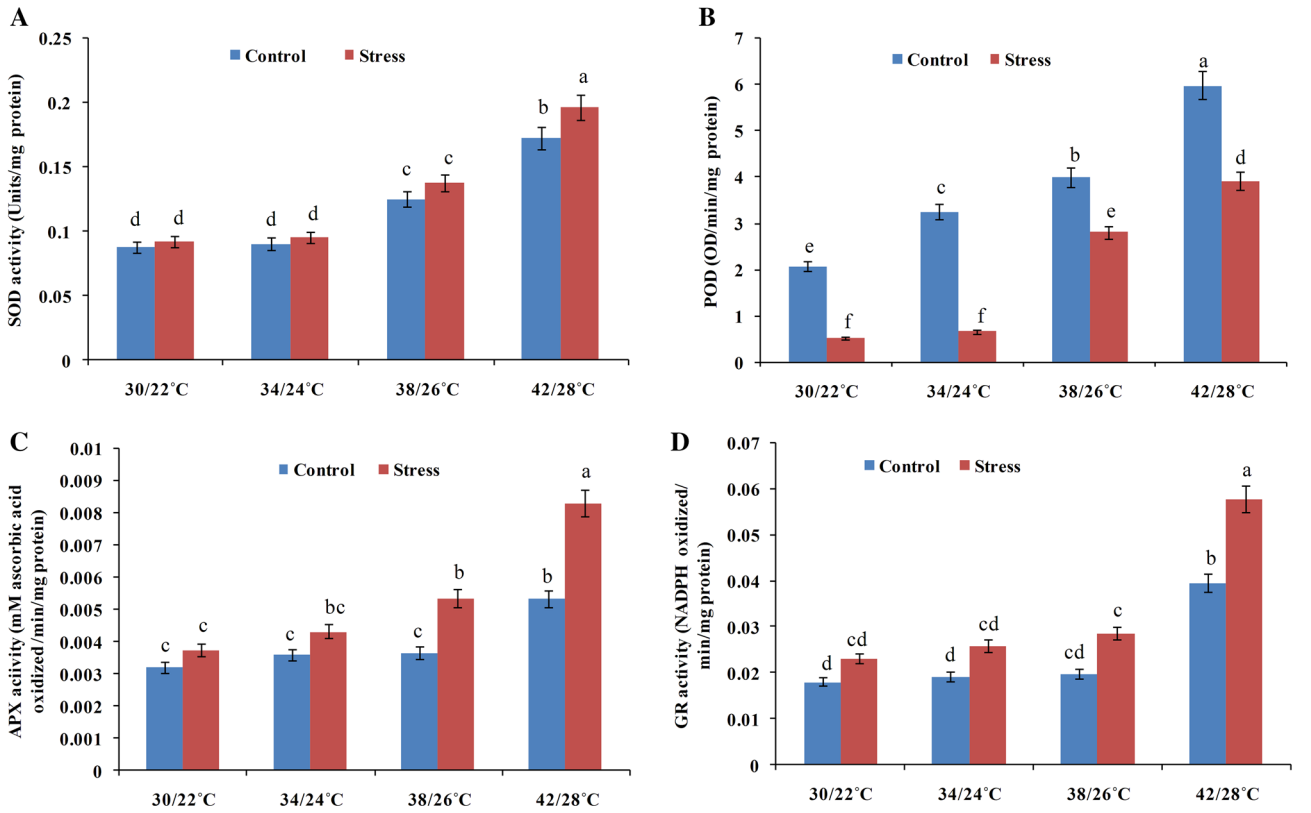

Fig. 5 Interactive effect of temperature and water stress on **a** SOD, **b** POD, **c** APX and **d** GR. The vertical bar indicates ± SE for mean. Values followed by the same letter are not significantly different at $P \leq 0.05$

stress indicated that the water stress treatments differently influenced the proline content when imposed at various temperatures (Table 1). The proline content was significantly more in water stressed plants at all the temperatures as compared to control plants. The average proline content was significantly higher in water stressed plants as compared to control plants by 149, 164, 167 and 178% at 30/22, 34/24, 38/26 and 42/28 °C, respectively (Fig. 6d).

Seed yield

The seed yield in soybean was significantly affected by temperature and water stress. The maximum average seed yield in soybean (10.8 g/pl) was observed in plants grown at 30/22 °C which was significantly declined by 13 (9.4 g/pl), 30 (7.6 g/pl) and 52% (5.2 g/pl) as the temperature increased to 34/24, 38/26 and 42/28 °C, respectively. The average yield of water stressed plants grown at 30/22, was 4.9 g/pl which was declined to 3.9 (20%), 2.9 (41%) and 1.8 g/pl (63%) when plants were water stressed at reproductive stage at 34/24, 38/24 and 42/28 °C, respectively. The interaction of temperature with water stress was significant indicating that plants responded differentially to water stress at four growing temperatures (Table 1). This indicated that in plants water stressed at temperatures 30/22, 34/24, 38/24 and 42/28 °C, the seed yield was reduced by 55, 59, 62 and 65% as compared to seed yield of unstressed plants at each temperature, respectively (Fig. 7).

Discussions

The impacts of abiotic stresses have mostly been studied independently while under field conditions, these stresses often occur in combination. Hence, it is important to understand the interactive effect of environmental stresses particularly drought and heat stress on crop plants, which have received far less attention. Thus, the present study was conducted with the aim to examine the combined effect of temperature and water stress on physiological and biochemical processes in soybean. The occurrence of drought and high temperatures conditions has often been observed in many soybean growing regions of India. Drought and high temperature are among the most important abiotic stresses that decline photosynthesis (Yordanov et al. 2000; Yamori et al. 2014) which would be reflected ultimately in growth and productivity (Ashraf and Harris 2013). However, it has been observed that PSII within the photosynthetic apparatus appears to be main

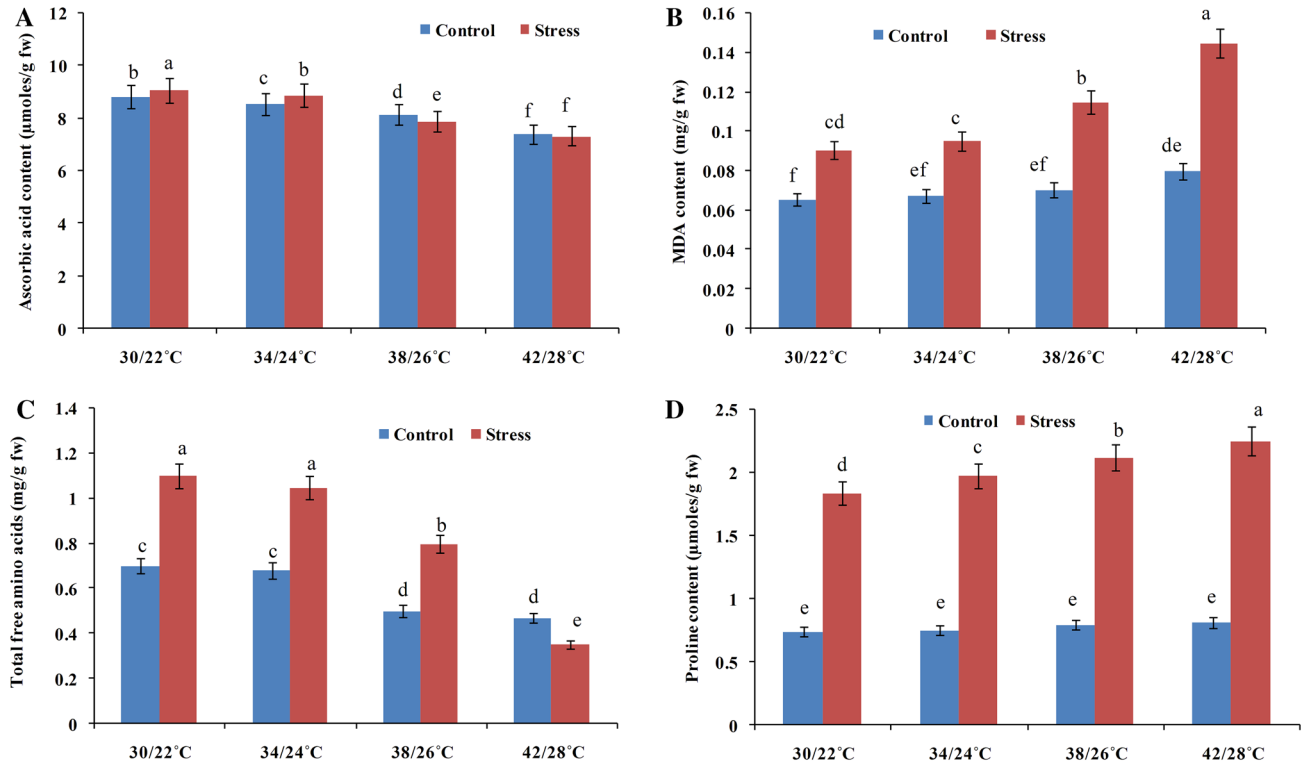

Fig. 6 Interactive effect of temperature and water stress on **a** ascorbic acid, **b** MDA, **c** total free amino acid and **d** proline content. The vertical bar indicates ± SE for mean. Values followed by the same letter are not significantly different at $P \leq 0.05$

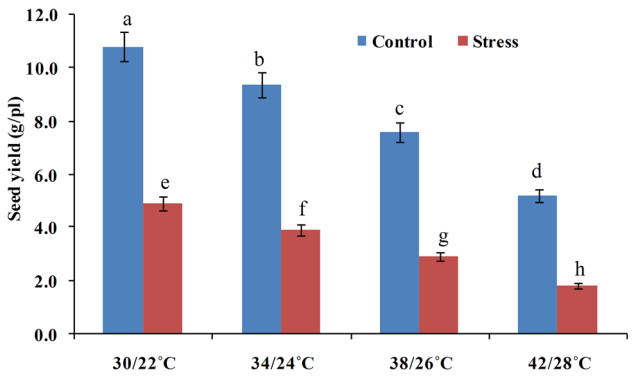

Fig. 7 Interactive effect of temperature and water stress on Seed yield. The vertical bar indicates ± SE for mean. Values followed by the same letter are not significantly different at $P \leq 0.05$

target of high temperatures. Our results showed that increasing temperatures as well as water stress had a significant negative effect on leaf photosynthesis and stomatal conductance. However, a significant increase in vapor pressure deficit was observed with increasing temperature conditions. In the present study with increasing temperatures, there was decline in stomatal conductance and increase in the VPDL to keep plants canopy cool (Ashraf 2004).

Chlorophyll fluorescence techniques are used to evaluate non-stomatal changes in photochemical efficiency and heat dissipation (Strasser et al. 2004). Significant change in chlorophyll fluorescence parameters in response to high temperature condition and drought such as Fv/Fm, PhiPSII, electron transport rate, photochemical and non-photochemical quenching has been reported in many crops (Garstka et al. 2007; Pshybytko et al. 2008; Zhang and Sharkey 2009). Our results indicated that significant but very little differences in the Fv/Fm (maximal quantum yield of PSII photochemistry) in response to water stress and high temperature conditions. However, there was a decline in photochemical quenching, electron transport rate and PhiPSII with the increase in temperatures and imposition of water stress. While non photochemical quenching was increased with increase in temperatures and imposition of water stress. To avoid the damage to PSII, if the energy absorbed it is not used for photochemical reaction, the absorbed energy needs to be dissipated by other means such as non-photochemical quenching. Thus, a high non-photochemical quenching may be a mechanism to down-regulate photosynthetic electron transport. Photochemical quenching represent the fraction of open PSII reaction centers and small decline in it suggests increased thermo stability of PSII (Jumrani et al. 2017). The processing of light through PS II was calculated by means of recording transients of chlorophyll fluorescence, and analyzed by Biolyzer software. Phenomenological leaf model showed

more active reaction centers in plants grown at lower temperatures as compared to high temperature. Imposition of water stress led to the closing of reaction centre at each temperature as compared to their respective control plants. Maintenance of chlorophyll in leaves is considered to be an important factor for maintaining higher rate of photosynthesis and hence better yields under environmental stresses such as drought and heat (Borrell et al. 2001). In the present study when considered independently, the deleterious effect of high temperature on chlorophyll content was relatively less as compared to water stress. Epicuticular wax was increased significantly both under high temperature and water stress conditions which can be used as a physiological trait for selecting genotypes tolerant to temperature and water stress.

Abiotic stresses leads to the production of ROS, which are extremely damaging and mechanisms of ROS detoxification exist in all plants (Herbinger et al. 2002). Increase in antioxidant enzymes activity has been reported to be associated with tolerance to abiotic stress in plants (Duan et al. 2012). In our study, with the increase in temperatures there was a significant increase in the activities of SOD, APX, POD and GR, indicating that there was enhanced production of ROS. Among the antioxidant enzymes the maximum increase was observed in POD activity indicating that under increasing temperatures this enzyme had played a key role in reducing the deleterious effect of ROS. With increasing temperatures, the ascorbate content was decreased, as ascorbate is the most abundant water soluble antioxidant, which reacts with ROS and is also the substrate for APX, its reduction at high temperatures reveals that it may be utilized for the detoxification of ROS. On the other hand plants subjected to water stress as compared to control plants did not show significant change in ascorbate while the activities of all the antioxidant enzymes except for POD, was increased.

Generation of ROS also leads to oxidative damage and lipid peroxidation. MDA content is commonly used as an indicator for assessing lipid peroxidation and its low levels has been associated with increased resistance in plants (Zhang and Kirkham 1994), but unfortunately, relative little is known on impact of combined effect of drought and high temperature on lipid peroxidation in plants. In the present study a significant increase in MDA content was observed in soybean genotypes in response to increase in temperature and water stress, which indicated that both these stresses leads to formation of free radicals and could have deleterious impact on cell membrane. Also, the production of MDA was more in water stressed plants and degree of increase also increased with increasing temperatures indicating that occurrence of water stress at elevated temperatures could lead to much more damage to plant cells than occurrence of individual stresses.

Under drought and heat stress conditions, the accumulation of free amino acids helps in osmotic adjustment of the plants (Good and Zaplachinski 1994; Rai 2002; Martinelli et al. 2007; Lugan et al. 2010) by altering both pattern and the concentrations of individual amino acids. Our study also indicated a significant impact of temperature and water stress on free amino acids content. Free amino acids content was decreased with increase in temperature this may be due to low availability of amino acids for synthesis of protein in plants grown at high temperature. Whereas, in plants which were water stressed there was an increase in free amino acids. Degradation of protein could have resulted in increase in free amino acids content under water stress conditions. Proline is accumulated as an adaptive response under stress condition (Maggio et al. 2002). Proline content was increased marginally under increasing temperature conditions while the increase was of very high magnitude when plants were water stressed as compared to unstressed plants. Also the magnitude of increase was much higher in water stressed plants at higher temperatures as compared to lower temperature indicating the combined effect of these two stresses was much more severe as compared to water stress alone. Genetic variation in epicuticular wax content or cuticular transpiration is found in many crops including soybean (Kim et al. 2007). Our results showed that epicuticular wax was increased significantly both under high temperature and water stress conditions.

In the present study results clearly indicated that plants exposed to extreme temperatures and water stress conditions use several physiological and biochemical mechanisms to prevent the destructive effects of oxidative stress. Enhanced level of antioxidant defense enzymes are correlated with stress tolerance. Genotypic differences do exist for physiological and biochemical parameters in response to temperature and water stress and hence, evaluation of more genotypes may help in identifying genetic sources, which are tolerant to temperature and water stress. Understanding the defense mechanisms in plants is important for developing drought and temperature tolerant genotypes. Seed yield declined with the increase in growing temperatures both in control and water stressed plants. However, the magnitude of reduction in water stressed plants was more at higher temperature as compared to low temperatures. Therefore, there is an urgent need to tackle the problem of both high temperature and drought stress in a holistic manner.

Conclusions

The results clearly indicated that when water stress occur in combination with high temperature, water stress exacerbate the adverse effects of high temperature. In India, most of the soybean growing regions fall under semiarid region and drought and high temperature stress are severe limitation for production of soybean. Therefore, understanding of combined effects of drought and high temperature on physiological traits and yield is of special importance for improving soybean productivity. High temperature is often accompanied with drought, so the primary aim of breeding must be to develop cultivars tolerating to both high temperature and drought. Understanding of combined effects of temperature and water stress on photosynthesis, chlorophyll fluorescence, antioxidant and other defense mechanisms therefore, has a special significance for breeding programme to improve productivity of soybean. Thus, there is need to develop dedicated research programs aimed at enhancing the tolerance to combination of different abiotic stresses and tolerance mechanisms for drought and heat stress may be different therefore; an integrated approach should be taken for cultivar development.

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