RESEARCH

Additive effects of combined heat and salt stress is manifested in an enhanced sodium ions accumulation and increased membrane damage in wheat seedlings

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ABSTRACT

Domestication of wheat (*Triticum aestivum* L.) in some tropical and subtropical regions may encounter both high temperature and soil salinity problems. This research aims to investigate how salt and heat stress combination affects growth and physiological characteristics of wheat seedlings to unravel their response mechanisms. The 14-d-old wheat seedlings of three cultivars were treated with salt stress (150 mM NaCl), heat stress (42 °C for 4 h) and combined stress (150 mM NaCl at 42 °C for 4 h). Growth and physiological parameters were monitored at 0 h, 24 h, 5 d and 10 d after stress exposure except for ion concentration, which was analyzed at 5 d post stress exposure. The combined stress inhibited shoot growth and compromised physiological parameters (e.g., relative water content [RWC], malondialdehyde [MDA], electrolyte leakage [EL], Na^+ content and Na^+/K^+ ratio) to a greater extent than a single salt or heat stress, demonstrating additive effects particularly on plant water status and membrane stability. After 10 d exposure to the combined stress, the most sensitive cultivar 'Fahng 60' exhibited the highest reduction in shoot dry weight (34%). Moreover, 'Fhang 60' showed the lowest RWC (36%) and the highest EL (75%) while 'Samerng 2' and 'Phrae 60' had RWC of 70% and 64%, and EL of 54% and 60%, respectively. The effect was remarkably evidenced after 5 d of stress exposure, suggesting a late onset of stress injury. Together, the results demonstrated that the effect of salt and heat stress combination was additive, which could be manifested in an enhanced Na^+ accumulation and membrane damage.

Key words: Heat stress, membrane stability, salt stress, stress combination, *Triticum aestivum*, wheat.

INTRODUCTION

Climate change has created detrimental effects for crop production worldwide and is projected to continue causing yield reduction in most regions of the world. Moreover, global warming leads to deterioration of many environmental parameters essential for plant growth. It is well recognized that global warming has increased soil temperature, reduced soil moisture regime and crop duration, increased soil evapotranspiration and, subsequently leading to increased water shortage and soil salinity (Bannari and Al-Ali, 2020). Heat, drought, and salinity are among some of the most devastating abiotic stresses for crops, leading to water deficit, metabolic disturbances, cellular damage, and yield losses (Bita and Gerats, 2013). Under natural conditions, plants are subjected to a few stressors simultaneously such as high light intensity, drought stress, heat stress and salt stress (Mittler, 2006). Studies on the response of plants to a single stress treatment

have been carried out extensively. However, the response of plants to a combination of stresses can be very different from the response of plants to each of the stress applied individually. Therefore, more attention should be paid to how stress combination, such as heat and drought or heat and salinity, impacts plant growth and yield (Li et al., 2011).

Globally, 20% of the dietary calories comes from wheat, second only to rice (Ortiz et al., 2008). Wheat (*Triticum aestivum* L.) is typically a temperate crop, but it is also grown in cool and high-altitude regions in many subtropical and tropical environments where its growth and yield may be more frequently affected by abiotic stresses (Kumar and Rai, 2014). The threshold temperatures for vegetative growth and reproductive development of wheat were reported to be 20-30 °C and 15 °C, respectively (Balla et al., 2019). Based on a testing of 30 different wheat crop models in which growing season mean temperatures ranged from 15 to 32 °C, it was estimated that global wheat production will fall by 6% for each 1 °C of further temperature increase (Asseng et al., 2015). In wheat, heat stress causes inhibition in seed germination and seedling establishment, alteration in plant water relations, reduction in chlorophyll and photosynthesis, production of reactive oxygen species (ROS), membrane damage, reduction in leaf growth and tiller formation, reduction in pollen viability, impaired grain growth and quality, and consequently yield reduction (Akter and Islam, 2017; Qaseem et al., 2019). Wheat genotypes differ in the ability to tolerate heat stress. Genotypes with higher heat tolerance exhibit multiple strategies including (1) enhanced stomatal conductance and changing leaf orientation to increase transpirational cooling, (2) maintenance of leaf chlorophyll and photosynthesis capacity, (3) enhanced activity of antioxidant enzymes, (4) enhanced membrane thermostability, and (5) activation of chaperones to protect protein denaturation and aggregation (Fahad et al., 2017; Sarkar et al., 2021).

Rising soil and water salinity also pose a serious threat to wheat production globally. High salt concentrations in the soil initially causes osmotic stress which induces water loss leading to inhibition of cell division and expansion. Subsequently, salt stress causes ion toxicity stress after plants uptake and accumulate Na^+ and Cl^- to a toxic level (Munns and Tester, 2008). Salt stress in wheat causes reductions in germination rate, leaf relative water content, chlorophyll content, photosynthesis rate, and uptake of essential elements particularly K^+. On the other hand, higher accumulation of Na^+ and increased Na^+/K^+ ratio led to ROS accumulation, lipid peroxidation, and membrane damage (Hasanuzzaman et al., 2013). The primary salt tolerance strategy found in wheat and other cereals is the Na^+ exclusion mechanism which prevent high Na^+ concentration in the shoots, and higher salt tolerance correlated with lower Na^+. However, some wheat genotypes which accumulate very high Na^+ concentration were found to have high level of salt tolerance suggesting that they exhibited the tissue tolerance mechanism, the ability to maintain cellular functions in the presence of high Na^+ (Munns et al., 2006; Genc et al., 2019). In salt-affected areas in the tropics and subtropics, wheat can be under threats by a combination of heat and drought, or heat and salinity. Qaseem et al. (2019) exposed 108 wheat genotypes to drought stress (D), heat stress (H), and drought and heat combination (DH) and found greater cellular damage, lower biomass, and yield reduction under DH than D or H. Damages to membrane, chlorophyll and proteins were higher under H than D while water status and sink strength were more affected by D. To the best of our knowledge there has been no published literature on physiological responses of wheat under combined heat and salt stress.

Thailand imports approximately 3 million tons of wheat for human consumption and animal feed but produces less than 3000 t mainly from the northern provinces in the cool season as a minor crop after rice harvest. However, in recent years, the demand for locally and organically grown wheat as health-promoting food has increased enormously. It is therefore important to explore the possibility of growing wheat in other geographic regions such as in the northeastern part of the country where some agricultural lands are affected by salinity. The present study aims to evaluate growth and physiological responses under salt stress (S), heat stress (H), and salt and heat stress combination (SH) in three wheat cultivars. Wheat breeding program in Thailand began since the 1960s from crossing of cultivars received from International Maize and Wheat Improvement Center (CIMMYT). The genotypes used in this study were approved as cultivars and released during 1983-1987. These cultivars resulted from numerous selections of breeding lines in upland and paddy field environments in the north and northeastern part of Thailand (Pradit et al., 2009). A better understanding of the combined effects of salt and heat stress may contribute to an identification of physiological traits useful as selection criteria for further improvement of wheat tolerant to multiple stress conditions. Furthermore, information from this study could be useful for the promotion of wheat growing in salt-affected lands in tropical regions.

MATERIALS AND METHODS

Plant materials and treatments

Three wheat (*Triticum aestivum* L.) cultivars namely 'Samerng 2', 'Fahng 60' and 'Phrae 60' were used. The seeds were obtained from Samoeng Rice Research Center, Chiang Mai, Thailand. The seeds were thoroughly washed with distilled water and germinated in the dark at ambient temperature for 2 d. Germinated seeds were placed in the holes in an extruded polystyrene foam (Styrofoam) lined at the bottom with plastic net, floating on the half strength nutrient solutions (Yoshida et al., 1976) in a plastic container (42.5 × 27.5 × 9 cm). Thirty-two germinated seeds of each cultivar were placed in each container. The seedlings were allowed to grow for 7 d. Thereafter, the concentration of the nutrient solution was raised to a full strength, and the seedlings were grown for the next 7 d. The plants were grown in the culture room at 25 ± 2 °C, exposed to light intensity of 33 μmol photon m^{-2} s^{-1} for 12 h per day. After 14 d in the solution culture, the plants were divided into four treatment groups, each with six replicates, as follows: Control (C), salt stress (150 mM NaCl) (S), heat stress (42 °C for 4 h) (H), and combination of salt (150 mM NaCl) and heat stress (42 °C for 4 h) (SH). For S, the nutrient solutions in the containers were replaced with the one containing 150 mM NaCl, and the plants were grown in the culture room for 10 d. For H, the solutions in the containers were refreshed, the containers were then placed in the temperature chamber at 42 °C for 4 h, returned to and grown in the culture room for 10 d. For SH, the solutions in the containers were replaced with the one containing 150 mM NaCl before being placed in the temperature chamber at 42 °C for 4 h, then returned to and grown in the culture room for 10 d. For C, the nutrient solution was refreshed, and the plants were grown in the culture room for 10 d.

Plant growth parameters

Plant growth parameters were measured as shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), and root dry weight (RDW) before and after 24 h, 5 and 10 d after the initiation of stress treatment.

Physiological variables

Relative water content (RWC). The RWC of leaves was measured before and after 24 h, 5 and 10 d exposure to stress by the modified method of Turner (1981). The leaf samples were cut to 1 cm long, and fresh weight (FW) of five pieces was immediately measured and recorded. Then the leaf pieces were put into a petri plate containing 10 mL distilled water under low light intensity for 4 h. After that the turgid weight (TW) was measured and recorded. Then the leaf samples were dried in a hot air oven at 70 °C for 7 d, and dried weight (DW) was recorded. The RWC was calculated using the equation:

$$RWC = [(FW - DW)/(TW - DW)] \times 100$$

where FW is fresh weight, DW is dry weight, and TW is turgid weight.

Chlorophyll content. Chlorophyll was measured before and after 24 h, 5 d and 10 d exposure to stress by the method of Arnon (1949). Fresh leaf samples (about 30 mg) were extracted for chlorophyll by soaking with 5 mL 80% (v/v) acetone for 48 h. The absorbance of chlorophyll extract was measured at 645 and 663 nm. Chlorophyll contents were calculated using the following equation:

$$\text{Total chlorophyll (mg g}^{-1}\text{ tissue)} = (20.2A_{645} + 8.02A_{663} \times V)/(1000 \times W)$$

where W is weight of leaf sample (g) and V is total volume (mL).

Malondialdehyde (MDA) content. The MDA content in wheat leaves was measured before and after 24 h, 5 and 10 d exposure to stress by thiobarbituric acid reactive substance (TBRS) assay based on the modified method of Sunohara and Matsumoto (2004). Approximately 0.1 g wheat leaves were ground in 4 mL 0.1% (w/v) trichloroacetic acid (TCA). Then, the sample (1 mL) was centrifuged for 20 min (10 000 rpm). The supernatant (0.5 mL) was transferred to a tube containing 0.5 mL 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) TCA and heated in water bath at 95 °C for 30 min. The tube was cooled on ice for 10 min. The absorbance was measured at 532 and 600 nm in a spectrophotometer. The MDA content was calculated using the equation:

$$MDA = (A_{532} - A_{600})/155$$

where the extinction coefficient of MDA is 155 mM^{-1} cm^{-1}.

Electrolyte leakage (EL). The EL of leaves was measured before and after 24 h, 5 and 10 d exposure to stress by the modified method of Dongsansuk et al. (2021). The freshly collected leaf was cut into 1 cm long pieces. Then, six pieces were placed in a test tube containing 10 mL deionized distilled water and kept for 24 h in the dark at room temperature to release cellular electrolytes. Electrical conductivity (EC) of the bathing solution was measured by a conductivity meter (PL-700PC, Gondo, Taipei, Taiwan), and the value was recorded as EC1. Then the test tube was boiled at 100 °C for 20 min, cooled to room temperature, then the EC was measured again and recorded as EC2. The EL was calculated using the equation: EL = EC1/EC2 ×100.

Ion concentration. Ion concentration in shoot of wheat seedlings was analyzed after 5 d exposure to stress. Shoot of seedlings was dried at 70 °C for 1 wk, and then ground to a fine powder. The samples (about 0.5 g) were digested with 10 mL nitric acid and 5 mL perchloric acid at 200 °C. The contents were covered to reflux acid fumes generated during digestion until the digest appeared translucent. After cooling down, 100 mL deionized water were added to each digestion tube. The contents were filtered, and the Na^+ and K^+ contents were analyzed by using an atomic absorption spectrophotometer (932AAA, GBC Scientific, Braeside, Victoria, Australia).

Statistical analysis

The experiment was conducted in randomized complete block design (RCBD). The data of six replicates were analyzed using a one-way ANOVA, and Tukey's test was used to compare means among treatments and time points. In any cases, $p < 0.05$ is considered significant. All data analyses were performed using SPSS analysis software (Version 28.0., IBM, Armonk, New York, USA). The principal component analysis (PCA) was done following logarithmic data transformation on MetaboAnalyst web-based platform (MetaboAnalyst 5.0, The Metabolomics Innovation Centre (TMIC), University of Alberta, Canada) (Pang et al., 2021).

RESULTS

Growth characteristics

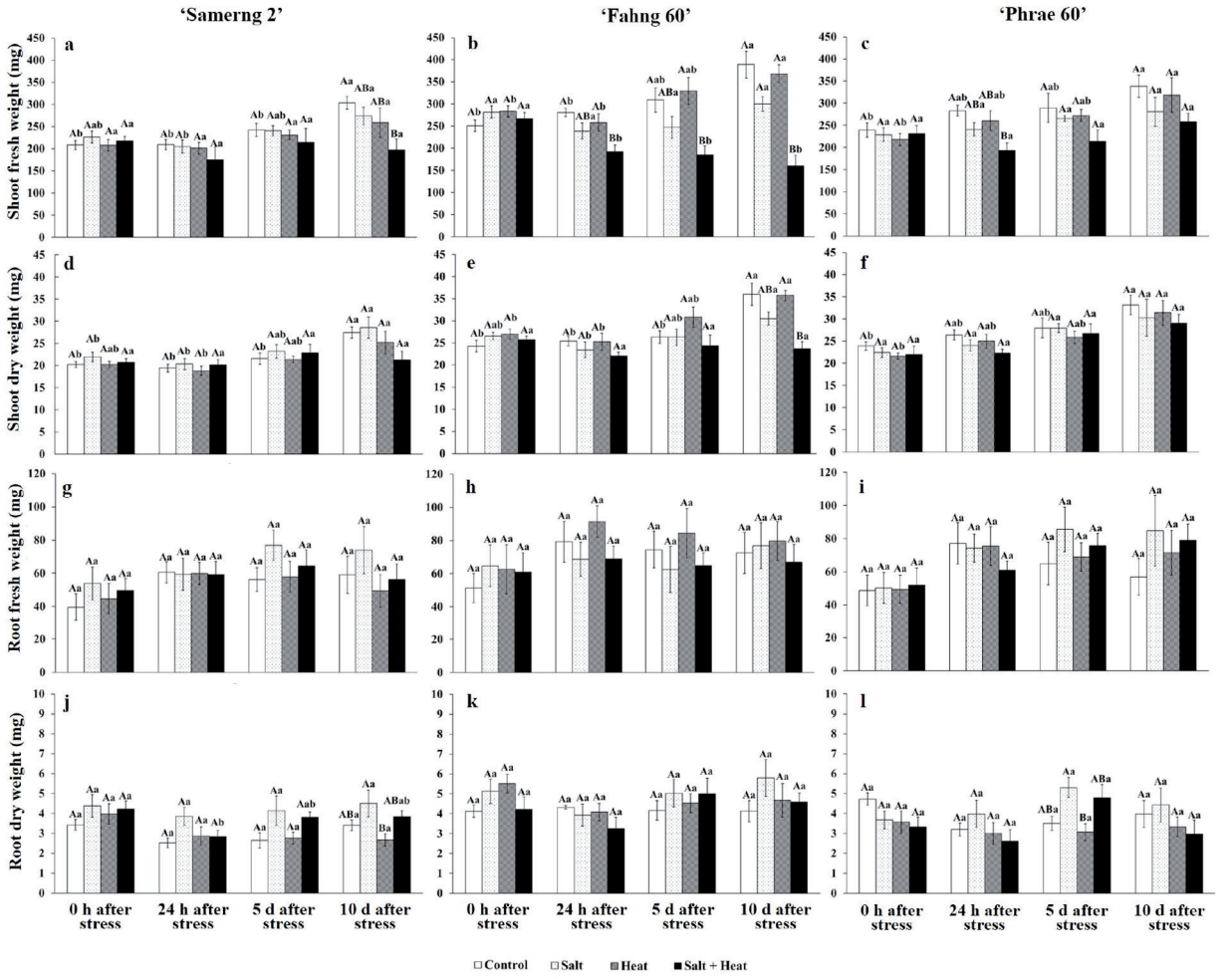
Shoot and root growth of the three wheat cultivars responded differently to salt (S), heat (H), and salt and heat stress combination (SH) (Figure 1). The inhibitory effects of the stresses on shoot fresh weight (SFW) were quickly observed after 24 h with the combined stress (SH) being the most damaging. For 'Fahng 60' and 'Phrae 60' treated with SH, the shoot fresh weights were 31% and 32% reduced from the control, respectively (Figures 1b, 1c). For 'Fahng 60', SFW continued to be inhibited 5 d and 10 d after stress with 40% and 59% reduction from the control, respectively (Figure 1b). It was notable that throughout the 10 d of stress exposure, H or S alone did not affect 'Fahng 60's SFW, but SH significantly reduced SFW, compared to the control, from 24 h to 10 d after stress induction. Shoot growth of 'Phrae 60' under stresses, on the other hand, tended to recover and its SFW after 5 and 10 d did not differ significantly from the control (Figure 1c). Significant effects of SH on SFW of 'Samerng 2' were observed only after 10 d stress exposure, showing 35% reduction compared with the control (Figure 1a). The effects of stresses on shoot dry weight (SDW) followed comparable trends as those of the (SFW). However, only 'Fahng 60' showed significant reduction in SDW after 10 d (Figure 1e) when subjected to SH (34% reduction), and nonsignificant reduction of 15% in response to S.

For root fresh weight (RFW; Figures 1g-1i), all three stress conditions did not have significant effects on all three cultivars. However, there was a tendency that root growth was insignificantly stimulated by salt stress. The only significant difference was observed after 10 d in 'Samerng 2' under H with significantly lower root dry weight (RDW) than that under S (Figure 1j). Similarly, for 'Phrae 60' after 5 d stress exposure (Figure 1l), H caused a significantly lower RDW than S. For 'Fahng 60', all three stress conditions did not significantly reduce either fresh or dry weight of roots compared with the control (Figure 1k).

Plant water status

Individual S or H stress, and combination SH variously affected the water status of wheat seedlings depending on cultivars, and time of stress exposure (Figure 2). The SH condition tended to have more negative effects on the RWC than either S or H alone. Significant reduction in RWC of 'Phrae 60' seedlings occurred as early as 24 h after SH treatment

Figure 1. Effect of salt, heat, and salt and heat stress combination on shoot fresh weight (a-c), root fresh weight (g-i), shoot dry weight (d-f), and root dry weight (j-l) of three wheat cultivars before stress (0 h), and 24 h, 5 d and 10 d after stress exposure.



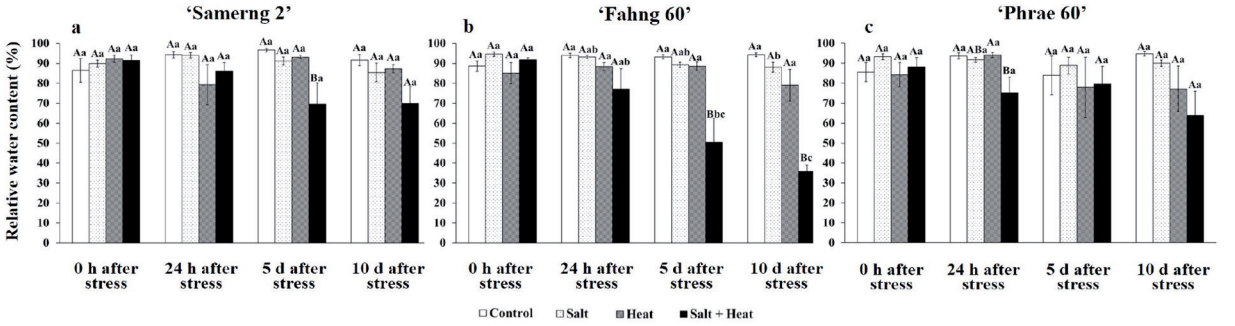
Data are mean ± SE. Means with different uppercase and lowercase letters are significantly different at $p < 0.05$ among different treatments at the same time point and among different time points of the same treatment, respectively, by Tukey's test (n = 6).

(Figure 2c). However, RWC of 'Phrae 60' remained stable after 5 and 10 d after SH stress and did not differ significantly from the control. For 'Samerng 2', a significant reduction in the SH-treated seedlings was observed only on day 5 after treatment (Figure 2a), and remained stable after 10 d. Among cultivars, 'Fahng 60' was the most severely affected by SH having RWC of only 50% after 5 d while RWC of the S- and H-treated plants had normal RWC of approximately 89% (Figure 2b). After 10 d, RWC of the H-treated 'Fahng 60' plants reduced to 79%, whereas RWC of the SH-treated plants dramatically declined to 36%.

Chlorophyll content

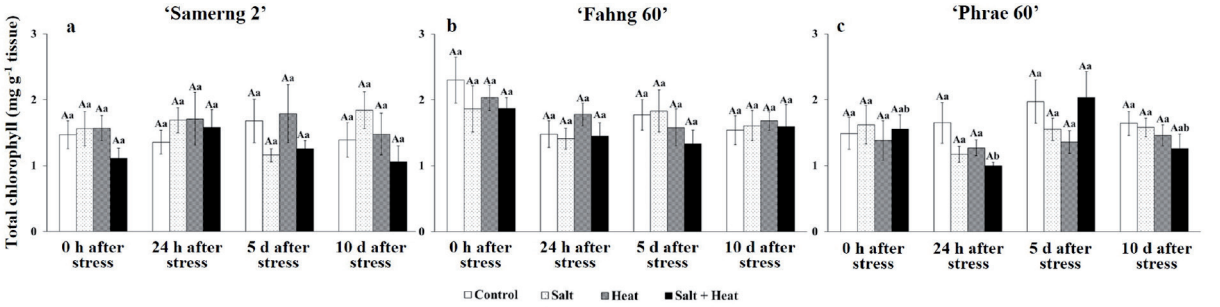
The levels and durations of stress imposed by salt, heat, and salt and heat combination had minimal nonsignificant effects on chlorophyll contents of the three wheat cultivars (Figure 3). The notable negative effects on chlorophyll contents were observed in 'Phrae 60' after 24 h of SH treatment (Figure 3c). However, after 5 and 10 d, these seedlings were able to recover and achieved higher chlorophyll contents.

Figure 2. Effect of salt, heat, and salt and heat stress combination on relative water content (RWC) of three wheat cultivars before stress (0 h), 24 h, 5 d and 10 d after stress exposure.



Data are mean ± SE. Means with different uppercase and lowercase letters are significantly different at $p < 0.05$ among different treatments at the same time point and among different time points of the same treatment, respectively, by Tukey's test (n = 6).

Figure 3. Effect of salt, heat, and salt and heat stress combination on total chlorophyll contents of three wheat cultivars before stress (0 h), 24 h, 5 d and 10 d after stress exposure.



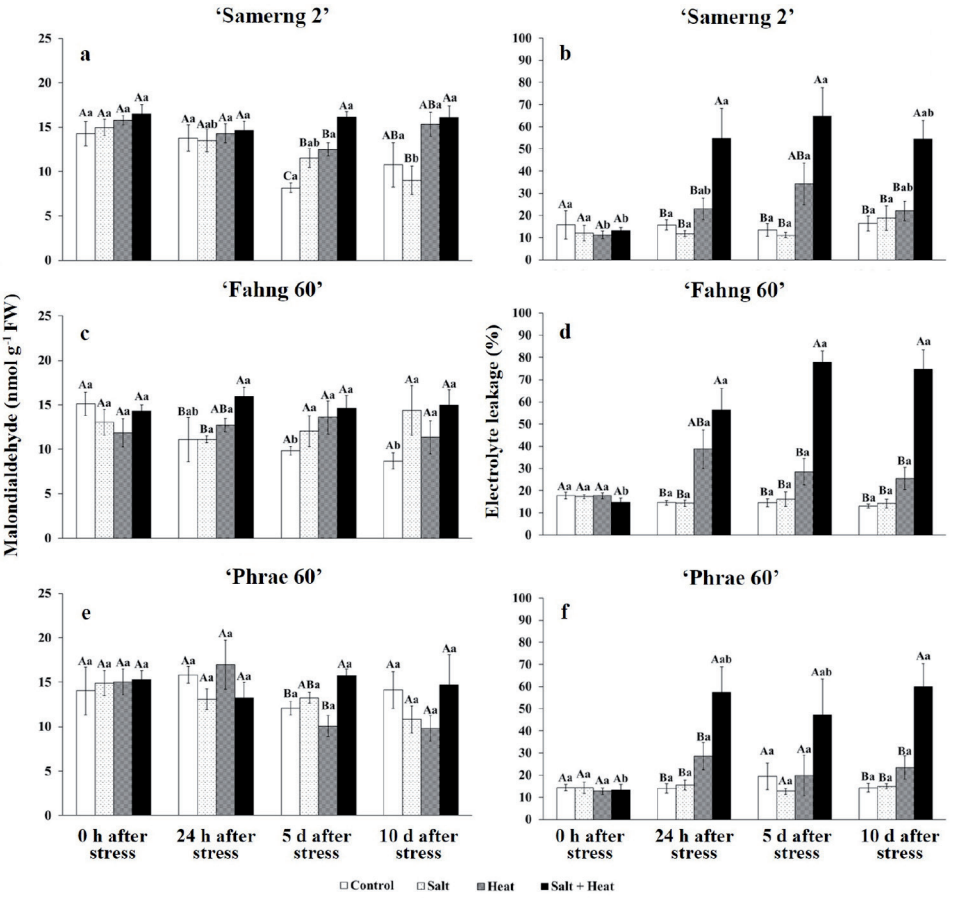
Data are mean ± SE. Means with different uppercase and lowercase letters are significantly different at $p < 0.05$ among different treatments at the same time point and among different time points of the same treatment, respectively, by Tukey's test (n = 6).

Malondialdehyde and electrolyte leakage

The earliest response to stress in relation to the MDA content, an indicator of lipid peroxidation, was observed in 'Fahng 60' after 24 h, when the SH-treated seedlings showed significantly higher MDA (15.92 nmol g^{-1} FW) than the control and the S-treated (11.10 nmol g^{-1} FW) plants (Figure 4c). The H-treated plants produced a moderate level of MDA (12.73 nmol g^{-1} FW). In 'Phrae 60' after 5 d stress exposure, the combined SH treatment also caused a significantly higher production of MDA (15.74 nmol g^{-1} FW) compared with the control (12.08 nmol g^{-1} FW), and the H (10.07 nmol g^{-1} FW) treatment (Figure 4e). Stronger effects of stresses on MDA occurred in 'Samerng 2', where 41%, 53% and 97% increase in MDA were observed 5 d after stress exposure in the S-, H-, and SH-treated seedlings, respectively, compared to the control (Figure 4a). After 10 d, the MDA contents of the H- and SH-treated seedlings remained high but did not differ significantly from the control.

In all three cultivars, salt stress did not cause any significant increase in EL at any time points during the experiment (Figure 4). However, responses to heat stress varied among cultivars. After 24 h, heat stress resulted in a large increase in EL only in 'Fahng 60' (2.66-fold increase from control) (Figure 4d). On the other hand, the salt and heat stress combination strongly affected EL of all cultivars, inducing 3- to 4-fold increases in EL within 24 h. In the following 5 and 10 d after SH stress exposure, the degrees of membrane leakage in 'Samerng 2' (Figure 4b) and 'Phrae 60' (Figure 4f) remained stable or slightly increased. In contrast, EL of the SH-treated seedlings of 'Fahng 60' (Figure 4d) dramatically increased from 56% at 24 h to 78% and 75% on day 5 and day 10, respectively.

Figure 4. Effect of salt, heat, and salt and heat stress combination on malondialdehyde content (a, c, e) and electrolyte leakage (b, d, f) of three wheat cultivars before stress (0 h), 24 h, 5 d and 10 d after stress exposure.



Data are mean ± SE. Means with different uppercase and lowercase letters are significantly different at $p < 0.05$ among different treatments at the same time point and among different time points of the same treatment, respectively, by Tukey's test (n = 6).

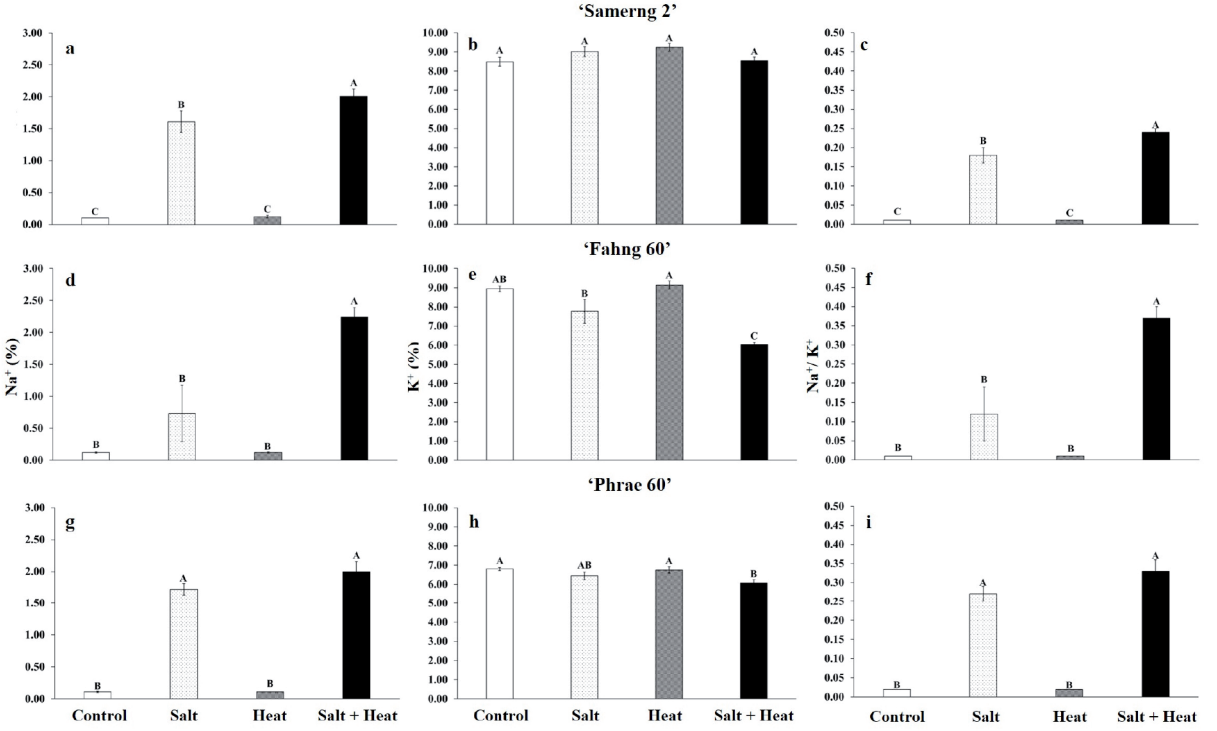
Concentration of Na^+ and K^+

Five days after stress exposure, salt treatment induced dramatic increases in Na^+ concentration in shoot tissues of all three wheat cultivars, resulting in 16.1-, 15.6-, and 6.1-fold increase in 'Samerng 2', 'Phrae 60' and 'Fahng 60', respectively (Figure 5a, 5g, 5d, respectively). The combination of heat and salt stress exacerbated the salinity effects leading to 20.1-, 18.2- and 18.7-fold increase in 'Samerng 2', 'Phrae 60', and 'Fahng 60', respectively. Salinity caused significant reduction in K^+ concentration only in 'Fahng 60', and the combined SH stress led to a further decline (Figure 5e). For 'Phrae 60', a slight but significant reduction in K^+ was observed only in the stress combination treatment (Figure 5h). Changes in the Na^+/K^+ ratios followed the same trend as that of the Na^+ concentrations (Figures 5c, 5f, 5i). Among cultivars, the highest Na^+/K^+ ratio of 0.37 was observed in 'Fahng 60' under the combined stress condition (Figure 5f) while the values for 'Phrae 60' and 'Samerng 2' were 0.33 and 0.24, respectively (Figures 5i, 5c).

Principal component analysis (PCA)

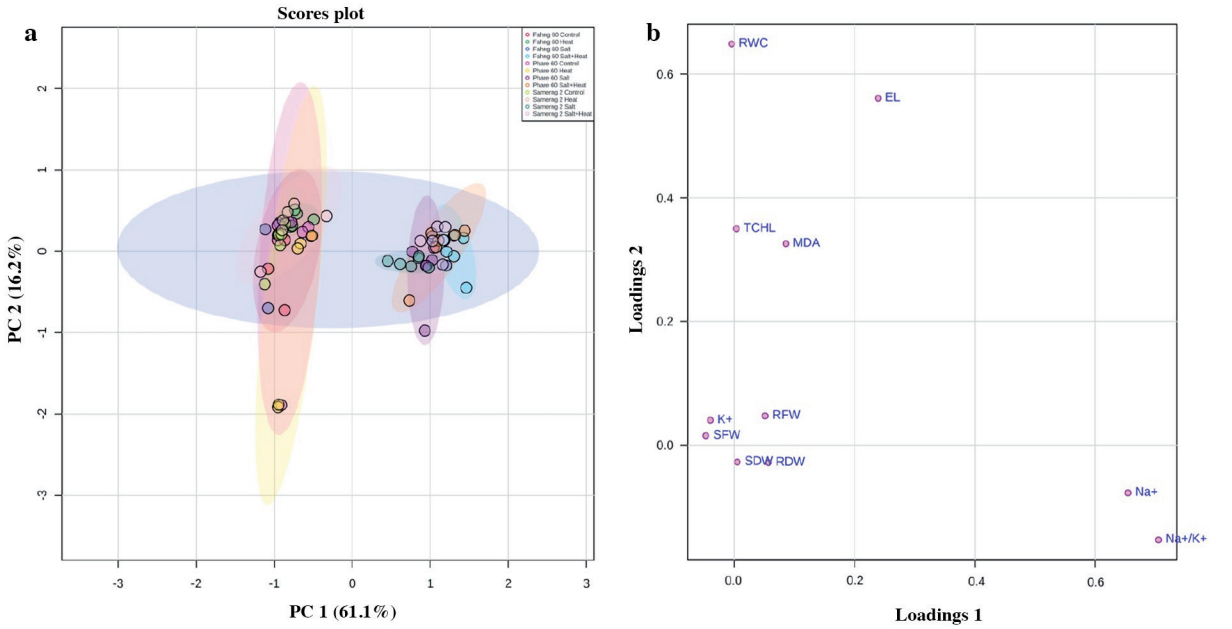
The growth and physiological parameters of the three wheat cultivars at 5 d after stress exposure were analyzed by PCA. The score plot (Figure 6a) showed two groups of data; group 1 consists of data pertaining to salt (S) and salt and heat stress combination (SH) treatments, and group 2 consists of data collected from control (C) and heat (H) treatments. In addition, the loading plot (Figure 6b) revealed that, among various physiological characteristics, Na^+ concentration and Na^+/K^+ ratios were the main parameters that separated the two groups of data.

Figure 5. Effect of salt, heat, and salt and heat stress combination on Na^+ concentration (a, d, g), K^+ concentration (b, e, h) and Na^+/K^+ ratio (c, f, i) of three wheat cultivars 5 d after stress exposure.



Data are mean ± SE. Means with different letters are significantly different at $p < 0.05$ by Tukey's test (n = 6).

Figure 6. 2D scores plot (a) and loadings plot (b) resulting from the principal component analysis of the growth and physiological data of three wheat cultivars 5 d after exposure to salt, heat, and salt and heat stress combination.



EL: Electrolyte leakage; MDA: malondialdehyde content; RDW: root dry weight; RFW: root fresh weight; RWC: relative water content; SFW: shoot fresh weight; SDW: shoot dry weight; TCHL: total chlorophyll content.

DISCUSSION

Seedlings of all three wheat genotypes were tolerant to 4 h heat treatment at 42 °C expressing nonsignificant reduction or even slight nonsignificant increases in shoot and root weights (Figure 1). As also shown in the PCA analysis (Figure 6a), growth and physiological responses of the seedlings treated with heat alone were similar and clustered in the same group as those of the control plants. The fact that wheat genotypes used in this study were being bred and selected under the field conditions in the tropical environments in Thailand could contribute to the observed heat tolerance ability (Pradit et al., 2009). However, Gupta et al. (2013) exposed 7-d-old wheat seedlings at 42 °C for 2 h and found a wide variation of reduction in SDW from 2% to 21% among 10 wheat genotypes collected from Rajasthan in India. On the other hand, genotypic variation was observed in this study when wheat seedlings were exposed to 150 mM NaCl. Among genotypes, 'Fahng 60' was the most salt-sensitive showing the highest reductions in SFW (23%) and SDW (15%) 10 d after salt stress exposure, compared with the control. Similarly, Hussain et al. (2021) treated seedlings of 40 locally grown wheat cultivars in Pakistan with 150 mM NaCl for 3 wk and found a range of 10% to 75% reduction in SDW.

The combined salt and heat stress imposed stronger negative effects on growth than salt stress and caused a significant reduction in SFW of 'Phrae 60' (32% reduction) only after 24 h stress exposure, but the plants were able to recover thereafter. In contrast, SFW of 'Fahng 60' was significantly reduced from 24 h (31%) to 5 d (40%) and 10 d (59%) after stress exposure, whereas significant reduction in 'Samerng 2' was observed only after 10 d (35%). This indicated that salt stress was the decisive factor causing significant negative effects on growth of wheat seedlings (Figure 1) especially on those of 'Fahng 60'. The damaging effects of salt on 'Fahng 60' were apparently related to the highest Na^+/K^+ ratio and lowest K^+ concentration (Figure 5). Wheat was reported to be moderately salt-tolerant and a salt excluder (Genc et al., 2019), and genotypes with lower Na^+, higher K^+ and lower Na^+/K^+ in the shoots exhibited lower growth reduction (Hussain et al., 2021; Saddiq et al., 2021). The dramatic increase in Na^+/K^+ under the combined salt and heat stress compared with the salt stress alone was previously observed in *Arabidopsis thaliana* which caused 60% reduction in plant survival compared with 100% survival under salt or heat stress individually (Suzuki et al., 2016). It was proposed that the harmful effects of salinity stress could be accelerated when salt stress was combined with heat stress because high temperature enhanced transpiration which in turn increased water loss and uptake of Na^+ into the shoot (Suzuki et al., 2014). In this study, it was clear from PCA analysis (Figure 6) that salt stress (at 150 mM) was harmful to growth and physiological processes in wheat seedlings and the salt stress effects were exacerbated by heat stress (42 °C for 4 h), making the S- and SH-treated seedlings being clustered in the same group (Figure 6a) and the Na^+ content and Na^+/K^+ ratio were the prominent discriminative physiological parameters separating them from the control (Figure 6b).

Salt or heat stress individually did not cause significant reductions in plant water status in any genotypes at any time points under investigation. In contrast, the combined salt and heat stress caused a rapid effect in 'Phrae 60' after 24 h of stress exposure (Figure 2c) leading to 18% reduction in RWC compared to the control, which corresponded with the significant reduction in SFW (Figure 1c). It could be deduced that 'Phrae 60' were sensitive to the osmotic effects of salt stress, hence the significant reduction in RWC and SFW within 24 h. The initial phase of growth response to salt stress which occurred within hours was the water loss due to the osmotic pressure of the external high salt concentration (Munns and Tester, 2008). Although salt stress on its own did not cause significant reduction in RWC, the osmotic effect was exacerbated by heat stress due to the adverse effect of high temperature on increasing membrane fluidity and permeability (Niu and Xiang, 2018) leading to significant reduction in RWC. The most damaging effect of the combined stress on plant water status was prominently observed in 'Fahng 60' after 5 and 10 d stress exposure (Figure 2b), which coincided with the reduction in SFW (Figure 1b). Dramatic water loss of 'Fahng 60' during this later phase was affected by the specific Na^+ toxicity indicated by the highest Na^+/K^+ ratio among genotypes (Figure 5). It was established that high Na^+ induced the production of ROS which attacked membrane lipids and proteins which impaired membrane structure and hence permeability (Mansour, 2013). The severe reduction in RWC of 'Fahng 60' was also related to the significant reduction in K^+ content in response to the combined stress. It has been well documented that maintaining adequate cellular K^+ concentration is essential for maintaining cell turgor and osmotic adjustment under salt stress (Wang et al., 2013).

Primary target of the environmental stress is the cell membrane, and stress-induced ion leakage from plant tissues has been widely used as a measure of abiotic stress tolerance. As the plant tissues become more stressed the more disintegrated cell membrane they have, which subsequently leads to an increased passive efflux of electrolytes from

cytosol to the bathing medium (Ilík et al., 2018). Measurement of electrolyte leakage was a reliable method for screening wheat cultivars for salt tolerance (Farooq and Azam, 2006; Jamali et al., 2015). However, in this study, NaCl at 150 mM did not significantly induce membrane damage to wheat genotypes despite the increased concentrations of Na^+ up to 16 times higher than the control (Figure 5). This indicated that these genotypes had high potential for tissue tolerance - the ability of the organ to maintain metabolic functions in the presence of elevated level of Na^+ (Munns and Tester, 2008). Heat stress tended to impose more membrane damage than salt stress as indicated by the higher EL at all time points (Figure 4b, 4d, 4f). Among genotypes, 'Fahng 60' showed the highest EL after 24 h heat exposure. Nevertheless, for all three genotypes, when salt and heat stress were combined the amount of EL increased three to four times that of the control (Figure 4b) relating to the significantly higher Na^+, and Na^+/K^+ under the combined stress compared with salt stress alone (Figure 5). In addition, 'Fahng 60' which had the highest EL after 5 and 10 d exposure to the combined stress (Figure 4d) showed the highest shoot growth reduction (Figures 1b, 1e). It could be proposed that heat stress was the primary factor causing membrane damage which was then exacerbated by salt stress. Heat stress primarily caused an increase in membrane fluidity and changes in the tertiary and quaternary structure of proteins, which enhanced membrane permeability and decreased cell membrane thermostability (Chaudhary et al., 2020). Additionally, heat stress also induced the production of ROS, which attacked membrane lipids through lipid peroxidation process further inducing solute leakage in wheat seedlings (Savicka and Skute, 2010). Lipid peroxidation, indicated by an increase in MDA as shown in Figure 4, could partly be responsible for membrane damage and increases in EL under the combined stress.

In contrast to the significant effects of the combined salt and heat stress on the cell membrane damage, these stress conditions seemed to have a marginal effect on the chlorophyll content (Figure 3). Similar to the results in this study, an exposure of seedlings of 10 wheat genotypes to heat stress at 42 °C for 2 h did not significantly reduce the chlorophyll content (Gupta et al., 2013). Moreover, after treating seven wheat cultivars at the vegetative stage with salt stress for 30 d, Jamali et al. (2015) reported a slight increase in chlorophyll contents. The effects of salt or heat stress on chlorophyll contents vary greatly depending on the intensity, duration, and plant growth stage (Kiani-Pouya and Rasouli, 2014). Most studies on the effects of salt or heat stress on wheat were conducted at the reproductive stages often with long duration of stress exposure, and the stress tolerant genotypes generally exhibited lower reduction in chlorophyll content which was associated to higher yield (Sarkar et al., 2021; Saddiq et al., 2021). Stress-induced reduction in chlorophyll content resulted from an increased activity of chlorophyll-degrading enzyme (chlorophyllase), degradation of chlorophyll-binding proteins, and disruption of chloroplast ultrastructure under severe stress (Hu et al., 2020). Small nonsignificant chlorophyll reduction or stable chlorophyll content under stress observed in this study could be explained by the short duration of stress treatments, the ability to synthesize protective chaperone proteins in chloroplasts (Hu et al., 2020), and the greater number of cells harvested for chlorophyll extraction due to the reduction in plant water content (particularly in the case of 'Fahng 60' after 10 d stress exposure).

CONCLUSIONS

In this study, heat stress did not affect growth and physiological performance of wheat seedlings, while salt stress induced marginal effects. Salt and heat stress combination prominently imposed additive effects resulting in growth inhibition, which could be attributed to an enormous increase in Na^+, Na^+/K^+ ratio, and membrane leakages. The overall effect of stress on growth and physiological characteristics of wheat seedlings was evidenced at 5 d stress exposure, suggesting the late onset of stress responses. Among genotypes, 'Fahng 60' was the most sensitive to the combined stress and may be less suitable for growing in the salt-affected regions with higher temperature such as the northeastern part of Thailand.

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