

## Agronomical, physiological and molecular evaluation reveals superior salt-tolerance in bread wheat through salt-induced priming approach

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### Abstract

Salt stress significantly limit wheat crop productivity worldwide. Exposure to non-lethal levels of salt stress, referred to as "salt-priming", allows plants to persist subsequent lethal conditions; the priming effect continues even after an extended salt stress-free period. This study attempted to evaluate the effectiveness of the salt-induced priming approach to cope with the toxic effects of long-term salinity stress in wheat. After 22 days of gradual salt acclimation to reach 250 mM NaCl, plants were recovered for eight days and finally shocked with 250 mM NaCl (priming+shock) for 7 days. After that, physiological parameters and gene expression of six salt-responsive genes were assessed. Additionally, 120 days after germination (at the end of the season), agronomic traits were recorded. Analysis of the agronomical traits revealed higher productivity in the salt-pretreated group (priming+shock) plants than the non-pretreated (shock only). Consistently, salt-pretreated plants maintained higher photosynthetic pigments level and decreased proline and MDA content than non-pretreated, suggesting enhanced salt tolerance. Moreover, salt-pretreated plants sustained high expressional levels of salt-responsive genes (*TaNHX1*, *TaSOS1*, *TaSOS4*, *TaHKT1*, *TaHKT2*, and *TaAKT1*) comparing with non-pretreated, indicating a vital role in ion homeostasis and conferring salt tolerance. Ultimately, this finding could facilitate novel smart approaches to improve wheat productivity under salt stress.

**Keywords:** gene expression; priming; NaCl; salt; stress; *Triticum aestivum*; wheat

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## Introduction

Bread wheat (*Triticum aestivum* L.) is one of the three most widely consumed cereals (maize, rice, and wheat) worldwide. It is grown in many countries and greatly participates in the global agricultural economy. According to FAO, wheat provides one-fifth of food calories and proteins to the world population (FAO 2011). By 2050, it is expected that wheat demand rises by 60% in the developing countries due to the expected increase in the global population (Bodirsky *et al.*, 2015). The gap between wheat production and consumption must be filled to meet increasing future food issues (Godfray *et al.*, 2010; Shiferaw *et al.*, 2011). The production of wheat as well as other cereals and several other crops is limited by various abiotic and biotic constraints (Majeed *et al.*, 2018).

Salinity represents one of the most severe abiotic challenges that must be overcome to fill this gap (Elshafei *et al.*, 2019). Almost 20% of total cultivated land and 33% of irrigated lands are affected by salinity stress, which is considered a leading cause of the loss of crop yields and production. It was estimated that salinized lands increase 10% annually, and consequently, it may reach 50% or more by 2050, which would be significantly reflected on the agricultural output worldwide. Different factors control this increase like the rapid growth of human population, land salinization, scarce water resources, high surface evaporation, low precipitation, irrigation with saline water, weathering of native rocks and poor cultural practices (Pitman *et al.*, 2002; Jamil *et al.*, 2011; Saade *et al.*, 2016; Soda *et al.*, 2016; Dawi *et al.*, 2021).

Crops grown on saline soils not only suffer from high osmotic stress, but also, nutritional disorders and toxicities, bad soil physicochemical conditions and consequently reduced crop productivity (Shao *et al.*, 2016). Besides exerting osmotic stress, soil salinity often creates water-deficit conditions in the form of physiological drought (Zhao *et al.*, 2016; Zhang *et al.*, 2017; El-Beltagi *et al.*, 2020a, b).

Soil salinity affects wheat plants in different aspects and stages; it suppresses seedling germination and emergence, disrupts many physiological processes like protein synthesis, enzyme activity, membrane integrity, cell division (Farooq *et al.*, 2015; Mohamed *et al.*, 2018). It may also speed up senescence with a gradual decrease in chlorophyll (Shoresh *et al.*, 2011). Consequently, bread wheat showed a decreased yield with the increase of salinity of the irrigation water. When the salinity of the irrigation water was 2-3 gm/L, the wheat yield was reduced by 7%-13% and was reduced by 13-24% when the salinity of the irrigation water was 3-5 gm/L.

Seeds priming is a physiological technique achieved by soaking the seeds in different solutions of different concentrations for different periods to enhance imbibition capacity and the pre-germinative metabolic process to ensure rapid germination, improved seedlings growth, vigor, and final yield under normal and stress conditions as well (Varier *et al.*, 2010; Paperella *et al.*, 2015; Salah *et al.*, 2015; Sano *et al.*, 2017). So, plant priming is a kind of hardening and sensitizing plants by exposing them to initial environmental stresses that function as reminders for plants to enter the primed state when exposed to the same environmental stress; primed plants have shown to be stimulated to provoke defensive processes faster than unprimed plants (Filippou *et al.*, 2013; Sani *et al.*, 2013). Recently, stress-induced priming and associated memory is an intriguing adaptive response in plants and has important implications for crop development and improvement.

Many reports proved that salt priming could improve plant tolerance to salt stress. Salt priming can help plants acclimate to lethal salinity by enhancing osmotic adjustment and repressing ionic toxicity, indicated by the lowered Na<sup>+</sup> concentration and increased accumulation of osmolytes in salt-pretreated plants. Adaptation strategies suitable for different crops and regions offer a simple alternative for the development of crops tolerant to abiotic stress, ensuring food security (Salah *et al.*, 2015).

'Priming' allows such acquired stress tolerance and offers many advantages: there is no introgression of an external genomic entity and it involves sub-lethal stress-mediated reprogramming of the molecular machinery to achieve enhanced tolerance; it is relatively fast; it is applicable for diverse stress conditions; and, with some optimization, it is capable of enhancing tolerance in a range of crops (Filippou *et al.*, 2013).

Plant intracellular balance of the K<sup>+</sup>/Na<sup>+</sup> ratio plays a crucial role in living cells' physiological processes and is very important for normal plant growth (Chen *et al.*, 2007; Shabala and Cuin, 2008). This is because the

optimum ratio of  $K^+/Na^+$  affects many cytosolic enzymes' activities, maintaining the ideal osmotic pressure and plasma membrane potential for different cell regulations (Zhu, 2003). Salinity stress disturbs the intracellular balance of this  $K^+/Na^+$  ratio, which consequently causes ionic toxicity, osmotic stress and oxidative stress in plants (Zhu, 2003; Chen *et al.*, 2007; Shabala and Cuin, 2008; Zhao *et al.*, 2020). Plants have evolved different strategies to maintain the optimal cytosolic  $K^+/Na^+$  ratio and prevent these harmful effects of salinity stress on plant growth and development.

Different known genes were isolated from halophytic plants like antiporters (NHX, SOS, HKT) and were then employed to develop salt stress-tolerant crop plants. Stress-responsive genes such as the Salt Overly Sensitive (casually named SOS) gene family which play a crucial role in ion homeostasis, therefore conferring salt tolerance (Liu *et al.*, 2000; Shi *et al.*, 2000; Oh *et al.*, 2010; Feki *et al.*, 2011). The signalling pathway of SOS is made of three main proteins, SOS1, SOS2, and SOS3. It was shown that overexpression of SOS1 confers salt tolerance in different plant species (Shi *et al.*, 2000; Ishitani *et al.*, 2000; Feki *et al.*, 2011), as it codes for plasma membrane  $Na^+/H^+$  antiporter which plays a significant role in the regulation of  $Na^+$  efflux in the cell and facilitates the transport of  $Na^+$  from the root system to the shoot system. HKT (histidine kinase transporter) family also play an important role in salt tolerance by regulating the transportation of  $Na^+$  and  $K^+$ , and thus prevent the excess accumulation and/or removal of excess  $Na^+$  in leaves, therefore offer protection of the photosynthetic tissues from  $Na^+$  toxic effect (Schroeder *et al.*, 2013). Intracellular NHX proteins are  $Na^+$ ,  $K^+/H^+$  antiporters involved in ions ( $Na^+$ ,  $K^+$ ,  $H^+$ ) homeostasis (Barragán *et al.*, 2012; Gálvez *et al.*, 2012). Consequently, it has become necessary to study and cover all agronomical, physiological, biochemical, and molecular aspects of salt tolerance and the efficiency of modern approaches such as salt-priming to improve wheat productivity smartly and safely (Sairam *et al.*, 2002; Gupta *et al.*, 2014).

Therefore, this study investigates the effect of the salt-induced priming approach to cope with the salinity stress on wheat plants by measuring agronomical and physiological traits and the gene expression of some salt-responsive genes in wheat.

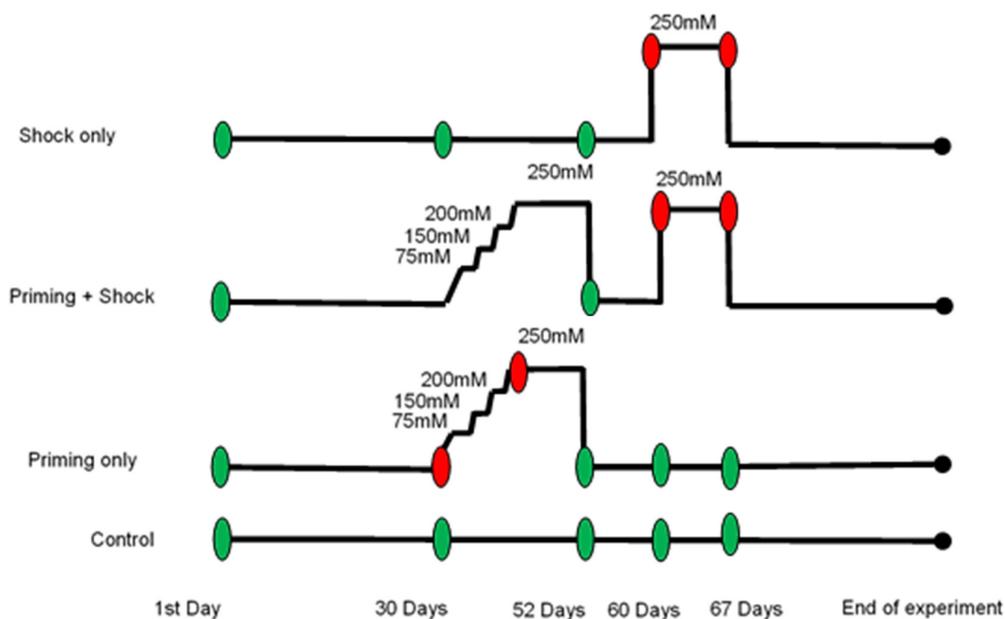
## Materials and Methods

### *Plant material and experimental design*

In order to test the effectiveness of salt-induced priming approach to improve the productivity of wheat plants, a pot experiment was carried out in the experiment farm of Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza, Egypt during the winter season of 2019/2020. Irrigation was scheduled based on crop water requirement and gap in rainfall.

In the present study, thirteen wheat cultivars were used (Table 1). Factorial experiment with two factors were designed in a completely randomized design with three replications. The treatments were designed as factor A: four different types of treatments in terms of salt stress application: control (Ctrl), priming only, priming+ shock and shock only, as shown in (Figure 1). Factor B: thirteen different wheat cultivars are varying from sensitivity to tolerance to salt stress.

Seeds were planted in plastic pots (30 cm in width/ 3 L), each containing a mixture of sandy soil and peat moss (1:1, v: v). Five wheat seeds were sown in each pot. Three pots were used as replicates per treatment. After the seedling establishment, four uniform and healthy plants were allowed to grow in each pot and fertilized regularly using a standard dose of N:P:K 20:20:20 (1 g/L). The salinity treatment was applied thirty days after sowing using a fixed amount of salt solution for each treated pot. The salt solution was freshly prepared by dissolving a calculated amount of NaCl with tap water. The salt solution was added in increment concentrations every three days until the final concentration of 250 mM NaCl was achieved to apply the salt stress in a gradual exposure approach (salt priming) as shown in (Figure 1). After one week, under the final concentration of 250 mM NaCl the plants were irrigated with tap water.



**Figure 1.** Scheme of the experimental setup

Horizontal lines represent different plant treatments as follows: control, priming only, priming + shock and shock only. Samples for physiological and gene expression analysis were collected at 67 days after sowing (at the end of the salt shock). Wheat yield was harvested at the end of experiments to calculate the total yield per plant

**Table 1.** Code of the 13 wheat cultivars, names and their origin

Code	Cultivar Name	Origin
Cv.1	'Sids14'	Sids, Egypt
Cv.2	'Giza171'	Giza, Egypt
Cv.3	'Giza168'	Giza, Egypt
Cv.4	'Maiaa'	Barida, KSA
Cv.5	'Henta Asmr'	Tamir, KSA
Cv.6	'Asmr'	Najran, KSA
Cv.7	'Molloaha Mokaom'	Alehsaa, KSA
Cv.8	'Samaa Baladi'	Elkharag, KSA
Cv.9	'Samaa Baladi'	Tamir, KSA
Cv.10	'Soariak'	Tamir, KSA
Cv.11	'Samaa'	Tamir, KSA
Cv.12	'Helba'	Barida, KSA
Cv.13	'Lokami'	Barida, KSA

### *Physiological parameters screening*

#### Photosynthetic pigments

Photosynthetic pigments (chlorophyll a, chlorophyll b, total chlorophyll and carotenoids) were determined using the spectrophotometric method (Atia *et al.*, 2020). 50mg fresh leaf tissues freshly grounded in liquid nitrogen and soaked in 0.5 ml 80% acetone to extract the pigments for one week. The slurry was centrifuged at 10000 g for 10 min and the debris was washed with 0.5ml 80% acetone ice cold. The supernatants were pooled and completed to a certain volume with acetone. The extract's absorbance was measured spectrophotometrically against a blank of 80% acetone at three different wavelengths, 440.5, 644 and 662 nm.

The concentration of each pigment was calculated using the following formula described by Sestak *et al.* (1971). The results were expressed as  $\mu\text{g pigment g}^{-1}$  FW.

$$\text{Chlorophyll a } (\mu\text{g g}^{-1} \text{ FW}) = 9.78 \text{ E662} - 0.99 \text{ E644}$$

$$\text{Chlorophyll b } (\mu\text{g g}^{-1} \text{ FW}) = 21.4 \text{ E644} - 4.65 \text{ E662}$$

$$\text{Chlorophyll (a + b)} (\mu\text{g g}^{-1} \text{ FW}) = 5.13 \text{ E662} + 20.41 \text{ E644}$$

$$\text{Carotenoids (Car)} (\mu\text{g g}^{-1} \text{ FW}) = 4.69 \text{ E440.5} - (\text{Ch a} + \text{Ch b}) \times 0.268$$

All extraction steps were carried out in dim light through a maximum of six hours to avoid the decomposition of pigments.

#### Lipid peroxidation (MDA)

Malondialdehyde (MDA) content of the flag leaf was estimated (Heath and Packer, 1968). The leaves were collected in liquid nitrogen and deposited at  $-80^{\circ}\text{C}$  till performing the MDA assay. OD600 values are subtracted from the MDA-TBA complex values at 532 nm and MDA concentration is calculated using the Lambert-Beer law with an extinction coefficient  $\epsilon_{\text{M}} = 155 \text{ mM}^{-1}\text{cm}^{-1}$ . Results are presented as nmols MDA  $\text{g}^{-1}\text{FW}$ .

#### Proline content

The proline content of the flag leaf was estimated according to Shabnam *et al.* (2016) and Abdelaziz *et al.* (2019). The leaves were collected in a paper bag and completely dried in the oven at  $70^{\circ}\text{C}$  till constant weight was obtained. The proline content was determined from a standard curve and calculated on a dry weight basis as follows:

$$\mu\text{moles proline g}^{-1} \text{ of fresh plant material} = \{(\mu\text{g proline mL}^{-1} \times \text{mL toluene}) / 115.5 \mu\text{g } \mu\text{mole}^{-1}\} / (\text{g sample}/5)\}$$

#### *Agronomical evaluation*

Agronomical traits were measured 120 days after germination (at the end of the season) before harvest. The traits included: plant height (cm), and number of tillers and spikes/plant, main spike length (cm), number of spikelets/spike, number of grains/plant, the total yield/plant and weight of 1000 grains (g).

#### *RNA isolation and qRT-PCR analysis*

Fifteen days' post priming application (67 days after germination), the leaves of control and treatment plants were collected from three replicates in liquid nitrogen. RNA was isolated with Trizol reagent and treated with DNase I (Cat Num.: EN0525, Thermo Scientific). The cDNA was synthesized using the SuperScript™ II Reverse Transcriptase as outline by the manufacturer's manual (Cat Num.: 18064014, Thermo Scientific). Salt-responsive genes (*TaNHX1*, *TaSOS1*, *TaSOS4*, *TaHKT1*, *TaHKT2*, and *TaAKT1*); and *Ta Actin* as a housekeeping gene were used for qRT-PCR analysis (Table 2). The qRT-PCR analysis was done using a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA, USA). The qPCR reaction in a final volume of 20  $\mu\text{L}$  contained 1  $\mu\text{L}$  of cDNA template, 0.5  $\mu\text{L}$  of gene-specific primers (10  $\mu\text{M}$ ), 10  $\mu\text{L}$  PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™), and 7.6  $\mu\text{L}$  ddH<sub>2</sub>O. The thermal cycles were  $94^{\circ}\text{C}$  for 30 s, 40 cycles of  $94^{\circ}\text{C}$  for 5 s and  $60^{\circ}\text{C}$  for 30 s and were followed by a dissociation stage. Each sample was repeated three times as technical repeats. The  $2^{-\Delta\Delta\text{Ct}}$  method was used for calculating the relative expression levels (Eisaa *et al.*, 2017; Mohammed *et al.*, 2017; Mokhtar and Atia, 2019). Three biological replicates (3 plants/treatment) were evaluated, and the mean and standard deviation values of statistics were measured.

**Table 2.** Gene names, primers sequences used for qRT-PCR analysis and their references

Gene Name	Primer F	Primer R	Ref
<i>TaNHX1</i>	GCCGGGTTTCAAGTAAAG	GGACTATCTTGCAATTGGG	(Zeeshan <i>et al.</i> , 2020)
<i>TaSOS1</i>	GTTGTGCGGTGAGGTGCGGAGGG	TCATCTTCTCCTACCGCCCTGC	(Ramezani <i>et al.</i> , 2013)
<i>TaSOS4</i>	ATCCAGTCCCACACCGTCCA	GCTGATTGCCATTGAGAACCTGTC	
<i>TaHKT1</i>	ACCTCGCCATCTTCATCATC	GCTTCCATGAAGGAAACCAA	(Kumar <i>et al.</i> , 2017)
<i>TaHKT2</i>	TATGTGATGAGTCGCAGCTTGAA	GCAACAAGAGGCCTGAATTCTTT	
<i>TaAKT1</i>	CGGATAATGCCGTGAATG	TTATACTATCCTCCATGCCT	(Zeeshan <i>et al.</i> , 2020)
Ta-Actin	GACAATGGAACCGGAATGGTC	GTGTGATGCCAGATTTTCTCCAT	(Zeeshan <i>et al.</i> , 2020)

### Statistical analysis

Experiments were carried out following a randomized complete block design with three replicates. Data normality and the homogeneity of variances were checked using the Kolmogorov-Smirnov test and Levene's test, respectively. All the data was subjected to one-way Analysis of Variance (ANOVA). Tukey's Multiple Comparisons Test ( $p \leq 0.05$ ) was carried out as the post-hoc test for mean separations. Also, Pearson correlation was calculated to determine the correlation between measured traits. All statistical tests were performed using the computer program SPSS statistics 25 (SPSS Inc., Chicago, IL, USA).

## Results

To fulfill our aim, we designed the experimental groups (control, priming, priming+shock and shock) to study the effects of the salt-induced priming approach on the agronomic traits (total yield/plant (g/plant), 1000 kernels weight (g), spike length (cm), number of kernels/spike, number of spikelets/spike, shoot dry weight (g), peduncle length (cm), and plant height (cm)), physiological parameters (proline, MDA, chlorophyll 'a', 'b', total chlorophyll, and carotenoids) and the expression level of some salt-responsive genes (*TaNHX1*, *TaSOS1*, *TaSOS4*, *TaHKT1*, *TaHKT2*, and *TaAKT1*). Thirteen wheat cultivars (three Egyptian cultivars and ten Saudi cultivars) were used to investigate and compare their performance under the salt-induced priming approach.

### Agronomic traits evaluation

A total of eight agronomic traits (peduncle length (cm), plant height (cm), 1000 kernels weight (g), spike length (cm), number of kernels/spike, number of spikelets/spike, shoot dry weight (g) and total yield/plant (g/plant)) were evaluated to reflect the usefulness of salt-induced priming approach to improving different yield component traits under salt stress. Comparative evaluation of the 13 wheat cultivars under control and salt stress conditions demonstrated that all the salt-stressed groups exhibited consistent patterns of decrease compared to the control, particularly for the salt-pretreated group (priming+shock), which always takes an intermediate value between the non-pretreated group (shock only) and the priming group (Figures 2A-H).

The correlation analysis between the eight agronomic traits revealed the highest correlation between the spike length and the number of spikelets/spike traits (0.833). Also, a high correlation was observed between the total yield/plant trait and both thousand-Kernels Weight and the number of Kernels/ Spike traits (0.682 and 0.678, respectively) (Table 3).

From another perspective, the heatmap manifests a panoramic appearance for the eight agronomic traits (Figure 3A). The heatmap clustered the priming group with the control group. Notably, among the thirteen wheat cultivars, the cultivar Cv.5 appeared to have the lowest values for the thousand-Kernels Weight, the

number of kernels/spike, the shoot dry weight, and the total yield/plant traits comparing with all other cultivars.

**Table 3.** Pearson correlation analysis between the eight agronomical traits

Trait	Total yield/plant	1000 kernels weight	Spike length	# Kernels /Spike	# Spikelets /Spike	shoot dry weight	Peduncle length	Plant height
Total yield/plant	1	0.682**	0.413**	0.678**	0.319**	0.443**	0.414**	0.404**
1000 kernels weight	0.682**	1	0.281**	0.358**	0.191*	0.444**	0.352**	0.322**
Spike length	0.413**	0.281**	1	0.454**	0.833**	0.360**	0.332**	0.437**
# Kernels/ Spike	0.678**	0.358**	0.454**	1	0.376**	0.324**	0.302**	0.339**
# Spikelets/ Spike	0.319**	0.191*	0.833**	0.376**	1	0.259**	0.303**	0.338**
Shoot dry weight	0.443**	0.444**	0.360**	0.324**	0.259**	1	0.423**	0.560**
Peduncle length	0.414**	0.352**	0.332**	0.302**	0.303**	0.423**	1	0.547**
Plant height	0.404**	0.322**	0.437**	0.339**	0.338**	0.560**	0.547**	1

\*\* Correlation is significant at the 0.01 level (2-tailed).

\* Correlation is significant at the 0.05 level (2-tailed)

#### *Physiological parameters screening*

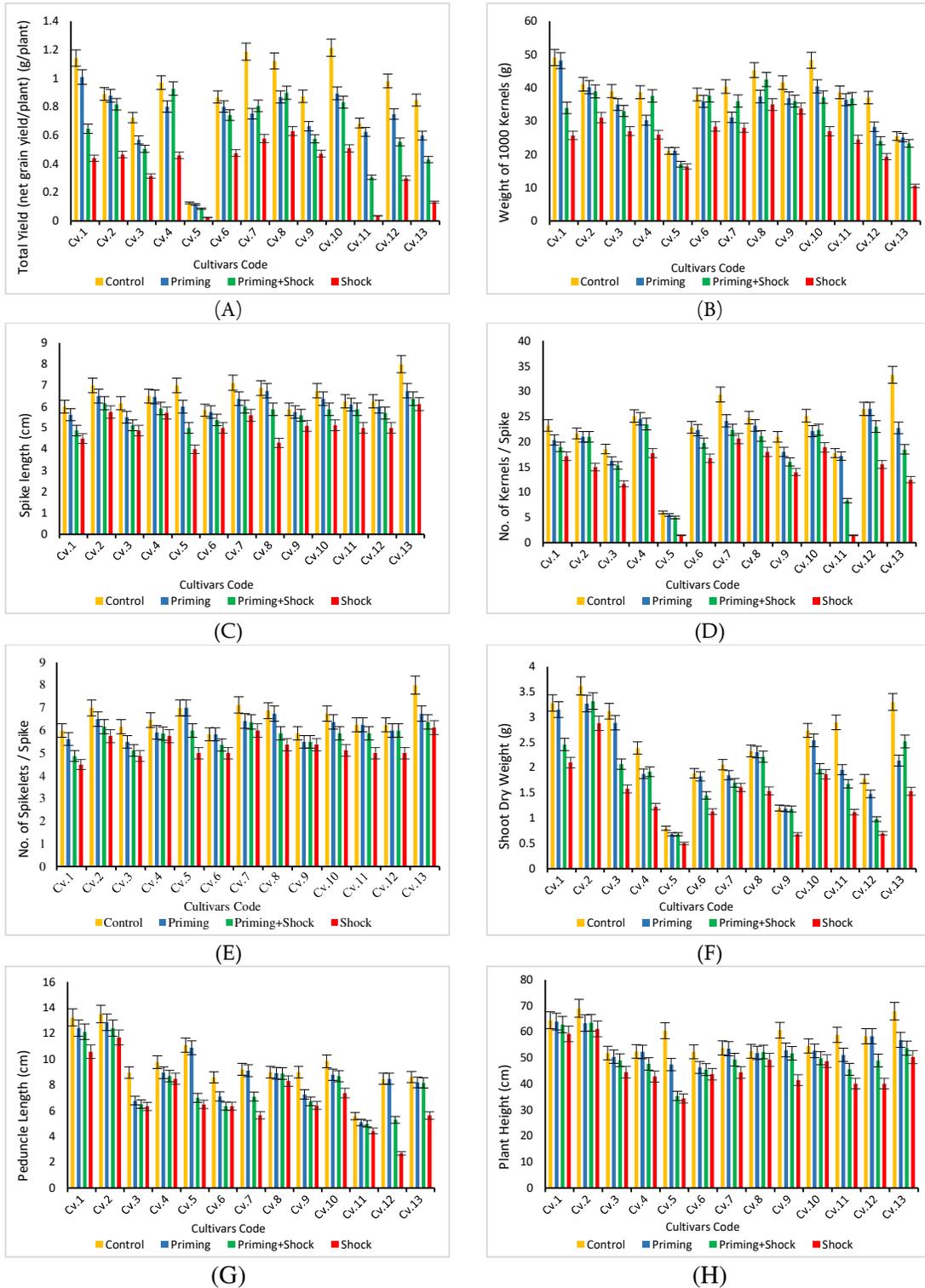
Regarding the effect of salinity stress on the physiological parameters (proline, MDA, chlorophyll 'a', 'b', total chlorophyll, and carotenoids), the shoot tissues of the experimental groups were collected after salt shock for seven days (at 67 days after germination).

#### Photosynthetic pigments

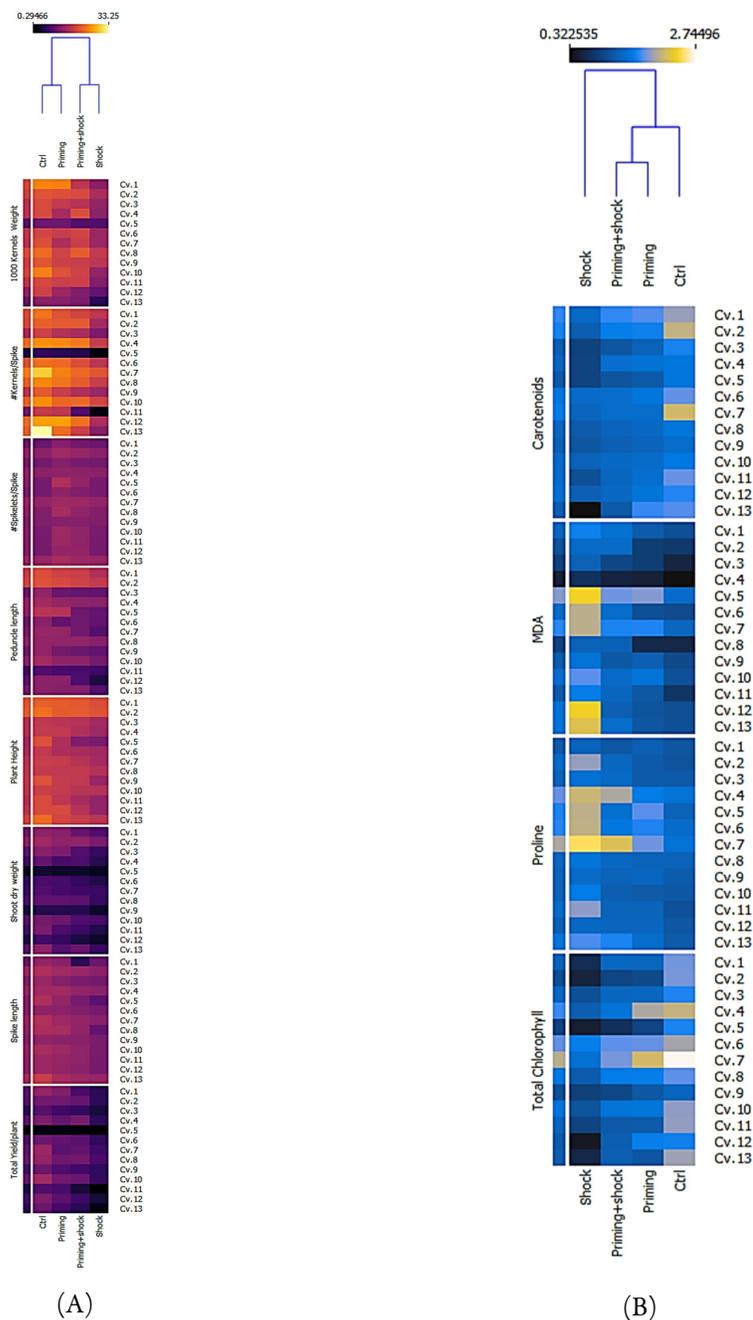
The photosynthetic pigments content of the wheat cultivars under study, including chlorophyll 'a', 'b', total chlorophyll, and carotenoids, were measured. The results revealed a decreased trend through the three salt-stressed groups (priming, priming + shock, and shock) compared to the control group. Although there is a general trend of decrease, the salt-pre-treated group (priming + shock) was outperforming the non-pre-treated group (shock only) with a relatively low decrease pattern in the chlorophyll 'a', 'b', total chlorophyll, and carotenoids contents (Figures 4A-D).

#### Lipid peroxidation (MDA)

Generally, increased lipid peroxidation (in terms of MDA level) content in plant tissues is a clear indicator of the reactive oxygen species levels and their damage to plant cells under salt-stress conditions. Notably, the cultivars Cv.5, Cv.6, Cv.7, Cv.12, and Cv.13 showed a significant increase (more than 2.5 nmol g<sup>-1</sup>FW) in the shock group compared to the control group. The priming group showed very close values to the control group. The values of the non-pre-treated group (shock only) were the highest among the three treatment groups. Also, the salt-pre-treated group (priming +shock) exhibited a trend of decreased MDA content than the non-pre-treated group (shock only) for all most of the cultivars. Except for the cultivars Cv.1, Cv.2, and Cv.8, there were insignificant differences between the priming + shock and the shock groups in the MDA level (Figure 4E).



**Figure 2.** Effect of salinity stress of the salt-treated groups (priming, priming+shock, and shock) on the eight agronomic traits: (A) total yield/plant, (B) 1000 kernels weight, (C) spike length, (D) number of kernels/spike, (E) number of spikelets/spike, (F) shoot dry weight, (G) peduncle length, and (H) plant height. Tukey's Multiple Comparisons Test was conducted to ascertain the significant difference between means (n=3) at a significant level of  $P < 0.05$  and represented as mean  $\pm$  standard deviation (SD).



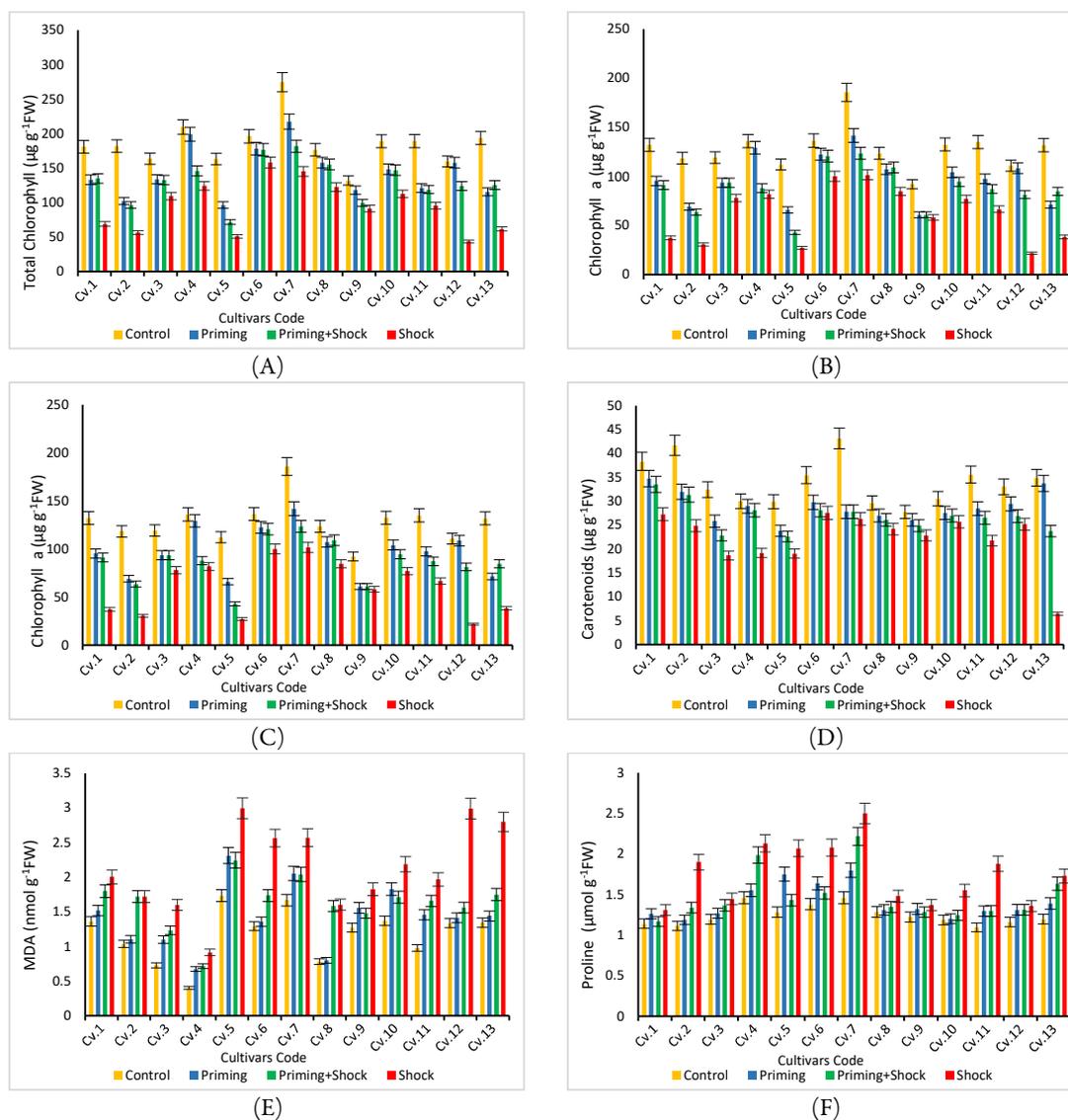
**Figure 3.** Two-dimensional heatmap visualization shows the interaction between the treatments (ctrl, priming, priming + shock and shock) and (A) the eight agronomic traits data, (B) the four physiological traits data of the 13 wheat accessions

Proline content

Generally, higher accumulation of the proline content is associated with salinity tolerance in wheat. The results showed a general trend of increase in the proline content in all groups compared to the control group, particularly in the shock group among the three salt-treated groups. The values of the salt-pretreated group (priming+shock) exhibited a general trend of decreased proline levels compared with the non-pretreated group (shock only). The priming group showed comparable levels for the control group. The results also revealed that

cultivars Cv.4, Cv.5, Cv.6, and Cv.7 of the shock group showed a significant increase (more than  $2 \mu\text{mol g}^{-1}\text{FW}$ ). While the salt-pretreated (priming+shock) group showed decreased values in all cultivars, except for the cultivars Cv. 4, Cv.9, and Cv.13, which showed insignificant decrease between the non-pretreated group and the salt-pretreated group (Figure 4F).

The correlation analysis of the four measured physiological traits disclosed a robust positive correlation between Carotenoids-Total Chlorophyll and MDA-proline (0.655 and 0.636, respectively). Meanwhile, the highest negative correlation was observed between Carotenoids-MDA (-0.550) (Table 4).



**Figure 4.** Overview of the changes in the physiological parameter's levels: (A) Total Chlorophyll, (B) Chlorophyll a, (C) Chlorophyll b, (D) Carotenoids, (E) MDA and (F) Proline of the salt-treated groups (priming, priming + shock, and shock)

Tukey's Multiple Comparisons Test was conducted to ascertain the significant difference between means ( $n=3$ ) at a significant level of  $P < 0.05$  and represented as mean  $\pm$  standard deviation (SD).

From a broad view, the relationship between physiological parameters, as translated in heatmap representation, revealed that the priming, priming+shock, and control groups were clustered together while the shock group was separated from the other groups. Above all, the cultivar 'Cv.7' appeared to have the highest proline content within the thirteen cultivars (Figure 3B).

**Table 4.** Pearson correlation analysis between the physiological traits (proline, MDA, total chlorophyll and carotenoids) data

	Proline	MDA	Total chlorophyll	Carotenoids
Proline	1	0.636**	-0.136	-0.340*
MDA	0.636**	1	-0.485**	-0.550**
Total chlorophyll	-0.136	-0.485**	1	0.655**
Carotenoids	-0.340*	-0.550**	0.655**	1

\*\* . Correlation is significant at the 0.01 level (2-tailed).

\* . Correlation is significant at the 0.05 level (2-tailed).

#### *Salt-responsive genes*

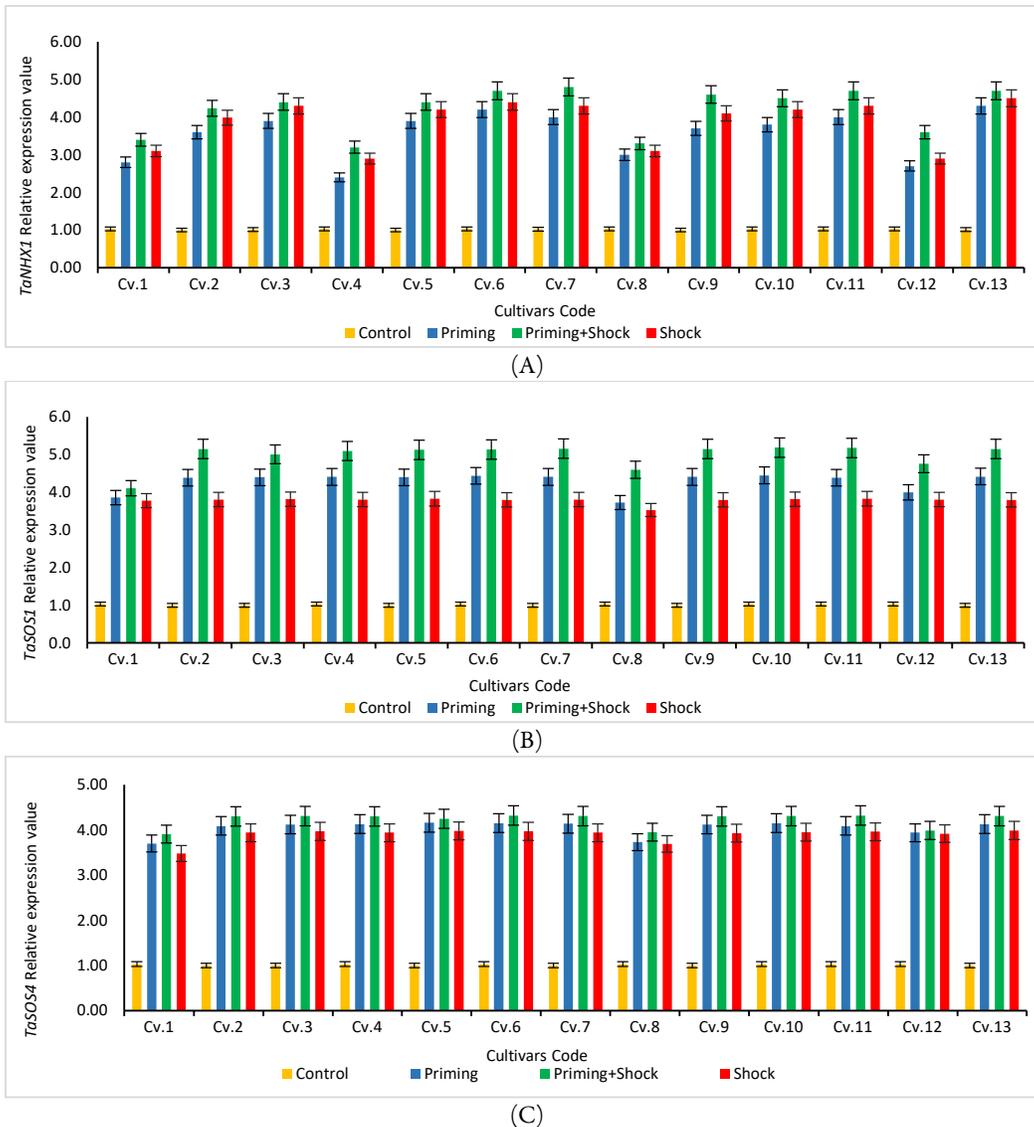
Quantitative Real-Time PCR (qRT-PCR) analysis was carried out to estimate the transcript levels of some of the salt-responsive genes, particularly genes participating in the ion transport process to achieve ion homeostasis. The expression patterns of the six antiporter genes named; *TaNHX1*, *TaSOS1*, *TaSOS4*, *TaHKT1*, *TaHKT2* and *TaAKT1* have quantitatively estimated in the leaves of wheat plants/groups subjected to long-term salinity stress as well as untreated control plants.

According to the expression levels of the *TaNHX1* gene, it was upregulated significantly in the salt priming group compared to the control group. Meanwhile, it was upregulated in the salt-pretreated group (priming+shock) compared to the non-pretreated group (shock only). Nevertheless, it was noticed that its expression level in the non-pretreated group (shock only) was higher than that in the salt priming group (Figure 5A).

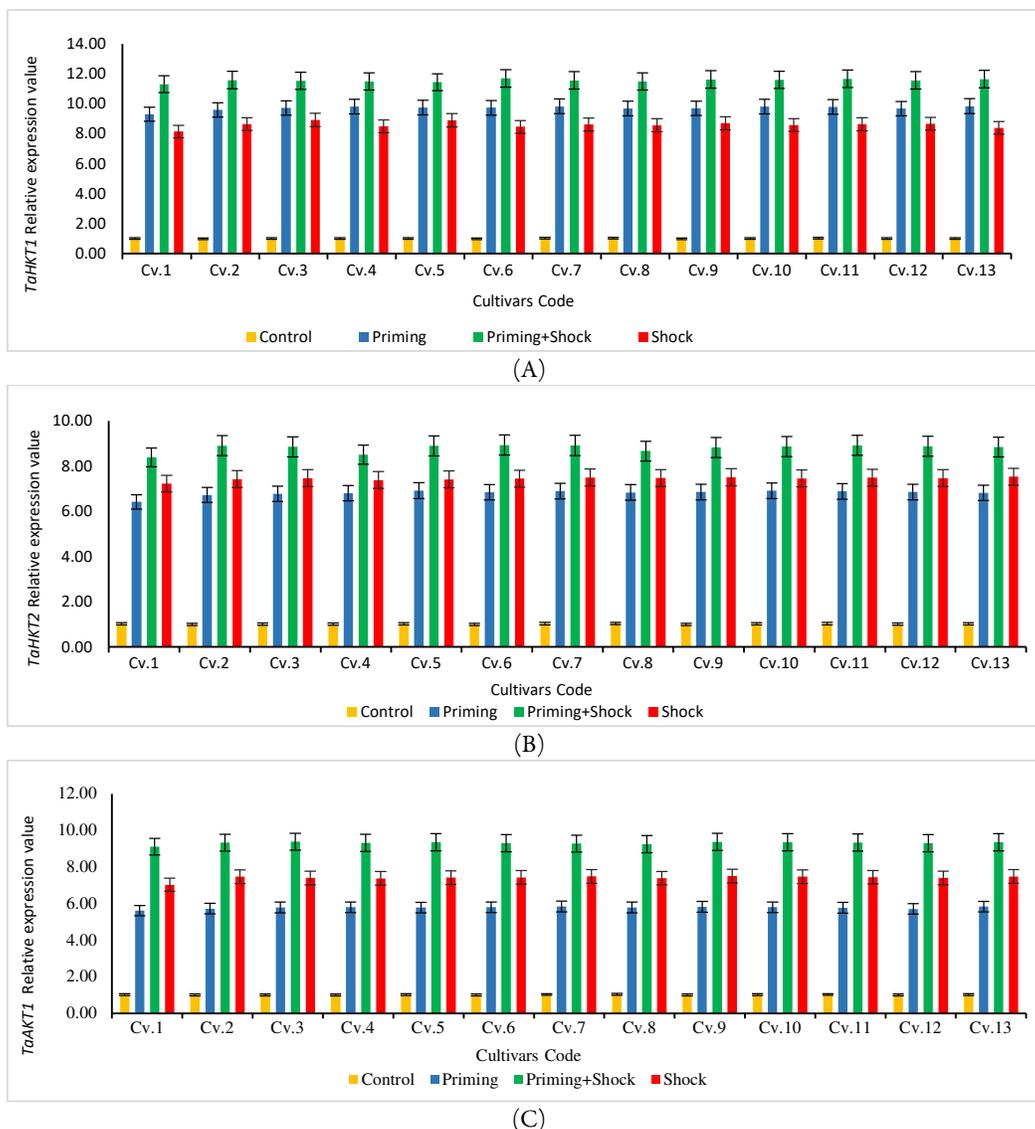
Concerning the expression levels of the Salt Overly Sensitive (SOS) pathway-related genes (*TaSOS1* and *TaSOS4* genes) in response to long-term treatment of salinity stress, we found that both genes showed a significant increase, especially in the salt priming group compared to the control group. Both genes also showed an increased expression level in the salt-pretreated group (priming+shock) compared to the non-pretreated group (shock only). However, this increase was significant in the expression level of *TaSOS1* and was not significant in the expression level of *TaSOS4* (Figure 5B-C).

Regarding *TaHKT1* and *TaHKT2* genes, the qRT-PCR results revealed a significant rise in their expression levels, especially in the salt priming group compared to the control. It was also significantly increased in the salt-pretreated group (priming+shock) compared to the non-pretreated group (shock only). Notably, the expression level of the *TaHKT2* was higher in the non-pretreated group (shock only) than in the salt priming group (priming only) (Figure 6A-B).

The expression level of *TaAKT1* was also significantly raised in the salt priming group (priming only) compared to the control group. Moreover, in the salt-pretreated group (priming+shock), the *TaAKT1* expression level showed a significant increase compared to the non-pretreated group (shock only) (Figure 6C).



**Figure 5.** Expression analysis of (A) *TaNHX1*, (B) *TaSOS1* and (C) *TaSOS4* antiporter gene in leaf tissues of the 13 wheat cultivars under the control, priming, priming + shock and shock conditions. Tukey's Multiple Comparisons Test was conducted to ascertain the significant difference between means (n=3) at a significant level of  $P < 0.05$  and represented as mean  $\pm$  standard deviation (SD).



**Figure 6.** Expression analysis of (A) *TaHKT1*, (B) *TaHKT2* and (C) *TaAKT1* antiporter gene in leaf tissues of the 13 wheat cultivars under the control, priming, priming + shock and shock conditions. Tukey's Multiple Comparisons Test was conducted to ascertain the significant difference between means ( $n=3$ ) at a significant level of  $P<0.05$  and represented as mean  $\pm$  standard deviation (SD).

## Discussion

Wheat (*Triticum aestivum* L.) is one of the world's most frequently consumed crop plants, which feeds an immense number of people. Attention in studying salt stress effects is overgrowing because salinity is now a major environmental factor limiting crop production (Alshehri *et al.*, 2020). Salt stress inhibits photosynthetic activity and reduces plant growth by inducing osmotic stress and ionic toxicity (Chavez and Oliveira, 2004; Tari, 2016). Numerous studies demonstrated that salt stress's osmotic and ionic components represent primary and secondary phases of the stress, respectively, where plants react to each component at different times (Shavrukov, 2013). The ionic effect is a continuous, long-term effect of cumulative processes since it is dependent on the intracellular salt ion levels, which increase with the duration of salinity stress. Therefore,

depending on the NaCl application method, whether in a single step or gradual, plants may experience either salt shock or salt stress, respectively.

Meanwhile, salt stress raises toxic ions concentration in plant cells, resulted in ion homeostasis disruption and consequently oxidative damage with excess production of reactive oxygen species (ROS) (Sani *et al.*, 2013); salt priming proved to help plants acclimation to lethal salinity by improving osmotic adjustment and attenuating the ionic toxicity harmful effects (Djanaguiraman *et al.*, 2018). Several approaches have been introduced to enhance the tolerance of plants against salinity, such as; osmo-priming with chemical compounds (polyethylene glycol (PEG), KNO<sub>3</sub>, K<sub>3</sub>PO<sub>4</sub>, MgSO<sub>4</sub>, KCl, and CaCl<sub>2</sub>), seed priming and salt priming (or gradual exposure of NaCl) (Yan, 2015). Many studies investigated the salt-induced priming approach for improving the salt tolerance of Sweet sorghum, Faba bean, and wheat (Yan, 2015; Qados, 2011).

To fulfill our aim, we designed our study to reveal the differences between the gradual exposure of salt stress (salt priming) and the sudden application of salt stress (salt shock) compared to the control (irrigated with tap water) and their impact on improving the salt tolerance of different wheat cultivars. Also, we investigated the effect of the salt priming without further salt shock compared to the control. Thirteen wheat cultivars from two countries (Egypt and Saudi Arabia) studied to compare Egyptian cultivars' performance versus the Saudi cultivars under our developed salt-induced priming approach. Also, we investigated the long-term salt stress effect on plant productivity by evaluating a robust set of agronomic traits, physiological parameters, and gene expression of six salt-responsive genes.

The harmful effect of salinity on the yield component traits was significant, depending on the salinity level and time of application (Qados, 2011). A close inspection of our obtained results revealed that the largest decrease in the total yield/plant, 1000 kernels weight, spike length, number of kernel/spike, number of spikelets/spike, shoot dry weight, peduncle length, and plant height traits were noted in the non-pretreated group (shock only) compared to the control. This performance might be due to the severe harmful effect of the sudden application of salinity stress. Furthermore, the salt-pretreated group (priming+shock) recorded a downward trend of decrease for the yield component traits than the non-pretreated group (shock only), supporting the hypothesis that salt-induced priming application before the shock seemed to provide a kind of salt-acclimatization. More precisely, the long-term application of salinity stress revealed an inverse relationship between salt concentrations and plants' productivity (Qados, 2011). Another previous supporting study reported that the shock-treated wheat plants experienced a higher stress level than the salt-induced primed in durum wheat (Almansouri *et al.*, 1999).

The effect of the application of sudden and gradual exposure of salt stress on the content of the photosynthetic pigments disclosed an inverse relationship between the salt-stressed groups and the photosynthetic pigments. The osmotic and ionic stresses and the loss in essential ions imposed by salinity resulted in significant disturbances in the photosynthetic pigments (El-Hendawy *et al.*, 2019). The photosynthetic pigments were declined by increasing salinity (negatively correlated with the salinity conditions) (Pervaiz *et al.*, 2002). The reduction in photosynthetic pigments might be due to the enhancement of chlorophyllase activity under salt stress conditions or the reduction in de novo chlorophyll synthesis (Hasson *et al.*, 1983). Our results revealed that wheat plants under salt stress seemed to have "decreased" photosynthetic pigments values compared to the control group, supporting the previous results. The salt-induced priming approach's effect appeared to be superior for improving the salt tolerance of wheat plants; the salt-pretreated group (priming+shock) was outperforming the non-pretreated group (shock only) in the decrease of the photosynthetic pigments' contents. Our results were supported by the study derived by Qados *et al.* (2011) as they revealed that salt stress was an inhibiting factor for the formation of carotenoids inside the stressed plants.

As a consequence of salt stress development within a plant, all the major processes such as protein synthesis, lipid metabolisms, energy, and photosynthesis are severely affected (Parvaiz and Satyawati, 2008). Salinity tolerance is frequently attributed to plants' ability to accumulate low MDA content as a stress marker, indicating that they do not suffer from a high oxidative stress condition (negatively correlated with salt tolerance) (Kumar *et al.*, 2017). Our results confirmed the negative correlation between the MDA content and

salt tolerance; all the stressed groups (priming, priming+shock and shock) showed increased MDA content compared to the control group. However, the elite salt-pretreated group (priming+shock) was outperforming the non-pretreated group (shock only) with a decreased MDA contents, supporting better salt tolerance effect of the salt-induced priming approach. Our results agreed with the study conducted by Zou *et al.* (2016), in which they recorded an increase in the MDA accumulation by approximately 63% after ten days of salt stress conditions. For the cultivars Cv.6, Cv.12, and Cv.13, there was a notable difference between the priming+shock and the shock groups in the percentage relative to the control group, supporting the positive effect of the salt-induced priming approach on enhancing salt tolerance of the wheat plants. The general trend of decreased contents for the salt-pretreated group (priming+shock) than the non-pretreated group (shock only) when compared to the control group for all the thirteen cultivars supported the effect of the salt-induced priming approach on enhancing salt tolerance of the wheat plants.

Proline accumulation is a well-known mechanism that evolved to cope with the drought or salinity stress in several plant species (Parvaiz and Satyawati, 2008). Proline plays a crucial role in protecting the subcellular structures and mediating osmotic adjustment in stressed conditions (Rao *et al.*, 2013). Our results revealed that the salt-pretreated group (priming+shock) seemed to be more salt-tolerant than the non-pretreated group (shock only; which is more susceptible to salt stress), supporting the positive effect of the salt-induced priming approach in improving the salt tolerance of wheat plants. Some cultivars recorded significant values; for the cultivars Cv.5, Cv.6, Cv.7, Cv.12, and Cv.13 the difference between the salt-pretreated group (priming+shock) and the non-pretreated group (shock only) was highly significant. Besides, Kanawapee *et al.* (2013) work on rice supported that the highly susceptible cultivars accumulated the highest proline level than the tolerant cultivars under salt stress.

The plant's ability to Na<sup>+</sup> compartmentalization into vacuoles provides an efficient mechanism to deal with the toxic effect of Na<sup>+</sup> in the cell cytosol level (Brini *et al.*, 2007). The NHX and SOS gene-families transporters have been reported in wheat (Brini *et al.*, 2005; Xu *et al.*, 2013). These families act as Na<sup>+</sup>/H<sup>+</sup> antiporter at the vacuolar level by transporting the Na<sup>+</sup> ions driven by the electrochemical proton gradient (Gaxiola *et al.*, 1999). Higher expression of these endogenous genes reflects vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters' levels and is significantly correlated with salinity tolerance in wheat genotypes (Saqip *et al.*, 2005; Benderradji *et al.*, 2011; Cuin *et al.*, 2011). Our results revealed that the expression of *TaNHX1* was upregulated in the salt-treated groups (priming, priming+shock and shock) compared to the control. Notably, the salt-pretreated group (priming+shock) was outperforming the non-pretreated group (shock only) by boosting the *TaNHX1* expression levels, supporting the salt-induced priming effect approach in enhancing the salt tolerance levels in wheat plants under salt-stress conditions. The results disclosed an agreement with the previous work of Zeeshan *et al.* (2020); as they treated the high tolerant wheat cultivar (Suntop) and the sensitive wheat cultivar (Sunmate) with 100 mM NaCl. They found that the expression level of the NHX1 gene was upregulated in Suntop (tolerant cultivar) while downregulated in Sunmate (sensitive cultivar). Also, its reported a 28-fold increment in the expression of the *TaNHX1* gene in the leaves of Cv. Kh65 (salt-tolerant), while it was about a 4-fold increase in Cv. HD2009 (sensitive) (Rana *et al.*, 2016).

Besides, the SOS signaling pathway is verified to have a vital regulatory role in salt tolerance either directly or indirectly, through controlling Na<sup>+</sup> ion homeostasis and mitigate osmotic stress caused by extreme salt conditions (Hasegawa *et al.*, 2000; Zhu, 2000). Among the SOS gene family, SOS1 (a trans-membrane Na<sup>+</sup>/H<sup>+</sup> antiporter) and SOS4 [a cytoplasmic pyridoxal (PL) kinase] genes were reported to play a critical regulatory role in salt tolerance. Our qRT-PCR results showed that the *TaSOS1* and *TaSOS4* genes generally recorded a significant up-regulation expressional levels between the salt-treated groups (priming, priming+shock and shock) compared to control. For *TaSOS1*, the expression levels exhibited significant differences among the salt-treated groups (priming, priming+shock and shock). Meanwhile, no significant differences were noted in the *TaSOS4* gene between the salt-treated groups (priming, priming+shock and shock). These consistent expression levels might be because *TaSOS4* is apparently involved in salt tolerance but has not been recognized as part of the SOS1, 2, and 3 pathways. Our results were in parallel with Liu *et al.*

(2019) work, as they studied the expression level of *TaSOS1* and *TaSOS4*. Their study found a significant difference in the expression level of the *TaSOS1* gene compared to control, while for *TaSOS4*, there was no significant difference. Likewise, Ahmadi *et al.* (2020) proved that salinity stress increased the relative expression of the *TaSOS1* gene in several ancestral and domesticated wheat genotypes.

High-affinity Potassium Transporters (HKTs) belong to an influential class of integral membrane proteins (IMPs) that promote cation transport across plant cells' plasma membranes. The HKT protein family is critical for salinity tolerance in commercially important crop species, particularly in wheat, by excluding Na<sup>+</sup> ions from sensitive shoot tissues in plants. Among the high-affinity K transporters (HKTs) gene family, *TaHKT1* and *TaHKT2* are necessary transporters that display specificity for K<sup>+</sup> over Na<sup>+</sup> (Assaha *et al.*, 2017; Kosová *et al.*, 2013). Our results for the *TaHKT1* and *TaHKT2* genes expression levels revealed significantly raise values in the salt-pretreated group (priming+shock) compared to the non-pretreated group (shock only). In agreement with our results, Ahmadi *et al.* (2020) recorded a significant increase in the *TaHKT1* expressional level by approximately 25-fold under salinity stress.

On the other hand, Wheat *TaAKT1* functions as a potassium ion transporter, the inward rectifier K<sup>+</sup> channel (AKT1) is considered an essential pathway for the uptake of K<sup>+</sup> in root cell (Wang and Wu, 2013). Our finding showed that the expression level of the *TaAKT1* gene in the salt-pretreated group (priming+shock) was higher than the non-pretreated group (shock only). This finding was in complete agreement with Zeeshan *et al.* (2020), in which they found that the expression of the *TaAKT1* gene was significantly upregulated under salinity stress in the tolerant wheat cultivar ('Suntop') while downregulated in the sensitive wheat cultivar ('Sunmate').

## Conclusions

The salt-induced priming approach improved salt acclimation capacity in bread wheat by enhancing osmotic balancing and mitigating ionic toxicity. Noticeably, all results obtained in this study (agronomic, physiological, and gene expression) presented strong evidence for the positive effects of the long-term salt-induced priming approach to increase wheat productivity. Our findings indicated that exposing wheat plants to a smart salt-priming system enhances the survival possibility under saline conditions and this approach could be successfully applied in exploiting coastal saline land.

## Authors' Contributions

Conceptualization, MAMA.; methodology, TKA and HA; software, MAMA; OA; TKA, HSE, and MAA; validation, TKA and HA; formal analysis, MAMA, OA, ME, MAA; investigation, MAMA, TKA and HA; resources, OA, MAA and MAMA; data curation, TK and MAMA.; writing-original draft preparation, TKA, HA, ME, HSE,OA, MAA and MAMA; writing-review and editing, MAMA; visualization, MAMA; TKA and OA; supervision, MAMA; project administration, MAMA and OA; funding acquisition, OA and MAMA. All authors read and approved the final manuscript.

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### Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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