J. Soil Sci. and Agric. Eng., Mansoura Univ., Vol. 5 (4): 455 - 470, 2014

HYDROGEN PEROXIDE AS A PRIMING AGENT FOR ALLEVIATING SOIL SALINITY STRESS ON WHEAT (*Triticum aestivum*) SEEDLINGS.

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ABSTRACT

This work investigated the potential of two concentrations of H_2O_2 (0.10 and 0.20 mM) applied through irrigation water, as a priming factor, in reducing the detrimental effects of soil salinity stress on wheat (*Triticum aestivum L.*). Two wheat genotypes, Gemaiza 9 (G9) as a moderately salt sensitive and Sakha 93 (S93) as a salt tolerant, were grown in pots containing a saline clay soil (EC_e of 7.35 dS/m) under greenhouse conditions for 45 days. The obtained results showed that H_2O_2 application at 0.10 and 0.20 mM stimulated the germination of G9 genotype by 10 and 20%, respectively. Both fresh and dry shoot weights of G9 had increased by 9.92 and 7.70 and 8.52 and 8.2% for 0.1 and 0.2 mM H_2O_2 treated plants, respectively, as compared to control treatment. In contrast, In S93 genotype, negative effects on germination and fresh and dry weight were recorded. The irrigation with 0.1mM H_2O_2-treated water markedly increased the tillers to about 100% and 22.2% in G9 and S93, respectively, while 0.2mM H_2O_2 treatment increased the tillers number to 28.6% more in G9 and decreased it 16.7% in S93. Addition of 0.2 mM H_2O_2 had a negative effect on chlorophyll A content in both cultivars. In G9, chlorophyll B and carotene content were promoted particularly by 0.2mM H_2O_2. Significant decreases in Na^+ content in both cultivars were observed with H_2O_2 application while potassium was not markedly affected. The K^+/Na^+ ratio of G9 was lower than that in S93 in all treatments and tended to increase in both genotypes with H_2O_2 applications. It can be conducted that application of H_2O_2 as a physiological priming factor may play a significant role in growth improvement of moderately salt sensitive wheat genotypes such as G9 through promotion of Chlorophyll B synthesis and reduction of Na^+ content.

INTRODUCTION

Soil salinity, one of the most severe abiotic stresses, limits the production of nearly over 6% of the world's land and 20% of irrigated land which represent about 15% of total cultivated areas and negatively affects crop production worldwide (Hasanuzzaman *et al.,* 2012). Plants are frequently exposed to adverse environmental conditions, termed abiotic stresses such as salinity, drought, heat, cold, flooding, heavy metals, ozone, UV radiation, etc. therefore, they pose serious threats to the sustainability of crop yield (Bhatnagar-Mathur *et al.* 2008) . Abiotic stresses remain the greatest constraint to crop production worldwide. It leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang *et al.* 2001). It has been estimated that more than 50% of yield reduction is the direct result of abiotic stresses (Acquaah 2007). However, the rapidity and efficiency of these responses may be decisive for the viability of the given species. Poor

germination and poor seedling establishment are the results of soil salinity, which adversely affects growth and development of crop plants and results low agricultural production (Garg and Gupta 1997). The adverse effects of salinity have been attributed to an increase in sodium (Na^+) and chloride (Cl^-) ions and hence these ions produce the critical conditions for plant survival by intercepting different plant mechanisms. Both Na^+ and Cl^- produce many physiological disorders in plants (Mahajan and Tuteja, 2005). A plant's response to salt stress depends on the genotype, developmental stage, as well as the intensity and duration of the stress. The outcome of these effects may cause the disorganization of cellular membranes, inhibit photosynthesis, generate toxic metabolites and decline nutrient absorption, ultimately leading to plant death (Mahajan and Tuteja 2005). In general, the response of a crop plant to salinity is reduced growth (Tavakkoli *et al.* 2011). Osmotic stress due to salinity leads to a slow growth rate and developmental characteristics such as vegetative development, net assimilation capacity, leaf expansion rate and leaf area index (Zheng *et al.* 2008 ; Hasanuzzaman *et al.* 2009). A reduction in photosynthesis is also one of the most conspicuous effects of salinity stress (Leisner *et al.* 2010 ; Raziuddin *et al.* 2011).

Wheat is grown in all types of soils and is classified as a moderate to salt tolerant crop (Mass and Hoffman, 1977). The effects of salinity at seedling stage of wheat range from reduction in germination percentage, fresh and dry weight of shoots and roots to the uptake of various nutrient ions. It is thought that the depressive effect of salinity on germination could be related to a decline in endogenous levels of hormones (Afzal *et al.,* 2006).

Hydrogen peroxide (H_2O_2), is one of the reactive oxygen species, plays two divergent roles in plants: at low concentrations, it acts as signaling molecule for the activation of defense responses under stresses, whereas at high concentrations, it causes exacerbating damage to cellular components (Hasanuzzaman *et al.,* 2012).

Several reports confirmed that enhanced antioxidant defense combats oxidative stress induced by abiotic stressors like salinity (Hasanuzzaman *et al.,* 2011a,b; Hossain *et al.,* 2011) and drought (Selote and Khanna-Chopra, 2010; Hasanuzzaman and Fujita, 2011)**.** Addition of H_2O_2 to the nutrient solution induces salt tolerance by enhanced activities of antioxidants and reduced peroxidation of membrane lipids in leaves and roots of maize as an acclimation response (Azevedo Neto et al., 2005). Several research works had been done on the use of H_2O_2 in seed pretreatments to alleviate the abiotic salt stress (Çavusoglu and Kabar, 2010; He et al., 2009; Abdul Wahida et al., 2007; Hameed *et al.,* 2004). To date, there is limited information on the use of H_2O_2, a stress signaling molecule for crop growth treatments. Here we investigate the effects of H_2O_2 application, as priming factor, with irrigation water on salt tolerance of winter wheat seedlings of salt-sensitive and salt-tolerant genotypes through studying of several growth and physiological parameters.

MATERIALS AND METHODS

Greenhouse growth experiment Two wheat (*Tritium aestivum* L.) cultivars; Gemaiza 9 (G9) and Sakha 93 (S93) were obtained from Crop Research Institute, Agricultural Research Center, MALR, Giza, Egypt. The selection of these two cultivars is based on the classification of wheat with respect to salt tolerance, where G9 and S93 are classified as salt sensitive and salt tolerant, respectively (El-Hendawy et al., 2005). The seeds were surface sterilized with 0.1 % (m/v) $HgCl_2$ for 10 min. then washed several times by distilled water (Abdul Galeel et al., 2008). Under greenhouse conditions, ten dried seeds were sown in plastic pots containing one kg of an air-dried saline alluvial soil. The main physic-chemical properties of the used experimental soil are presented in Table 1. Before seeds sowing, one-third of recommended doses of phosphorus (45 kg P_2O_5/Fed.) and potassium (50 kg K_2O/Fed.) in the forms of single-super phosphate (15.5% P_2O_5) and potassium sulfate (50% K_2O) were added and mixed with soil. The pots were irrigated

Table(1).The main physico-chemical properties of soil used in the study.

Property	Value
Paricle size distribution:	
Sand (%)	6.21
Silt (%)	17.20
Clay (%)	76.59
Soil texture:	Clay
Total carbonate (%)	3.98
Cation exchange capacity (CEC, cmol/kg)	55.61
Electrical Conductivity (EC, dS/m)	7.37
pH	7.20
Water soluble cations (meq/L)	
Ca^{2+}	46.40
Mg^{2+}	37.60
Na^{+}	15.00
K^{+}	4.00
SAR	2.31
Water soluble anions (meq/L)	
Cl^{-}	19.50
HCO_3^{-}	14.40
SO_4^{2-}	34.56

with tap water containing 0.0, 0.1 and 0.2 mM H_2O_2 for the first 30 days of sowing for both cultivars using three replicates to minimize the experimental error. Seeds in pots were watered by the mentioned solution to field capacity and the frequent irrigations were conducted by the compensation of weight loss.

At harvest, number of tillers was recorded for all treatments, and then the fresh weight of plant shoots for each treatment was measured and calculated for each plant. The harvested plants were washed several times

with distilled water, and divided into two parts. One part was oven dried for 24 hours at 70 ^{o}C (Jonse and Case, 1990). The dry weight was recorded and calculated for each plant. From the other part, 0.500 gram of fresh shoot was separated to chlorophyll (a and b) and carotene determinations.

Chlorophyll and carotene content

Extraction and determination of chlorophyll A (ChlA) and B (ChlB) and carotene (CAR) was performed according to the method of Arnon (1949) where 0.500 g of fresh shoot material was ground with 10 ml of 80% acetone and centrifuged at 2500 rpm for 10 minutes at 4°C. This procedure was repeated until the residue became colorless. The extract was transferred to a graduated tube and made up to 10 ml with 80% acetone and assayed immediately. Three milliliters aliquots of the extract were transferred to a cuvette and the absorbance was read at 645, 663 and 480 nm with a spectrophotometer (UV/VIS double beam JENWAY model 6850) against 80% acetone as a blank. Chlorophyll content was calculated using the formula of Arnon and expressed in mg g^{-1} fresh weight (FW) as follow:

Total chlorophyll (mg/ml) = (0.0202) × (A.645) + (0.00802) × (A.663)

ChA (mg/ml) = (0.0127) × (A.663) – (0.00269) × (A.645)

ChB (mg/ml) = (0.0229) × (A.645) – (0.00468) × (A.663)

CAR content was estimated using the formula of Kirk and Allen (1965) and expressed in mg g^{-1} FW.

Carotene = A.480 + (0.114 × A.663 – 0.638 × A.645).

Sodium and potassium content

For the determination of sodium (Na^{+}) and potassium (K^{+}), the oven-dried plant material of 0.500 g of ground and sieved (0.5 mm) shoots were transferred into porcelain crucibles and subjected to dry ashing at 550 ^{o}C for 5 hours in muffle furnace (Chapman and Pratt, 1961). The cold plant ash was dissolved in 5.0 mL 2.0N HCl and complete with distilled water to 50 mL then filtered using Whatmann No. 42 filter paper. The concentrations of Na^{+} and K^{+} were maeasured using flame photometer (JENWAY model PFP7/C) and their contents in plant shoots were calculated.

The obtained results were statistically analyzed and analysis of variance was conducted using Costat under windows software (CoHort Software, *http://www.cohort.com/index.html*)

RESULTS AND DISCUSSION

Effect of H_2O_2 treatment on germination

Figure (1) represents the early growth or seed germination of Gemaiza 9 (G9) and Sakha 93 (S93) under salinity conditions in the absence and presence of H_2O_2. It is important to mention that the salinity of the saturated extract of soil was 7.35 dSm^{-1} (Table 1) and that the level of soil salinity (control) had reduced seed germination percentage to 80% of G9 while did not affect S93 (100% germination). Treatment with both concentrations of H_2O_2 (0.1 and 0.2 mM) improved the growth of G9 by the same percentage but those treatments decreased the germination of S93 by 10 and 20% respectively (Fig. 1). The negative response of S93 to H_2O_2

stimulation may be attributed to that S93 has a defense strategy against the mentioned level of salinity rather than G9 and, therefore, is classified as salinity tolerant cultivar. Recent studies showed that S93 is more salt tolerant than G9 (e.g., Mahmoud, 2009 and El-Hendawy et al., 2005) therefore the germination improvement action of H_2O_2 was effective with the latter cultivar. In the same time, other studies pointed to the role of H_2O_2 in alleviating the hazardous effect of salinity stress on wheat seed germination where H_2O_2 was able to promote germination (Christophe *et al.* 2008) or formation and development of adventitious roots (Li *et al.* 2009). Similar concentrations of H_2O_2 (50 – 200 uM H_2O_2 led to significant increase in the germination rate of the seeds of drought sensitive wheat cultivars whereas and vice versa with drought tolerant ones (Lu et al., 2013).

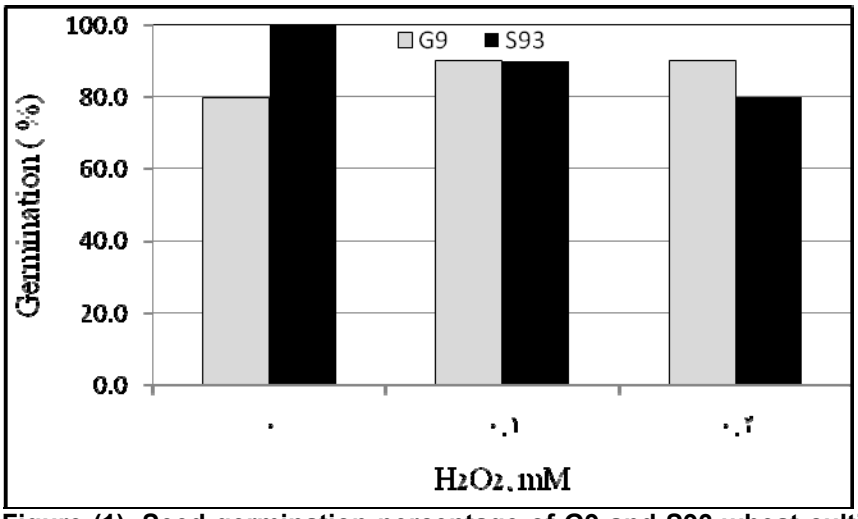

Figure (1). Seed germination percentage of G9 and S93 wheat cultivars as a result of H_2O_2 treatment.

Fresh and dry weight of seedlings

Figure (2) represents the priming effect of H_2O_2 on the fresh weight (FW) and dry weight (DW) of 45-days old shoot of G9 and S93 wheat cultivars. Water treatments with H_2O_2 increased both FW and DW of the shoot of G9 by 9.92 and 7.70 and 8.52 and 8.2 for 0.1 and 0.2 mM H_2O_2 treated plants, respectively, comparing to control treatment. While H_2O_2 application had a negative effect on the FW and DW of S93 shoot except that 0.1 mM H_2O_2 treatment increased the FW by 11.6% (Fig. 2).

Effect of H_2O_2 treatment on tillers formation

Tillering stage in wheat growth cycle starts after 20 days of planting date and continued to the end of experiment. As shown before, the saturation extract of the used soil used for wheat growth had electrical conductivity (EC_e) of 7.37 dSm^{-1} which consequently had an influence on the growth and tillering development. According to Fig. (3), number of tillers, at forty fife days

of growth, reached to 7 and 18 per pot for G9 and S93, respectively, for control treatment. The irrigation with 0.1mM H_2O_2-treated water markedly increased the tillers to about 14 (100%) and 22 (22.2%) per pot in G9 and S93, respectively. While the 0.2mM H_2O_2 treatment increased the tillers number to 9 (28.6% more) in G9 and decreased it to 15 (16.7% less) per pot in S93 (Figure 3). The adverse effect of high concentration of H_2O_2 (0.2 mM) on S93 wheat cultivar as compared to the control (irrigation water without H_2O_2) may be due to the effect of exogenous H_2O_2, as one of the reactive oxygen species (ROS), on salt-tolerant genotypes of wheat such as S93 which is considered as another stress (oxidative stress). The enhanced ROS concentration under salt stress induces phytotoxic reactions such as lipid peroxidation, protein degradation, and DNA mutations (Tanou *et al.* 2009). Furthermore, these physiological disorders may be reflected on the growth parameters such as tillering formation in salt tolerant genotypes (Hasanuzzaman et al., 2012).

Chlorophyll and carotene contents

The ChlA, ChlB and CAR contents in plant shoots were determined after 45 day of plantation. It was observed that, addition of H_2O_2 had a negative effect on ChlA content in both wheat cultivars particularly with 0.2 mM H_2O_2 treatment (Fig. 4). ChlB and CAR content increased in 0.2mM H_2O_2-treated G9 and decreased in S93 (Fig. 4). The data of total chlorophyll content showed that H_2O_2 stimulated the syntheses of chlorophyll in G9 wheat cultivar, while in S93 cultivar, it had not effect (0.1mM level) or negative (0.2mM level) as compared to the H_2O_2-non treated plants (Fig. 5). On the other hand, the calculated ratio of ChlA/ChlB indicated that there was a difference between the two tested wheat cultivars with respect to their response to hydrogen peroxide treatments (Fig. 6). In G9 cultivar, H_2O_2 application led to decrease the ChlA/ChlB ratio while seedlings of S93 showed reverse behavior Fig. (3). The tillers number of G9 and S93 wheat cultivars as a result of H2O2 application with irrigation water.

particularly in 0.2 mM H_2O_2-treated plants. It seems that, in salt sensitive plants, the pretreatment with H_2O_2 promotes the synthesis of ChlB under salt stress and therefore, the ratio of ChlA/ChlB had decreased (Yasmeen et al., 2013). As shown from the obtained results, increasing the concentration of ChlB, and subsequent increasing total chlorophyll and decreasing the ratio of ChlA/ChlB, in the shoot of the moderately-salt tolerant G9 cultivar is considered as an exogenous promoting adaptation mechanism against salt stress in saline soil by H_2O_2.

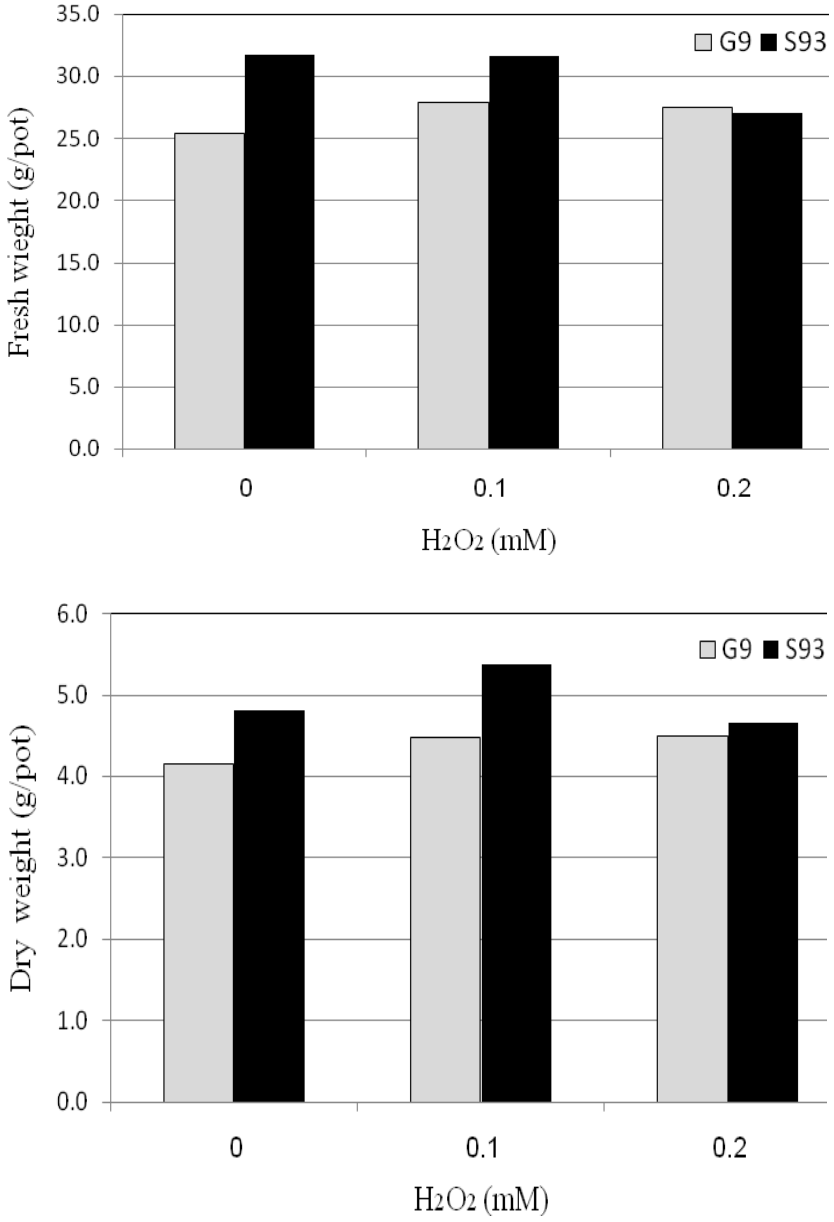

Fig. (2). The fresh and dry weight of 45-days old two wheat cultivars as influenced by H_2O_2 treatments.

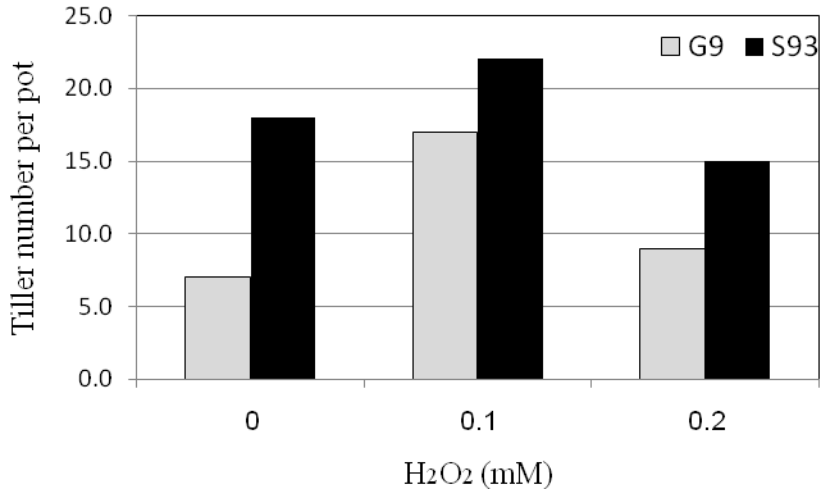

Fig. (3). The tillers number of G9 and S93 wheat cultivars as a result of H2O2 application with irrigation water.

Sodium and potassium uptake

Figure (7) demonstrates the shoot content of Na^+ and K^+ and the shoot K^+/Na^+ ratio in G9 and S93 genotypes of wheat as a result of H_2O_2 treatment. In general, Na^+ content in S93 was less than in G9 in the control and H_2O_2 treatments (Fig. 7 A). Irrigation with H_2O_2-containing water (0.1 and 0.2 mM H_2O_2) led to significant decreases in Na^+ content in both cultivars. Potassium content, on the other hand, was not markedly affected but, in general, it tended to slight decrease with the treatments of H_2O_2 (Fig. 6 B). The ratio of shoot K^+/Na^+ in G9 was lower than in S93 in all treatments (Fig. 7 C) and tended to increased with H_2O_2 applications. It is known that one of the key features of salt-tolerant plant was the ability for cells to maintain high K^+/Na^+ ratio (Tester and Davenport, 2003) as shown with the seedlings of S93 cultivar comparing to G9 (less salt-tolerant genotype) in the control treatments. The H_2O_2 treatments maintained a higher K^+/Na^+ ratio in the salt tolerant genotype (S93) compared with the salt sensitivity seedlings (G9) with both concentrations of hydrogen peroxide. Cuin *et al.*(2003) concluded that high K^+/Na^+ ratio is more important for many species than maintaining a low concentration of Na^+. The current results of Na^+ and K^+ content showed that addition of H_2O_2 with irrigation water, for the first 30 day after sowing, significantly decreased Na^+ (chiefly in the moderately sensitive wheatG9) while approximately maintained the level of K^+ in both genotypes (Fig. 7).

Data of analysis of variance (Table 2) demonstrate that the measured variables, which significantly related to H_2O_2 application, were the tillering development, sodium content and the K^+/Na^+ ratio whereas the fresh and dry weight of shoots, tillering development, Na^+ and K^+/Na^+ ratio were highly related to the type of wheat cultivar.

Fig. (4) Chlorophyll A and B and carotene content in the shoots of 45-day old wheat seedling cultivars; G9 and S93 as influenced by H_2O_2 application with irrigation water.

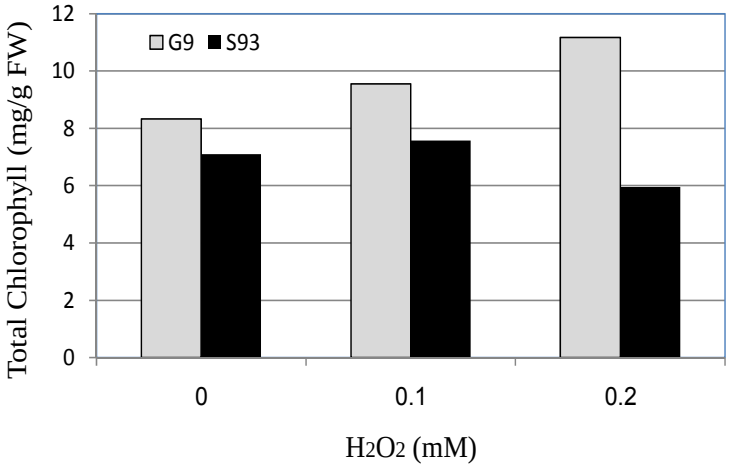

Fig. (5)Total Chlorophyll content in the shoot of 45-day old wheat seedling cultivars G9 and S93 as a function of H_2O_2 application with irrigation water.

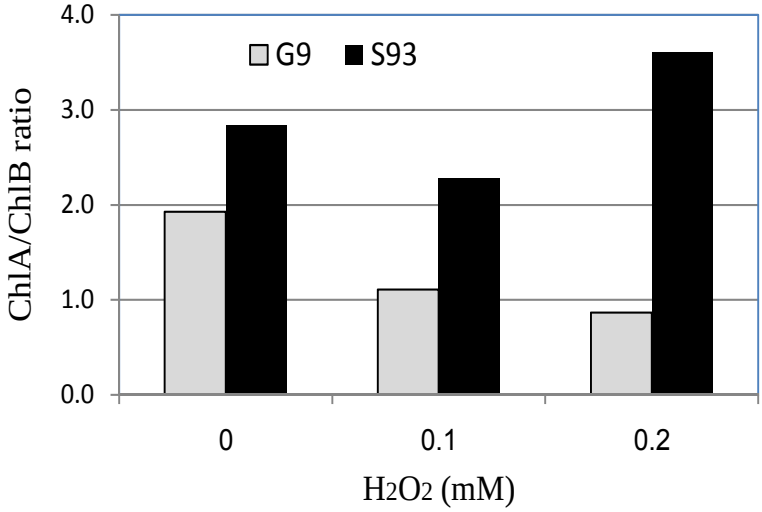

Fig. (6) Chlorophyll A/B ratio in the shoot of 45-day old wheat seedling cultivars G9 and S93 as a function of H_2O_2 application with irrigation water.

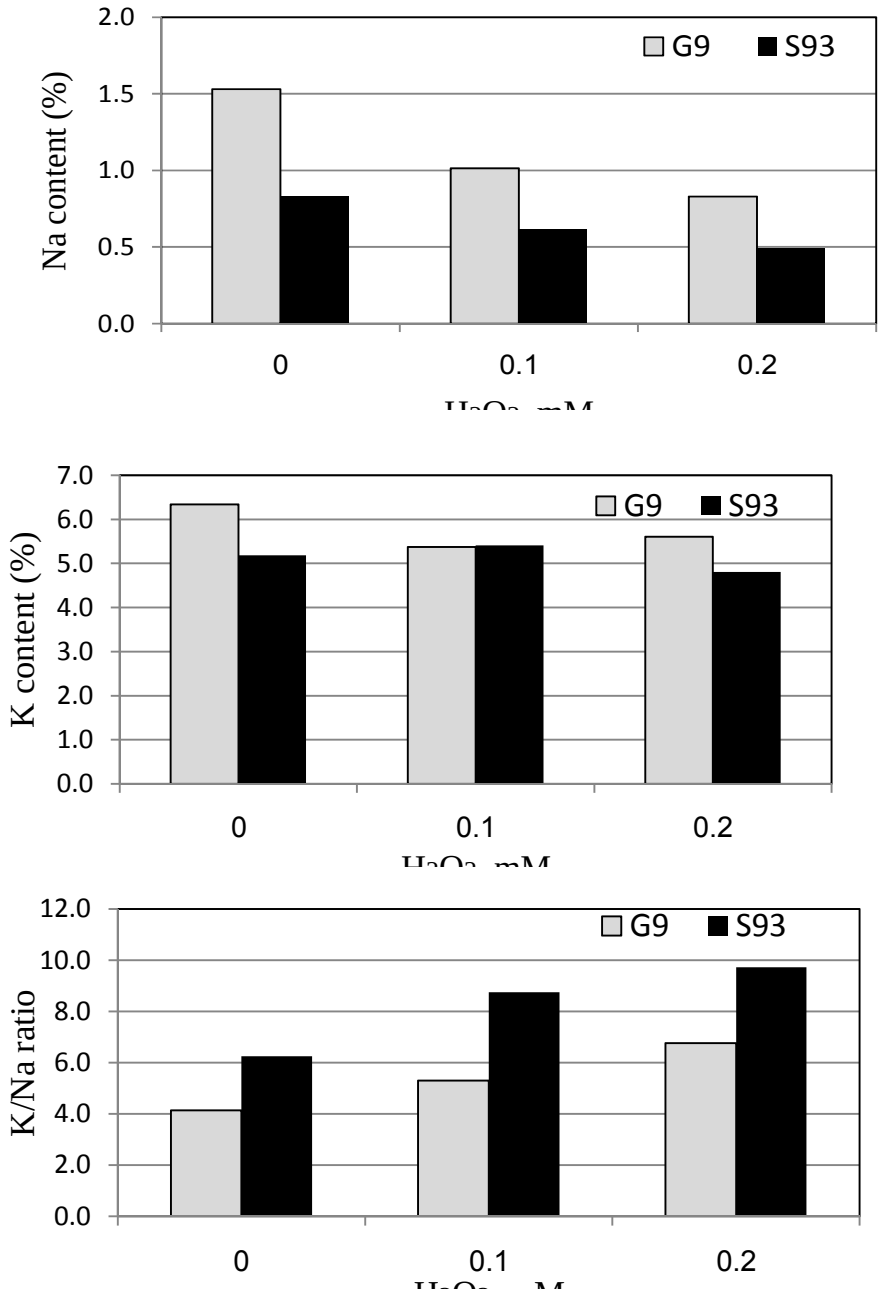

Fig. (7) The concentrations of sodium and potassium and K:Na ratio in shoots of G9 and S93 wheat cultivars after 45 days of treatment with H_2O_2.

Table(2)ANOVA analysis of the effect of H_2O_2 application and wheat cultivars on the means of fresh and dry weight of shoot, tillering, pigments content and sodium and potassium ionic balances.

Factor	Treatment	FW (g/pot)	DW (g/pot)	Tellers (No/pot)	Ch.A (mg/g)	Ch.B (mg/g)	CAR (mg/g)	ChA/B	Na (%)	K (%)
H_2O_2										
	0	28.58 a	4.485 a	12.667 b	5.353 a	2.342 a	4.910 a	2.798 a	1.152 a	5.420 ba
	0.1 mM	29.78 a	4.928 a	18.000 a	5.138 a	2.297 a	4.688 a	2.314 a	.817 b	5.392 a
	0.2 mM	27.28 a	4.565 a	12.333 b	4.922 a	1.528 a	4.970 a	3.284 a	.663 a	5.207 a
	$LSD_{0.05}$	2.807	0.533	3.911	0.726	1.512	1.45	0.88	0.212	1.391
Cultivar										
	G9	29.96 b	4.377 a	10.111 b	5.227 a	2.299 a	5.213 a	2.545 a	1.126 a	5.774 a
	S93	30.11 a	4.942 a	18.556 a	5.052 a	1.812 a	4.499 a	3.052 a	.629 b	4.904 a
	$LSD_{0.05}$	2.292	0.435	3.193	0.592	0.989	1.188	0.719	0.173	1.136
Significance										
	H2O2	ns	ns	*	ns	ns	ns	ns	***	ns
	Cultivar	*	*	***	ns	ns	ns	ns	***	ns
	Interaction	ns	ns	ns	ns	ns	ns	ns	ns	ns

Conclusion

From the obtained results, it can be concluded that

1-Application of H_2O_2 at low concentrations with irrigation water during the germination and seedling development stages of wheat plants can improve the germination percentage and growth of moderately sensitive cultivars such as gemaiza 9.

2-The low concentration treatment of H_2O_2 (0.10 mM) applied with water of irrigation stimulate the tillers number in both moderately salt sensitive (G9) and salt tolerant (S93) wheat cultivars.

3-The higher concentration of applied H_2O_2 (0.20 mM) stimulated G9 genotypes to compensate the reduction in ChlA concentration by increasing the concentration of ChlB and subsequent increasing the total chlorophyll content in the shoot.

4-The irrigation with H_2O_2-containing water significantly decreased Na^+ content and increased K^+/Na^+ ratio in both tested wheat cultivars which reflected on the reduction of Na^+ phytotoxic effect on certain physiological processes, such as plant growth and total chlorophyll content.

5-The obtained results may be a basis for improving abiotic stress tolerance in salinity sensitive and moderately sensitive wheat cultivars.

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فوق أوكسيد الهيدروجين كعامل محفز لتقليل اجهاد ملوحة التربة على بادرات القمح

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تبحث هذه الدراسة امكانية استخدام فوق أوكسيد الهيدروجين مع مياه الرى كعامل تحفيز فسيولوجى يعمل على تقليل الآثار الضارة لملوحة التربة على نمو صنفين من نباتات القمح هما جميزة ٩ (متوسط الحساسية للأملاح) وسخا ٩٣(متحمل للأملاح) فى أصص تحتوى على أرض طينية ملحية (التوصيل الكهربى لمستخلص عجينة الارض ٧.٣٥ ديسيسيمنز / متر) فى الصوبة لمدة ٤٥ يوم. بينت النتائج المتحصل عليها أن اضافة فوق أوكسيد الهيدروجين بتركيزات ٠.١ و ٠.٢ مللى جزيئى فى مياه الرى فى ال٣٠ يوم الأولى من الزراعة عملت على تحفيز الانبات لصنف جميزة ٩ بنسبة ١٠ و ٢٠% على التوالى. كما زاد كل من الوزن الرطب والوزن الجاف فى صنف جميزة ٩ بنسب ٩.٩٢ و ٧.٧ و ٨.٥٢ و ٨.٢% فى النباتات المعاملة بتركيز ٠.١ و ٠.٢ مللى جزيئى على التوالى مقارنة بمعاملة الكنترول. وعلى عكس ذلك، تأثر كل من الانبات والوزن الرطب والجاف سلبا بالنسبة لصنف سخا ٩٣ نتيجة تلك المعاملات. أدى إضافة ٠.١ مللى جزيئى من فوق أوكسيد الهيدروجين الى ماء الرى الى زيادة معنوية فى التفريعات وصلت الى ١٠٠% فى صنف جميزة ٩ و ٢٢% فى سخا ٩٣ بينما أدى الرى بماء محتوى على ٠.٢ مللى جزيئى من فوق أوكسيد الهيدروجين الى زيادة قدرها ٢٨.٦% فى جميزة ٩ وانخفاض قدره ١٦.٧% فى سخا ٩٣ مقارنة بالنباتات غير المعاملة (الكنترول). انخفض تركيز الكلوروفيل (أ) فى كلا الصنفين نتيجة اضافة التركيز المرتفع من فوق أوكسيد الهيدروجين بينما عمل هذا التركيز على تحفيز تخليق مزيد من الكلوروفيل (ب) والكاروتين فى صنف جميزة ٩. أوضحت النتائج أيضا حدوث انخفاض معنوى فى الصوديوم الممتص وعدم تغير فى البوتاسيوم وزيادة كبيرة فى نسبة البوتاسيوم الى الصوديوم فى كلا الصنفين نتيجة المعاملة بفوق أوكسيد الهيدروجين. خلصت الدراسة الى أن اضافة فوق أوكسيد الهيدروجين مع ماء الرى يمكن أن يكون له دور ايجابى فى تحسين ظروف نمو أصناف القمح متوسطة الحساسية لملوحة التربة مثل جميزة ٩ عن طريق تحفيز تخليق كلوروفيل (ب) وخفض امتصاص الصوديوم.

قام بتحكيم البحث

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