Investigation of wheat response to salinity stress can help to better understand the effective defense mechanisms of salinity stress tolerance. For this purpose, biochemical and physiological traits related to salinity tolerance in wheat cultivars were evaluated at Gorgan University of Agricultural Sciences and Natural Resources in 2019. Experimental factors, included two wheat crop cultivars (Sarc and Chinese spring as tolerant and susceptible wheat cultivars, respectively) and sampling time series (zero or control, 24, 48, 72, and 96 h) were examined in a factorial experiment based on a completely randomized design with three replications. Salinity stress was applied with sodium chloride at a concentration of 250 mM to uniform 10-day-old seedlings at the two-leaf stage, followed by sampling of shoot tissue. The studied traits were hydrogen peroxide (H\(_2\)O\(_2\)), chlorophyll a, chlorophyll b, total chlorophyll, chlorophyllase, carotenoids, proline, and total carbohydrates. Results of analysis of variance (ANOVA) indicated significant effects of genotype, time, and interaction of genotype × time (except H\(_2\)O\(_2\)) on all the studied traits. Results of interaction of genotype × time showed although the trend of changes in the studied traits, depending on the type of cultivar and the sampling time were different, but generally, the susceptible Chinese spring cultivar contained higher levels of chlorophyllase and carotenoids than the control time at the end of sampling time and also higher H\(_2\)O\(_2\) levels than the Sarc tolerant cultivar, while the Sarc tolerant cultivar, on the other hand, contained higher levels of chlorophyll a, chlorophyll b, total chlorophyll, and proline than the control time at the end of sampling time and also greater total carbohydrates than the susceptible Chinese spring cultivar. The results confirm the higher capacity of the antioxidant defense system of Sarc tolerant cultivar than the susceptible Chinese spring cultivar. Therefore, the osmolytes of proline and total carbohydrates are reliable for crop screening, particularly wheat crop, in salinity stress studies.

**Keywords**: Sodium chloride, Proline, Chlorophyllase, Chlorophyll, Carotenoids

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**Introduction**

Wheat has nowadays become the most important strategic crop worldwide (Shiri, Tafiti et al., 2019). In Iran, it is also one of the most important cereals (Alipour et al., 2019) and provides 47% of daily calories for people (Hosseini et al., 2007). In Iran, the area under wheat cultivation is 6.70 million hectares (FAO, 2019) while the total area of agricultural lands with different salinities of soil, water or both is estimated to be 3.5 million hectares (Banaei et al., 2004). According to forecasts, more than 50% of the world arable land will be saline by 2050 (Mohamed et al., 2006). A high level of sodium chloride is the main cause of soil salinity in most of these areas (Tejera et al., 2006).

On the other hand, it is estimated that the average crop yield reduction may reach more than 50% in saline areas (Qureshi et al., 2007). In these conditions, there are generally two methods to deal with salinity. The first method is to improve saline soils, which is based on the use of drainage and irrigation systems that are high-cost and require fresh water. The second is the biological method in which tolerance to salinity in plants can be increased by cultivating resistant plants or using physiological information, selection criteria, breeding methods, and biotechnological techniques (Almansouri et al., 2001; Munns, 2002; Blum, 1988).

In general, salinity stress induces various biochemical and physiological responses in plants and affects almost all plant functions from photosynthesis to growth and crop production (Daneshmand & Oluomi, 2014). One of the important biochemical changes that occurs upon exposure of the plant to saline environment is an increase in the production of reactive oxygen species (ROS) (Wang et al., 2003). The types of oxygen free radicals include superoxide (O\(_2^−\)), hydroxyl radical (OH\(^−\)), and hydrogen peroxide (H\(_2\)O\(_2\)) (Sairam & Tyagi, 2004). Elevation of these radicals lead to the oxidation of lipids, changes in the structure of proteins,
inactivation of enzymes, discoloration of chlorophyll, and degradation of nucleic acids (Nayyar & Gupta, 2006).

Plants have a high-performance defense system, including enzymatic and non-enzymatic mechanisms, to deal with induced oxidative stress (Loggini et al., 1999). Carotenoids are considered as non-enzymatic systems (Ozkur et al., 2009) and include key pigments of the antioxidant system in plants being very sensitive to oxidative damage (Kafi et al., 2011). These pigments are involved in neutralization singlet oxygen (Ashraf & Mc Neilly, 2004). In addition to carotenoids, chlorophylls are also affected by salinity stress, so that the degradation of chlorophyll molecule is another damage of oxidative stress (Yasar et al., 2006). Degradation of chloroplast structure and reduction of chlorophyll content are influenced by increased chlorophyllase activity due to altered nitrogen metabolism associated with the production of such compounds as proline that play a role in osmotic regulation (Borzouei et al., 2011). Reduction of leaf total chlorophyll under salinity stress generally results in decreased leaf photosynthetic efficiency and consequently plant growth (Emadi et al., 2009).

In addition to the antioxidant enzymatic defense mechanism, compatible compounds (osmolytes), such as proline and carbohydrates, improve plant tolerance to salinity (Heydari et al., 2010). Accumulation of compatible compounds helps to detoxify ROS, and chaperone-like activities of these compounds maintain and stabilize the structure and function of proteins and cellular structures (Apse & Blumwald, 2002). An increase in proline concentration is the most frequent and constant response observed upon the development of stress (Suriyan & Chalermpol, 2009). As a soluble substance, proline increases cellular osmotic potential, preserves cell turgor, and stabilizes the shape of proteins, thereby protecting the stability of cell membranes (Verslues et al., 2006). Stress-resistant plants have a greater ability to synthesize proline and, consequently, have more membrane stability, which results in less water loss through cell membranes (Valentovic et al., 2006).

Accumulation of soluble sugars as compatible osmolytes also increases the resistance of plants to salinity stress (Setayesh Mehr & Esmaeizadeh Bahabadi, 2013). Degradation and hydrolysis of larger molecules, such as starch, and their conversion into sugar compounds, such as sucrose, and then smaller molecules, such as glucose and fructose, due to salinity stress cause more negativity of water potential in cells and osmotic regulation (Bartels & Sunkar, 2005).

Wheat cultivars respond very differently to salinity stress and the study of defense mechanisms is obviously of paramount importance. The amounts and variations of photosynthetic pigments, H2O2, and compatible compounds were compared and evaluated in the present study to better understand the effect of salinity stress on susceptible and tolerant cultivars of wheat crop.

Materials and Methods

Planting and sampling methods

This research was conducted in the laboratory of the Faculty of Plant Production in Gorgan University of Agricultural Sciences and Natural Resources during 2019. Experimental factors, namely two crop wheat cultivars (Sarc 6 and Chinese spring as tolerant and susceptible wheat cultivars, respectively) as the first factor, and five sampling time series (zero or control, 24, 48, 72, and 96 h) as the second factor were examined in a factorial experiment as a completely randomized design with three replications. To plant and apply salinity stress at the seedling stage, seeds were first disinfected using a solution of sodium hypochlorite and 70% ethanol. The uniformly germinated seeds were then transferred to hydroponic growth conditions using Hoagland's solution (Hoagland & Arnon, 1950). Planting containers were placed in a controlled environment with 16 h light at 25 °C and 8 h darkness at 20 °C. The nutrient solution was changed every three days and its pH was adjusted between 5.5 and 6.5 using sodium hydroxide (NaOH). Salinity stress was applied with NaCl at a concentration of 250 mM to uniform 10-day-old seedlings at the two-leaf stage. Calcium chloride (CaCl2) was added to NaCl solution to maintain a Na/Ca ratio of 10: 1. To measure the traits, leaf samples of each cultivar were harvested in three replications before salinity stress at time zero and then at 24, 48, 72, and 96 h after salinity stress.

Extraction and measurement of H2O2

The amount of H2O2 was measured using a spectrophotometer based on absorbance at 390 nm (Sergiev et al., 1997) and expressed in micromoles per gram of fresh weight.

Extraction and measurement of chlorophyll and carotenoids

Chlorophyll and carotenoids were measured based on the adsorption values of the solutions through spectrophotometry at 480 and 510 nm for carotenoids and 645 and 663 nm for chlorophyll a and b (Arnon, 1949), and calculated using the following formulas:

\[
\text{Chl. a (mg/g FW)} = \frac{12.7 \times (A_{465}) - 2.69 \times (A_{645})}{V/W} \\
\text{Chl. b (mg/g FW)} = \frac{12.9 \times (A_{465}) - 4.68 \times (A_{663})}{V/W} \\
\text{Total Chl. (mg/g FW)} = \frac{20.2 \times (A_{465}) + 8.02 \times (A_{663})}{V/W} \\
\text{Car. (mg/g FW)} = \frac{7.6 \times (A_{480}) - 1.49 \times (A_{510})}{V/W}
\]

In the above equations, A463, A464, A480, and A510 are the absorbance read at 663, 645, 480, and 510 nm, respectively, V is the final volume (ml) of consumed acetone, and W is the weight of fresh plant tissue. The contents of chlorophyll and carotenoids were expressed in mg/g of fresh weight.

Extraction and measurement of chlorophyllase

Chlorophyllase was extracted using the modified method of Fernandez-Lopez et al. (1992), followed by measuring chlorophyllase through calculation of chlorophyllide a spectrophotometrically at a wavelength of 665 nm using an extinction coefficient of 54.1 mmol/cm (Tanaka et al., 1982). The amount of chlorophyllase was then expressed in nanomoles per gram of fresh weight.

Measurement of proline content

Proline content was measured using the method described by Bates (1973). According to this method, the upper phase is harvested from two phases formed in the reaction solution, and finally proline content in the samples was determined quantitatively using a standard curve spectrophotometrically at a wavelength of 520 nm. Proline
content was then expressed in micromoles per gram of fresh weight.

**Extraction and measurement of total carbohydrates**

Total carbohydrate was extracted using the phenol-sulfuric method (Dubois et al., 1956). Accordingly, total carbohydrate was measured spectrophotometrically using glucose as a standard solution based on the absorbance at 490 nm. Total carbohydrate content was then expressed in micromoles per gram of fresh weight.

**Statistical analysis**

Data were analyzed statistically, including analysis of variance (ANOVA) and comparison of mean values of the studied traits, using SAS software. Mean values were compared based on the LSD method and significant differences were considered at levels of 5% and 1%.

**Results and Discussion**

**Hydrogen peroxide**

According to ANOVA results (Table 1), salinity stress had a significant effect on $\text{H}_2\text{O}_2$ contents in the studied cultivars as well as on sampling time series. However, the interaction of genotype and time was not significant.

**Table 1 : Analysis of variance on the studied traits under salinity stress**

<table>
<thead>
<tr>
<th>S.O.V</th>
<th>df</th>
<th>$\text{H}_2\text{O}_2$</th>
<th>Chlorophyll a</th>
<th>Chlorophyll b</th>
<th>Total Chlorophyllase</th>
<th>Carotenoid</th>
<th>Proline</th>
<th>Carbohydrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>1</td>
<td>0.406</td>
<td>0.00032 $a$</td>
<td>0.009 $a$</td>
<td>0.0015 $a$</td>
<td>288.9 $a$</td>
<td>0.000086 $a$</td>
<td>461.4 $a$</td>
</tr>
<tr>
<td>Time</td>
<td>4</td>
<td>8.93 $a$</td>
<td>0.0054 $a$</td>
<td>0.018 $b$</td>
<td>0.0112 $b$</td>
<td>139.6 $a$</td>
<td>0.00064 $a$</td>
<td>142.1 $a$</td>
</tr>
<tr>
<td>Genotype x Time</td>
<td>4</td>
<td>0.038 $a$</td>
<td>0.0009 $a$</td>
<td>0.0019 $b$</td>
<td>0.0050 $b$</td>
<td>3.84 $a$</td>
<td>0.00032 $a$</td>
<td>17.61 $a$</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>0.019</td>
<td>0.000065</td>
<td>0.000066</td>
<td>0.00003 $a$</td>
<td>1.17 $a$</td>
<td>0.000002 $a$</td>
<td>0.923 $a$</td>
</tr>
<tr>
<td>C.V (%)</td>
<td></td>
<td>4.25</td>
<td>4.88</td>
<td>7.08</td>
<td>6.41</td>
<td>4.39</td>
<td>6.49</td>
<td>5.49</td>
</tr>
</tbody>
</table>

ns: not significant; $a$ and $**$: significant at probability levels of 5% and 1%, respectively

**Chlorophyll and chlorophyllase contents**

Based on the results of ANOVA, the amounts of chlorophyll a, chlorophyll b, total chlorophyll, and chlorophyllase in the studied genotypes, sampling times and interactions of genotype and time were affected significantly by the salinity stress. Comparison of mean interactions between chlorophylls a and b contents (Figs. 2 and 3) revealed that the Sarc tolerant cultivar contained the highest chlorophyll a content at 96 h, which was not significantly different from the susceptible Chinese spring cultivar at the same time. The lowest amount belonged to Chinese spring cultivar at 72 h, which was not significantly different from both cultivars at 48 h and also the control time of Chinese spring cultivar. The highest chlorophyll b content belonged to Sarc cultivar at control time (zero) and the lowest content was recorded in Chinese spring cultivar at 72 h after salinity stress, which was not different significantly from Sarc cultivar at 24 h. For total chlorophyll trait, the interaction results showed that the uppermost and lowermost total chlorophyll levels belonged to Sarc and Chinese spring cultivars at 96 h and 72 h after salinity stress, respectively.

**Fig. 1 : Comparison of mean simple effects of genotype (A) and sampling time (B) on $\text{H}_2\text{O}_2$ trait in wheat cultivars**
Evaluation of salinity stress effects on changes in photosynthetic pigments, hydrogen peroxide and osmolytes in sensitive and tolerant cultivars of wheat crop

In general, the results of genotype and sampling time interaction on chlorophyll a, chlorophyll b, and total chlorophyll showed that the application of salinity stress led which was attributed to no regular decrease or increase relative to the control time. The general trend, however, indicated the elevated contents of chlorophyll a, chlorophyll b and total chlorophyll at 96 h after the stress compared with other sampling times, particularly at zero time (except chlorophyll b levels in Sarc cultivar, its value was less than zero, despite an increase in 96 h). Elevation of traits in the Sarc tolerant cultivar was more than that of the sensitive Chinese spring cultivar.

Comparison of mean chlorophyllase interactions revealed that Chinese spring cultivar contained the highest chlorophyllase level at 96 h after stress and the lowest level belonged to Sarc cultivar at 24 h after stress. In general, the results of genotype and sampling time interaction on this trait showed that the general trend of changes in this trait was regular after salinity stress. After a significant reduction of this enzyme at 24 h after stress (compared to time zero), the changes had an increasing trend until the end of sampling and finally the highest amount of enzyme was obtained in both cultivars at 96 h. However, the amount of this enzyme was significantly higher in the susceptible Chinese spring cultivar than the tolerant Sarc cultivar at all the sampling times.

Both chlorophyll a and b are believed to be sensitive to stress (Farooq et al., 2009). Salinity stress leads to changes in the amounts of these molecules in plant cells (Arvin, 2015). Salinity stress causes chloroplast degradation, chlorophyll decomposition, and photosynthetic pigmentation reduction through decreasing the activity of enzymes involved in chlorophyll synthesis (Vieira Santos, 2004), stimulating chlorophyllase production by increasing growth regulators such as abscisic acid and ethylene (Drazkiewicz, 1994), and increasing nitrogen utilization by proline synthesis (Bybordi, 2012). However, the stability of photosynthetic pigments under salinity stress conditions is considered as a Criteria for plant resistance to salinity stress (Sevengor et al., 2011). In addition, chlorophyll concentrations increase in mild salinity stresses and decrease in severe stresses (Nemati et al., 2013).

Researchers believe that plants respond differently to osmotic potential and its effect on the minimum or maximum reduction of photosynthetic pigments during salinity stress (Vojodi Mehrabani et al., 2017). There are currently various reports of decreasing or increasing chlorophyll content in plants under salinity stress. These include decreasing chlorophyll content in wheat (Ehsanzadeh et al., 2009),
safflower (Siddiqi et al., 2009), sugar beet (Emadi et al., 2009), and rice (Kanawapee et al., 2012), and increasing chlorophyll content in wheat (Jam Barandozi et al., 2012), tobacco (Locy et al., 1996), sugar beet (Dadkhah, 2011), and safflower (Karimi et al., 2015) under salinity stress. Movahhed Dehnavy et al. (2004) attributed the reason an increase in chlorophyll is the decreased leaf surface area and accumulation of chlorophyll at a lower leaf surface area, while Borzouei et al., (2011) explained decreased leaf surface area and an increase in chlorophyll content as a stress prevention mechanism. Papp et al. (1983) also reported that leaf thickness increased at all salinity levels and this change in leaf thickness increased chlorophyll levels. In similar results to this study, Sadat Musavizadeh et al. (2018) reported significant effects of genotype, time, and their interactions on chlorophyll a and chlorophyll b contents of rice under salinity stress. They also observed no regular trend of changes in these traits at sampling times of 0, 6, 24, 48, 120, 72, and 168 h after salinity stress, but most of these traits occurred at the final time (168 h) after stress.

On the other hand, the results of this study showed that despite the increased chlorophyllase levels in both tolerant and sensitive cultivars, the chlorophyll content increased at the final time of stress. This indicates the effectiveness of defense and antioxidant mechanisms of wheat and chlorophyll stability in dealing with salinity stress given the decreasing trend of H₂O₂. However, chlorophyll stability is considered as an indicator of plant resistance to salinity stress so that tolerant and sensitive cultivars have higher and lower stability indices, respectively (Mohan et al., 2000).

It should also be emphasized that salinity stress tolerance is not a function of a plant organ or trait, but a result of most plant traits (Akhari Ghogdi et al., 2011). Undoubtedly, several enzymatic and non-enzymatic mechanisms contribute to the resistance or sensitivity of plants to salinity stress (Kordrostami et al., 2016).

Carotenoid content

The results of ANOVA revealed that salinity stress had significant effects on the carotenoid content in the studied genotypes, sampling time, and the interaction of genotype and sampling time. Accordingly, comparison of mean interactions of carotenoid content (Figure 4 A) indicated that the Chinese spring cultivar contained the highest and lowest carotenoid content at 96 and 72 h after stress, respectively. The results of genotype and sampling time interaction on this trait showed that the general trend of changes in this trait was different and opposite in the studied cultivars after applying salinity stress. After a significant reduction of this enzyme in Sarc cultivar at 24 and 48 h after stress (compared to time zero), the changes had an uptrend until the final time of sampling. In Chinese spring cultivar, on the other hand, a significant increase in this enzyme at 24 h after stress (compared to time zero) was followed by declining changes until the penultimate time of sampling. Similarly, Sadat Musavizadeh et al. (2018) reported an increase in rice carotenoid content at final times (120 and 168 h after stress) after a decreasing trend at initial times of sampling. Karimi et al. (2015) reported an increase in carotenoid concentrations in safflower cultivars at different levels of salinity stress, and concluded that elevated carotenoid concentrations was part of the plant defense mechanisms against salinity stress. Jam Barandozi et al. (2012), In the study of salinity resistance of wheat cultivars, reported that carotenoid content increased in some wheat cultivars and decreased in others by applying salinity stress in comparison to control conditions. On the other hand, as fat-soluble antioxidants in chloroplast membranes, carotenoids play an important role in plant processes, including tolerance to oxidative stress (Lovdel et al., 2010). In this study, the susceptible Chinese spring cultivar seems to be more inclined to use this defense mechanism to overcome oxidative stress while being exposed to higher H₂O₂ and chlorophyllase levels than Sarc tolerant cultivar. It is believed that genotypes select different antioxidant activities in response to salinity stress and this difference in defense mechanisms varies not only in different species, but sometimes in the genotypes and cultivars of a single plant species (Dastneshan & Sabokdast, 2020).

Content of osmolytes

According to the results of ANOVA, proline and carbohydrate contents in the studied genotypes and sampling time were affected significantly by salinity stress. However, the interaction of genotype and time was significant on proline but not on total carbohydrates. Comparison of mean interactions for proline content (Fig. 4 B) showed the highest proline content belonged to the Sarc tolerant cultivar at 96 h, which was not significantly different from that of 72 h, and the lowest level was recorded in the Chinese spring cultivar at time zero. In general, the results of genotype and sampling time interaction on proline revealed that salinity stress led to an increasing trend in proline changes of the Sarc tolerant cultivar, which was more regular than that of the sensitive Chinese spring cultivar. However, proline content increased in the Chinese spring cultivar compared to the control time (similar to Sarc cultivar) at the time series, but it decreased after 72 h of stress application. Due to the non-significant interaction between genotype and time on carbohydrate content, simple effects diagrams of genotype and sampling time factors (Fig. 5) showed that carbohydrate content increased from time zero to the final times by applying salinity stress. It reached the uppermost level at 96 h after salinity stress and was less abundant in the susceptible Chinese spring cultivar than in the Sarc tolerant cultivar.

Researchers reported similar results on increased proline (Martin et al., 1993; Heydari et al., 2010) and soluble carbohydrates (Hamada & Khalea, 2010; Farhoudi, 2014) in wheat under salinity stress. In a similar research on rice plant, proline content increased significantly with increasing after stress time and the tolerant cultivar contained higher levels than the sensitive cultivar (Sadat Musavizadeh et al., 2018). Kerepesi and Galiba (2000) stated increased carbohydrate concentrations in wheat seedlings to be a criterion for the selection of salinity-tolerant wheat cultivars. During salinity stress, resistant plants are able to maintain cellular turgescence by producing osmotic compatible compounds such as proline and sugars (Ashraf & Harris, 2004).
Evaluation of salinity stress effects on changes in photosynthetic pigments, hydrogen peroxide and osmolytes in sensitive and tolerant cultivars of wheat crop

Fig. 4 : Comparison of mean interactions of carotenoid (A) and proline (B) contents in wheat cultivars under salinity stress

These osmolytes support plants by detoxification of ROS and stabilization of the quaternary structure of proteins (Chinnusamy et al., 2006). As such, proline is considered as a source of energy, carbon, and nitrogen for damaged tissues during stress (Najafi et al., 2010) while insoluble sugars are broken down and decrease the risk of cellular dehydration through the production of soluble sugars (Parvaiz and Satyawati, 2008).

Conclusion

In this study, salinity stress caused physiological and biochemical changes in wheat cultivars so that it had significant effects on all traits in the studied cultivars and on the sampling time series. According to the results, although tolerant and susceptible genotypes of wheat utilize various defense mechanisms to overcome the effects of exposure to salinity stress, the Sarc tolerant cultivar had a higher capacity, efficiency, and ability to utilize defense mechanisms, in particular the non-enzymatic antioxidant system, than the sensitive Chinese spring cultivar. Therefore, the biomarkers of proline and soluble carbohydrate are reliable for crop screening, particularly wheat crop, in salinity stress studies.

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