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ARTICLE

The response of wildtype and mutant cultivars of soybean to salt stress - comparing vegetative and reproductive phases on the basis of leaf biochemical contents, RWC, and stomatal conductance

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ABSTRACT

Soybean, as a major oilseed crop, is cultivated in many parts of the world including saline-affected regions. A four-replicated factorial experiment based on a completely randomized design with five salinity levels (control, 50, 100, 150, and 200 mM NaCl) and five wild-type and new mutant cultivars of soybean (Williams, Clark, M-4, M-7, and M-9) was done at two vegetative and reproductive phases to study leaf oxidants, antioxidants and osmolytes under salinity stress. The vegetative phase demonstrated higher K\textsuperscript{+}:Na\textsuperscript{+} ratio, cell membrane stability, relative water content (RWC) and stomatal conductance to H\textsubscript{2}O (gs) accompanied with lower oxidative stress, antioxidants and osmolytes contrasting to reproductive phase. At both phenological phases, saline stress stimulated antioxidative defense owing to the enhancement of oxidative stress. The results indicated that mutants possessed a better antioxidative system than their wild-types.

Introduction

Today, considering water shortage, the usage of poor-quality water sources is almost inevitable. In addition to industrial and agricultural wastewater, the sewerage and detergents of spreading cities have salinized a considerable part of underground and surface water sources in many regions. It is clear high-quality water is principally allocated to city consumption in all around the world; hence, crops have to be probably irrigated with saline water in the future.

The major common problem in saline circumstance for plant production is excessive salt concentration causing osmotic stress, reduction in soil water availability, cell dehydration, and toxicity or imbalance of specific solutes (Esan et al. 2017). The end consequence of these effects is the oxidative stress (Munns 2002; Jovelina Da Silva et al. 2017). Superoxide radical (O\textsubscript{2}\textsuperscript{-}) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), are the main reactive oxygen species (ROS) produced within oxidative stress (Munns 2002; Batkova et al. 2008). The worst aspect of oxidative stress is hydroxyl radical (OH\textsuperscript{-}), an extremely dangerous reactive product of these two ROS, initiates the peroxidation of membrane lipids, gene mutations, DNA breakage, chlorophyll degradation, protein destruction, etc. (Dias et al. 2016). Although H\textsubscript{2}O\textsubscript{2} is able to cross biomembranes and attack biomolecules, its reactivity is limited and most of the oxidative damage is a consequence of H\textsubscript{2}O\textsubscript{2} conversion into other more reactive species such as hydroxyl radicals (Dias et al. 2016). Malondialdehyde (MDA)
production, as an indicator of membrane lipid peroxidation, is used to study cell membrane stability (Weisany et al. 2011; Cunha et al. 2016).

Plants have developed a combined antioxidative defense system to alleviate the injuries caused by ROS (Khan et al. 2009). The antioxidative system includes a complex of (1) non-enzymatic low molecular mass antioxidants such as carotenoids, ascorbate, glutathione, α-tocopherols, phenols together with (2) enzymatic antioxidants like superoxide dismutase (SOD, EC 1.15.1.1.), catalase (CAT, EC 1.11.1.6.), peroxidase (POD, EC 1.11.1.7) in addition to enzymes involved in ascorbate-glutathione cycle, viz., ascorbate peroxides (APX, EC 1.11.1.1.) and glutathione reductase (GR, EC 1.6.4.2.) (Batkovka et al. 2008; Dias et al. 2016). There is good evidence that the alleviation of oxidative injuries and salt-tolerance is mostly correlated with an efficient antioxidative defense (Khan et al. 2009).

Superoxide is scavenged by SOD to produce H$_2$O$_2$, which is extremely injurious to the chloroplasts, nucleic acids and proteins. Subsequently, it is eliminated chiefly by POD, CAT and APX (Khan et al. 2009; Islam et al. 2016). POD catalyzes H$_2$O$_2$ to H$_2$O by oxidation of co-substrates like phenolic compounds and/or other antioxidants (Dias et al. 2016). CAT is the key scavenger in peroxisomes, converting H$_2$O$_2$ to H$_2$O and molecular oxygen (Hidri et al. 2016). APX, as the first enzyme of the ascorbate-glutathione cycle, catalyzes the reduction of H$_2$O$_2$ to H$_2$O in association with ascorbate (Asc) as a reductant that has high specificity and affinity with APX (Courtney et al. 2016). It has been reported that APX activity may have a crucial role in the mechanism of salt tolerance in plants (Islam et al. 2016).

GR, the final enzymatic part of the ascorbate-glutathione cycle, catalyzes the NAD(P)H-dependent reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). When APX reduces H$_2$O$_2$ into H$_2$O, Asc is used as an electron donor and malon dehydroascorbate (MDA) is formed (Bashir et al. 2007). Then, malon dehydroascorbate is recycled back to Asc by malon dehydroascorbate reductase (MDAR), which produces dehydroascorbate (DHA) (Islam et al. 2016). DHA is also converted to Asc by dehydroascorbate reductase (DHAR) using reduced glutathione (GSH) as an electron donor and resulting in GSSG formation (Batkovka et al. 2008; Dias et al. 2016). Conversion of GSSG to GSH is done by NAD(P)H-dependent GR (Ahmed et al. 2015). Hence, GR catalyzes reactions that maintain GSH and ascorbate pool in the H$_2$O$_2$ scavenging pathway in chloroplasts (Hidri et al. 2016).

Changes in leaf water potential, relative water content (RWC) and stomatal conductance to H$_2$O (gs) are some of drought impacts, imposed by salinity stress, leaf cells withstand by synthesizing and accumulating organic compounds such as proline, soluble proteins, and soluble sugars (Vaseva et al. 2012; Jovelina Da Silva et al. 2017).

The impact of salinity on the nutrient composition of plant tissues, particularly calcium (Ca$^{2+}$) and potassium (K$^+$) content, has been widely researched, and numerous investigations have confirmed that the detrimental effects of salinity on plant growth may occur through an ionic imbalance, mainly Ca$^{2+}$ and K$^+$ ions (Hussein et al. 2015). In saline soils, plants must maintain relatively great concentrations of Ca$^{2+}$ and K$^+$ in addition to accumulate fewer toxic ions like Na$^+$ and Cl$^-$ for a successful growth (Munns 2002; Cunha et al. 2016). Selective ion uptake by discrimination between chemically similar ions like Na$^+$ and K$^+$ is one of the salt-adaptive strategies in soybean (Umezawa et al. 2000, 2002; Hidri et al. 2016).

Soybean [Glycine max (L.) Merril] as an industrial oil and seed crop is moderately salt-tolerant (Phang et al. 2008). A large abundance of soybean wild-type genotypes and their plentiful derived mutants have enriched the genetic diversity of this valuable crop for different environmental conditions (Phang et al. 2008). Modern cultivars of soybean have been selected mainly for their most important traits such as high yield and good quality; however, their productivity can be severely affected by environmental stresses (Ahmed et al. 2015). There are a few studies about comparing soybean wild-type genotypes and their derived mutants. Williams and Clark are two greatly famous soybean cultivars with several derived commercial mutants. M-4, M-7 and M-9, which are predicted to perform better than their wild-type genotypes, are some mutants of these two famous cultivars. Results of this study are notable as they provide whether the biochemical compounds of wild-type and mutant cultivars differ in severe conditions such as salinity stress. The
present work was, therefore, carried out to screen wild-type and newly released mutant cultivars of soybean for salt tolerance characteristics, based on variability in antioxidative system and to examine the influence of salinity on the major osmolytes and enzymatic antioxidants in some mutants and their wild-type cultivars of soybean.

Materials and methods

Growth conditions

A pot-cultured study was conducted in a climate-controlled greenhouse (30/25 °C day/night temperature; 40/50% day/night RH; 1500 µmol m\(^{-2}\) s\(^{-1}\) midday light, 14:10 h day/night photoperiod) at the College of Agriculture and Natural Resources, University of Tehran (Karaj, Iran), June to November, 2011. Additional lighting was supplied by tungsten (100 W m\(^{-2}\)) and white light fluorescent (23 W m\(^{-2}\)) lamps after eliminating all the artificial sources of illumination during night. A factorial experiment based on completely randomized design with four replications was performed in this research.

Five commercial soybean cultivars including Williams and Clark as the wild-type cultivars, M-4 as the new mutant cultivar of Williams, and M-7 and M-9 as the new mutant cultivars of Clark, were investigated in this assay (Seed & Plant Improvement Institute, Karaj, Iran). As their wild-type cultivars, M-4 is in maturity group III while M-7 and M-9 are in maturity group IV. However, the mutant cultivars are more adapted than their wild-types to cool nights of temperate highland regions (Supplementary information 1).

Saline water containing sodium chloride and calcium chloride (Merck KGaA, Darmstadt, Germany) in 10:1 M ratio (Na\(^{+}\):Ca\(^{+}\)) was applied in five concentrations: control (tap water as irrigation water), 50, 100, 150, and 200 mM NaCl corresponding to electrical conductivities (EC): control (Supplementary information 2), 5, 10, 15, and 20 dS m\(^{-1}\) two weeks after cultivation and persisted up to the end of growing term. In order not to impose a sudden, deathful stress to new-emerged seedlings, 100 mM and higher salinity concentrations were progressively treated through 50 mM NaCl increases every 3 days toward the final saline concentration. The soil ECs, testified after the trial finished, were 14, 47, 87, 132, and 181 mM NaCl, in turn. Soil and water details are exhibited in supplementary information 2.

15 kg plastic bags (30 × 20 cm) with holes at the bottom were filled with soil (Supplementary information 2) and 0.5 kg small gravels. Then, ten rhizobium-inoculated seeds were planted in each pot and the seedlings were thinned to four uniform ones per pot thereafter. The pots were fertilized by 50 and 40 mg N and P kg\(^{-1}\), respectively, while there was no need to K fertilizer according to the soil test.

All measurements were conducted on the youngest fully expanded leaf of three plants in each replication at both vegetative (V5: growing of the fifth node and the fifth trifoliate leaf) and reproductive (R5: beginning of seed filling) phases determined by the Iowa State University of Science and Technology (Ritchie et al. 1985).

\(\text{Na}^{+}\) and \(\text{K}^{+}\) contents, relative water content (RWC), and stomatal conductance to \(\text{H}_2\text{O}\) (gs)

\(\text{Na}^{+}\) and \(\text{K}^{+}\) were determined by flamephotometry according to the method of Wolf (1982). RWC was calculated as: RWC [%] = [(FM – DM)/(TM – DM)] × 100 where FM, DM and TM are abbreviated for fresh matter, dry matter and turgid matter, respectively (Muranaka et al. 2002). The leaf stomatal conductance to \(\text{H}_2\text{O}\) (gs) was measured by a portable leaf porometer (Decagon SC-1, Decagon Devices, Pullman, WA, USA) between 10:00 and 14:00 h on a sunny day.

\(\text{H}_2\text{O}_2\) and MDA contents

To determine \(\text{H}_2\text{O}_2\), leaf samples (0.2 g FW) were homogenized in liquid nitrogen, mixed with trichloroacetic acid (TCA; 2 ml, 0.1 % w/v), and centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant (0.4 ml) was added to ice-cold potassium phosphate buffer (0.4 ml, 10 mM, pH 7.0) and
potassium iodide (0.8 ml, 1 M). The absorbance of the supernatant was measured at 390 nm (Turan & Tripathy 2012).

To determine MDA, leaf samples (0.1 g FW) were homogenized in liquid nitrogen, mixed with TCA (1.5 ml, 20% w/v), and centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant (0.3 ml) was added to thiobarbituric acid reagent (TBA; 1.2 ml 0.5%, w/v), heated at 95 °C for 30 min, cooled, and centrifuged at 10,000 × g for 10 min at 4 °C. The absorbance was read at 532 and 600 nm (Kramer et al. 1991).

**Antioxidative enzyme contents**

For enzymatic assays, frozen leaf samples were homogenized in liquid nitrogen, extracted with ice-cold potassium phosphate buffer (50 mM, pH 7.0), centrifuged at 20,000 × g for 30 min at 4 °C. The supernatant was used as the crude enzyme extracts (Dionisio-Sese & Tobita 1998).

SOD (EC 1.15.1.1) was determined according to Beauchamp and Fridovich (1971). The crude enzyme extract (2.5 ml) was added to the reaction mixture (3 ml) containing potassium phosphate buffer (50 mM, pH 7.0), ethylene diamine tetraacetic acid (EDTA; 0.1 mM), methionine (13 mM), nitro-blue tetrazolium (NBT; 63 μM), and riboflavin (1.3 μM). The reaction was allowed to proceed in tubes covered with a black cloth in a dark room at 25 °C for 15 min, and then illuminated with two 20 W fluorescent tubes at 25 °C for 15 min. The absorbance was read at 560 nm.

POD (EC 1.11.1.7) was measured according to Chandlee and Scandalios (1984). The crude enzyme extract (0.3 ml) was added to the reaction mixture (0.2 ml) consisted of potassium phosphate buffer (50 mM, pH 7.0), guaiacol (1%) and H2O2 (0.4%). The absorbance was measured at 470 nm.

APX (EC 1.11.1.11) was assayed as described by Nakano and Asada (1981). The crude enzyme extract (0.1 ml) was added to the reaction mixture (2.5 ml) containing potassium phosphate buffer (50 mM, pH 7.0), EDTA (0.2 mM), ascorbate (0.5 mM), and H2O2 (0.25 mM). The reaction was started at 25 °C by adding H2O2. The absorbance was recorded at 290 nm for 1 min.

CAT (EC 1.11.1.6) was assayed according to the method of Huang et al. (2004). The crude enzyme extract (0.2 ml) was added to the reaction mixture (2.5 ml) containing potassium phosphate buffer (50 mM, pH 7.0) and H2O2 (10 mM). The absorbance was measured at 240 nm for 3 min.

GR (EC 1.6.4.2) was determined according to Halliwell and Foyer (1978). The crude enzyme extract (0.3 ml) was added to the reaction mixture (1 ml) consisted of Tris-hydrochloric acid (Tris-HCl; 100 mM, pH 7.8), EDTA (21 mM), NADPH (0.005 mM), oxidized glutathione (GSSG; 0.5 mM). NADPH was added to start the reaction. The absorbance was read at 340 nm.

**Osmolyte contents**

To determine free proline, leaf samples (0.5 g FW) were homogenized in sulfosalicylic acid (SSA; 10 ml, 3% w/v), centrifuged at 3000 × g for 5 min at 4 °C. The supernatant (2 ml) was heated in water bath at 100 °C for 1 h after adding ninhydrin (2 ml) and glacial acetic acid (2 ml). Reaction was stopped using an ice bath and the reaction mixture was extracted by toluene (4 ml). The absorbance was read at 520 nm (Bates et al. 1973).

Total soluble proteins were determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard. Leaf samples (0.6 g FW) were homogenized in ice-cold potassium phosphate buffer (12 ml, 50 mM, pH 7.8) containing EDTA (0.1 mM), and centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant (0.02 ml) was homogenized by the reaction solution (3 ml) containing Coomassie brilliant blue G-250 (100 mg l−1), ethanol (50 ml, 95 %) and phosphoric acid (100 ml, 85 %). The absorbance was recorded at 595 nm after 2 min.

Total soluble carbohydrates were determined using the anthrone method (Hewitt 1958). Leaf samples (0.3 g FW) were homogenized in ethanol (5 ml, 95 %) and centrifuged at 4500 × g for 15 min. The supernatant was gathered and the residue was resuspended in ethanol (5 ml, 95 %). This procedure was done four times up to gather 20 ml supernatant, concentrated by heating to
5 ml. The supernatant (50 μl) was added to the anthrone-sulfuric acid solution (3 ml) and distilled water (150 μl), stirred for 5 min, heated in a boiling water bath (100 °C) for 20 min, and cooled in water (4 °C). The absorbance was determined at 625 nm.

Statistics

The data was analyzed according to GLM procedure of SAS 9.1 (SAS Institute, Cary, NC, USA) and Fisher's protected least significant difference (LSD) test was used at P ≤ 0.05 level to define significant differences among means ± SE (n = 4). The significant interactions for K⁺:Na⁺, RWC, gs, SOD, APX, CAT, and GR were calculated and compared using LSD test. The mean values of other traits, which had no significant interactions, were analyzed according to LSD test. All the traits of cultivars' roots under salinity levels between two phenological phases (vegetative phase vs. reproductive phase) were compared using single degree-of-freedom orthogonal contrasts.

Results and discussion

*K⁺:Na⁺ ratio, relative water content (RWC), and stomatal conductance to H₂O (gs)*

Salinity caused a significant decrease in leaf K⁺:Na⁺ ratio of soybean cultivars at both vegetative and reproductive phases; however, the wild-type cultivars showed lower K⁺:Na⁺ ratio in all saline levels (Figure 1). The reproductive phase showed significantly lower K⁺:Na⁺ ratio in comparison with the vegetative phase representing that sodium accumulated in leaves over growing season (Table 1 & Figure 1). K⁺:Na⁺ ratio decreased by 99% of the control in the highest salinity either at vegetative or reproductive phase of all cultivars (Figure 1).

(Ahmed et al. 2015), (Courtney et al. 2016), and (Jovelina Da Silva et al. 2017) observed decreased K⁺ and increased Na⁺ of soybean leaf tissues under increasing salinity. Plant uptake of Na⁺ is related to the level of salinity as high electrical conductivity of the soil contributes to greater Na⁺ accumulation and the type of salinity does not affect Na⁺ uptake (Cunha et al. 2016). Umezawa et al. (2000) confirmed that ion accumulation, for example Na⁺ and Cl⁻, was one of the factors attributed to the salt tolerance of soybean and plant survival rate was dependent on ion accumulation. It was suggested that selective mechanisms of ion transport existed on xylem/symplast boundary in soybean (Courtney et al. 2016).

Accumulation of inorganic ions for osmotic adjustment is an energy-effective way; notwithstanding, leaf K⁺ content was less at higher salinity levels that is a result of Na⁺ and K⁺ competition on the absorptive sites of cell membranes (Hussain et al. 2015). In another study, Umezawa et al. (2002) could show that Na⁺ accumulated in leaf tissue hourly but accumulation of Na⁺ in the salt-tolerant cultivar might be restricted by the Na⁺ exclusion mechanism, activated between 10 and 48 h after the initiation

![Figure 1. K⁺:Na⁺ of soybean leaves affected by interactions ± SE (n = 4) of salinity levels and wild-type/mutant cultivars according to LSD test (P ≤ 0.05). * indicates statistical significance in comparison with the control. Veg: vegetative phase; Rep: reproductive phase.](image-url)
of salt stress. Soybean acclimation to salinity stress is a result of the K\textsuperscript{+}:Na\textsuperscript{+} selection system at xylem transport and/or retranslocation mechanisms of Na\textsuperscript{+} from leaves (Umezawa et al. 2000).

Na\textsuperscript{+} accumulation takes place at the expense of K\textsuperscript{+} accumulation (Bhattarai & Midmore 2009). Although Na\textsuperscript{+} assists in maintaining turgor pressure, it is never a substitute for Ca\textsuperscript{2+} and/or K\textsuperscript{+}, which specifically function in enzyme activation and protein synthesis (Jovelina Da Silva et al. 2017). Tester and Davenport (2003) assumed the inhibition of Na\textsuperscript{+} transport to leaves was genetically controlled and conditioned by the variations in net sodium absorption arising generally from variations of the net rate of Na\textsuperscript{+} loading in xylems. Moreover, Esan et al. (2017) found different Na\textsuperscript{+} accumulation in leaves of salt-affected soybean cultivars due to different root system characteristics as better root growth, better root osmotic adjustment, higher root pressure, and finally higher Na\textsuperscript{+} and Cl\textsuperscript{−} exclusion ability could increase salt tolerance in some soybean cultivars.

Varieties that can accumulate fewer toxic ions and maintain greater profitable ions, i.e., K\textsuperscript{+} and Ca\textsuperscript{2+}, tolerate saline conditions better (Munns 2002). Na\textsuperscript{+} accumulation in salt-sensitive and salt-tolerant soybean leaves, subjected to even low saline condition, was raised during the growth period (Ahmed et al. 2015). Bhattarai et al. (2006) proposed K\textsuperscript{+}:Na\textsuperscript{+} as an ion regulation index for indicating the salt stress and declared it was a better indicator rather than Na\textsuperscript{+} concentration alone due to Na\textsuperscript{+} concentration was not sufficient to determine the plant salt tolerance.

According to Table 2, relative water content of leaves fell especially in high levels of salinity at both phases as it was 13% and 16% lower in 200 mM NaCl in comparison with the control at vegetative and reproductive phases, respectively. While leaf RWC decreased, gs also declined to restrict water loss from the stomata. However, gs decline showed a slight trend by 0.8% and 2.2% at vegetative and reproductive phases, respectively (Table 2). Contrarily, increase in salt concentration up to 200 mM NaCl did not influenced leaf RWC and gs of soybean in Bhattarai and Midmore (2009) observations. On one hand, Hussain et al. (2015) expressed that salinity

### Table 1. Analysis of variances (ANOVA) of orthogonal contrasts between vegetative and reproductive phases.

<table>
<thead>
<tr>
<th>Trait</th>
<th>MS</th>
<th>Trait</th>
<th>MS</th>
<th>Trait</th>
<th>MS</th>
<th>Trait</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>K\textsuperscript{+}:Na\textsuperscript{+}</td>
<td>4617.93 **</td>
<td>SOD</td>
<td>22.79 **</td>
<td>Free proline</td>
<td>31777.20 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RWC</td>
<td>7234.84 **</td>
<td>POD</td>
<td>179.68 **</td>
<td>Total Soluble proteins</td>
<td>67988.65 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gs</td>
<td>1016980.43 **</td>
<td>APX</td>
<td>7.06 **</td>
<td>Total Soluble carbohydrates</td>
<td>45725.30 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H\textsubscript{2}O\textsubscript{2}</td>
<td>783.49 **</td>
<td>CAT</td>
<td>22.64 **</td>
<td>H\textsubscript{2}O\textsuperscript{2}</td>
<td>783.49 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA</td>
<td>1244.90 **</td>
<td>GR</td>
<td>8.57 **</td>
<td>APX</td>
<td>7.06 **</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** refers to significant at P ≤ 0.01; MS: mean square of the orthogonal contrast between vegetative and reproductive phases.

### Table 2. RWC and gs of soybean leaves affected by salinity levels and wild-type/mutant cultivars*.

<table>
<thead>
<tr>
<th>Salinity (mM NaCl)</th>
<th>RWC (%)</th>
<th>gs (mmol H\textsubscript{2}O m\textsuperscript{-2} s\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Veg</td>
<td>Rep</td>
</tr>
<tr>
<td>Control</td>
<td>89.94 ± 0.63 a</td>
<td>80.32 ± 0.27 a</td>
</tr>
<tr>
<td>50</td>
<td>89.29 ± 0.48 a</td>
<td>79.04 ± 0.47 b</td>
</tr>
<tr>
<td>100</td>
<td>89.73 ± 0.48 a</td>
<td>70.81 ± 0.37 c</td>
</tr>
<tr>
<td>150</td>
<td>78.63 ± 0.48 b</td>
<td>67.65 ± 0.33 d</td>
</tr>
<tr>
<td>200</td>
<td>78.02 ± 0.48 b</td>
<td>67.64 ± 0.33 d</td>
</tr>
<tr>
<td>LSD (P ≤ 0.05)</td>
<td>1.44</td>
<td>1.01</td>
</tr>
<tr>
<td>Cultivar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Williams</td>
<td>83.30 ± 0.62 c</td>
<td>71.35 ± 0.45 c</td>
</tr>
<tr>
<td>M-4</td>
<td>84.83 ± 0.49 b</td>
<td>72.78 ± 0.33 b</td>
</tr>
<tr>
<td>Clark</td>
<td>84.46 ± 0.48 bc</td>
<td>72.41 ± 0.33 b</td>
</tr>
<tr>
<td>M-7</td>
<td>85.56 ± 0.49 a</td>
<td>74.51 ± 0.32 a</td>
</tr>
<tr>
<td>M-9</td>
<td>86.45 ± 0.72 a</td>
<td>74.40 ± 0.49 b</td>
</tr>
<tr>
<td>LSD (P ≤ 0.05)</td>
<td>1.40</td>
<td>0.99</td>
</tr>
</tbody>
</table>

* In each column, mean values ± SE (n = 4) followed by at least one similar letter were not significantly different according to LSD test (P ≤ 0.05). Veg: vegetative phase & Rep: reproductive phase.
distinctly affected leaf water relations and osmotic potential. Negative water potential in salt-affected leaves of soybean contributed to stomatal closure, either directly or via hormonal signals (Tong & He 2010). However, plants try to withstand saline stress and following moisture stress through a wide range of strategies like stomatal/leaf cuticle characteristics and stomatal closure (Tong & He 2010). The decrease in salt-affected leaf water potential leads to cell turgidity reduction and/or cell wall rheological changes (Munns 2002; Hidri et al. 2016), which may lead to stomatal closure.

Mutant cultivars displayed higher RWC and gs than wild-type cultivars at either vegetative or reproductive phases (Table 2). According to Table 2, M-4 showed 1.8% RWC and 3.2% gs higher than Williams at vegetative phase while it exhibited 2% RWC and 3% gs more at reproductive phase. M-7 and M-9 exhibited 2.5% and 2.4% RWC together with 1.5% and 1.7% gs, respectively, further than Clark at vegetative phase whereas they displayed 2.9% and 2.7% RWC together with 1.1% and 1.1% gs, respectively, more at reproductive phase (Table 2).

Moreover, RWC and gs significantly decreased at the reproductive phase rather than the vegetative phase (Table 1 & 2). A decrease in RWC (Weisany et al. 2011) and an increase in osmotic potential (Esan et al. 2017) of soybean leaves was reported along salinity enhancement while water absorption was better in the salt-tolerant cultivar owing to better growth, osmotic adjustment and osmotic pressure in its root system. Hussain et al. (2015) pronounced adaptation to harsh environmental circumstances varies within the germplasm of crops.

**H$_2$O$_2$ and MDA contents**

A significant increase was observed in H$_2$O$_2$, followed by cell membrane stability reduction, as a result of high salinity at both vegetative and reproductive phases (Figure 2). H$_2$O$_2$ rose to 208% and 227% of the control (vegetative phase) and to 114% and 119% of the control (reproductive phase) for Williams and Clark as wild-type cultivars in the highest level of salinity while it

![Figure 2](image-url)
slightly increased to 205%, 194%, and 168% of the control, respectively (vegetative phase) and to 107%, 96%, and 86% of the control, respectively (reproductive phase) for M-4, M-7 and M-9, as mutant cultivars (Figure 2). The same trend was seen in the case of MDA as it increased to 155% and 167% of the control (vegetative phase) and to 81% and 84% of the control (reproductive phase) for Williams and Clark as wild-type cultivars (Figure 2). The same trend was seen in the case of MDA as it increased to 151%, 140%, and 125% of the control, respectively (vegetative phase) and to 74%, 65%, and 59% of the control, respectively (reproductive phase) for M-4, M-7 and M-9, as mutant cultivars (Figure 2).

An increase in MDA and relative electrolytic leakage of soybean leaves was reported by Weisany et al. (2011) in response to saline stress. In another study, Bhattarai and Midmore (2009) recognized enhancement in electrolyte leakage ratio by increasing in salinity. Cell and organelle membranes are among the targets of $\text{H}_2\text{O}_2$, producing MDA by decomposition of membrane polyunsaturated fatty acids (Ahmed et al. 2015). Peroxidation of membrane lipids is a sign of membrane damage and leakage as a result of saline stress (Cunha et al. 2016).

Mutant cultivars especially M-7 and M-9, which were the mutants of Clark, were less influenced by salinity than wild-type cultivars (Williams and Clark) at both phenological phases (Figure 2). An increase in MDA content of both salt-tolerant and salt-sensitive soybean cultivars was distinguished by Khan et al. (2009) along with increasing in salinity levels. However, Khan et al. (2009) defined that lower MDA content was remarkable in salt-tolerant soybean cultivars. Dias et al. (2016) mentioned the variability in lipid peroxidation of genotypes was owing to the difference in their efficiency to scavenge ROS through a capable antioxidative defense system. The increase of $\text{H}_2\text{O}_2$ and decrease of cell membrane stability were more considerable at the reproductive phase vs. vegetative phase (Table 1 & Figure 2). During the time, MDA content rose in salt-sensitive and salt-tolerant soybean leaves, even in low saline circumstance (Ahmed et al. 2015).

Antioxidative enzyme contents

All of the antioxidative enzymes increased along with increasing in salinity levels at both phenological phases, which were considerably greater in two high saline levels (Figure 3). Enhancement of enzymatic antioxidants of salt-affected soybean leaves has been previously reported by Khan et al. (2009). Also, Jovelina Da Silva et al. (2017) remarked the enhancement of SOD, POD, and CAT in leaf tissues of salt-sensitive and salt-tolerant soybean even in plants grew under low saline conditions. Enhancement in GR activity was in correlation with the increase in APX activity (Khan et al. 2009). Antioxidant enzyme activity is dependent on injuries to macromolecules, cellular structures, and the level of lipid peroxidation (Vaseva et al. 2012). These enzymatic scavengers remove superoxide radicals and $\text{H}_2\text{O}_2$ so that hydroxyl radicals are not formed (Phang et al. 2008; Dias et al. 2016).

Wild-type cultivars produced more antioxidative enzymes particularly in two last saline levels at both vegetative (117% to 409%) and reproductive (95% to 349%) phases (Figure 3). Additionally, all soybean cultivars produced more antioxidative enzymes at the reproductive phase compared to the vegetative phase (Table 1 & Figure 3). Khan et al. (2009) declared enhancement of antioxidation enzymes caused by increasing salinity seemed to be a common response in all salt-sensitive and salt-tolerant soybean genotypes; whereas, there were apparent variations in the induction of enzymatic antioxidants among salt-sensitive and salt-tolerant genotypes. Batkova et al. (2008) announced the antioxidative defense system of cultivars relied on the plant age and crop cultivar.

Osmolyte contents

At both vegetative and reproductive phases, the whole of leaf osmolytes namely free proline (40% and 28%), total soluble proteins (39% and 23%), and total soluble carbohydrates (32% and 22%)
increased by increasing the salinity (Table 3). These findings are consistent with the results of Tong and He (2010). Proline concentration of all tolerant and sensitive cultivars of soybean sharply rose by salinity stress in Khan et al. (2009) study. Cunha et al. (2016) found out proline biosynthesis was associated with the production of NADP for the stimulation of the pentose phosphate pathway.
Table 3. Free proline, total soluble proteins and total soluble carbohydrates of soybean leaves affected by salinity levels and wild-type/mutant cultivars*.

<table>
<thead>
<tr>
<th>Salinity (mM NaCl)</th>
<th>Free Proline (µg g(^{-1}) FW)</th>
<th>Total Soluble Proteins (mg g(^{-1}) FW)</th>
<th>Total Soluble Carbohydrates (mg g(^{-1}) FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Veg Rep</td>
<td>Veg Rep</td>
<td>Veg Rep</td>
</tr>
<tr>
<td>Control</td>
<td>160.06 ± 1.88 c 191.10 ± 2.12 d</td>
<td>110.73 ± 1.62 c 150.53 ± 3.51 b</td>
<td>66.81 ± 1.87 b 97.51 ± 2.93 b</td>
</tr>
<tr>
<td>50</td>
<td>162.06 ± 0.66 c 193.30 ± 0.80 d</td>
<td>110.94 ± 1.05 c 152.18 ± 1.78 b</td>
<td>67.16 ± 0.78 b 97.42 ± 0.85 b</td>
</tr>
<tr>
<td>100</td>
<td>215.00 ± 0.98 b 236.25 ± 0.54 c</td>
<td>112.94 ± 0.98 c 153.79 ± 0.77 b</td>
<td>66.92 ± 0.73 b 99.16 ± 0.80 b</td>
</tr>
<tr>
<td>150</td>
<td>220.38 ± 1.45 a 241.63 ± 0.54 b</td>
<td>150.91 ± 0.97 b 182.17 ± 1.77 a</td>
<td>87.79 ± 0.73 a 115.54 ± 0.81 a</td>
</tr>
<tr>
<td>200</td>
<td>223.49 ± 1.77 a 244.76 ± 0.54 a</td>
<td>154.05 ± 0.98 a 185.29 ± 1.75 a</td>
<td>88.40 ± 0.72 a 118.67 ± 0.80 a</td>
</tr>
<tr>
<td>LSD (P ≤ 0.05)</td>
<td>3.30</td>
<td>3.09</td>
<td>3.00</td>
</tr>
<tr>
<td>Cultivar</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Williams</td>
<td>196.28 ± 1.96 a 221.32 ± 2.07 a</td>
<td>130.38 ± 1.34 a 167.79 ± 1.99 a</td>
<td>77.70 ± 1.45 a 108.82 ± 2.38 a</td>
</tr>
<tr>
<td>M-4</td>
<td>196.51 ± 0.86 a 221.76 ± 0.66 a</td>
<td>126.92 ± 1.39 b 162.38 ± 2.91 b</td>
<td>74.50 ± 1.38 a 104.15 ± 1.87 b</td>
</tr>
<tr>
<td>Clark</td>
<td>196.98 ± 0.86 a 222.22 ± 0.65 a</td>
<td>128.33 ± 0.83 ab 165.50 ± 1.27 ab</td>
<td>75.87 ± 0.75 ab 106.02 ± 0.82 ab</td>
</tr>
<tr>
<td>M-7</td>
<td>195.65 ± 0.85 a 220.93 ± 0.65 a</td>
<td>127.04 ± 0.83 b 164.20 ± 1.25 ab</td>
<td>74.57 ± 0.74 a 104.71 ± 0.82 ab</td>
</tr>
<tr>
<td>M-9</td>
<td>195.57 ± 1.30 a 220.80 ± 0.96 a</td>
<td>126.91 ± 1.25 b 164.07 ± 1.83 ab</td>
<td>74.44 ± 1.11 b 104.59 ± 1.22 ab</td>
</tr>
<tr>
<td>LSD (P ≤ 0.05)</td>
<td>3.41</td>
<td>3.12</td>
<td>2.67</td>
</tr>
</tbody>
</table>

* In each column, mean values ± SE (n = 4) followed by at least one similar letter were not significantly different according to LSD test (P ≤ 0.05). Veg: vegetative phase & Rep: reproductive phase.
while Vaseva et al. (2012) stated that proline biosynthesis might be relevant to the regulation of cytosolic pH. Changes in the proline content due to salinity stress displays membrane permeability is probably influenced (Khan et al. 2009).

Soluble carbohydrates take part in decreasing water potential and lead to maintain the structure of proteins and membranes under the osmotic stress (Dias et al. 2016). In addition to osmoregulant role of soluble sugars accumulated under saline stress, they preserve energy as carbon sources that are dependent on the photosynthesis (Hidri et al. 2016).

Although lower total soluble proteins and carbohydrates in mutant cultivars (M-4, M-7 and M-9) at vegetative phase, no distinct trends were observed among wild-type cultivars (Williams and Clark) and mutant cultivars at reproductive phase (Table 3). High salt-tolerant cultivars possess more ability for biosynthesis and accumulation of compatible compounds specifically when subjected to intense stresses (Tong & He 2010). Proline is accumulated in salt-tolerant cultivars more than salt-sensitive ones (Khan et al. 2009).

From these results it can be concluded that salinity stress stimulated antioxidative defense of mutant and wild-type cultivars of soybean. The antioxidants and osmotic compounds of soybean leaves was upgraded to decline oxidative and drought stresses by increasing in Na⁺ content and decreasing in RWC, gs and cell membrane stability. Despite improved performance of mutated varieties to salt content, other physiological parameters are necessary to investigate the screening of mutant and wild-type varieties of soybeans.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**References**


