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# Comprehensive Morphological and Proteomic Insights into Salinity Stress in Soybean (*Glycine max*): Elucidating Tolerance Mechanisms and Biomarker Discovery

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**ABSTRACT**

This study aimed to investigate the effects of salinity stress on growth parameters and proteomic responses in soybean. The experiment was conducted as a completely randomized design with three replications and four salinity levels (0, 3, 6, and 9 $dS \cdot m^{-1}$) under controlled greenhouse conditions at the Faculty of Agriculture, Mohaghegh Ardabili University, in 2019. The results indicated that salinity stress significantly and negatively affected morphological traits. The intensity of these effects varied by genotype, with the DPX cultivar exhibiting the least reduction and the highest tolerance. The traits were stem length, root length, leaf number, and total seedling dry weight. DPX showed the highest tolerance. According to the results of the three-way ANOVA (sampling time × salinity level × genotype), salinity stress significantly affected all evaluated traits, with differences being significant at the 1% and 5% probability thresholds. In the proteomic analysis, two-dimensional electrophoresis (2-DE) of soybean leaves revealed that salinity stress induced significant changes in the expression of several key cellular proteins. Proteins such as Glutathione S-transferase, Ferritin, ATPase, and Glutamine synthetase were upregulated in the DPX genotype, while the expression of Rubisco and Phosphoribulokinase was reduced in the sensitive cultivar, Arian. These results indicate the activation of defense mechanisms, antioxidant responses, ion regulation, and metabolic balance maintenance in the salt-tolerant DPX genotype. Accordingly, the DPX cultivar can be considered a salt-tolerant genotype for use in breeding programs and cultivation in saline soils. Moreover, the identified proteins may serve as potential biomarkers for screening salt-tolerant genotypes and developing molecular-level breeding strategies. These findings contribute to the understanding of soybean salinity tolerance mechanisms and support the integration of proteomic markers into molecular breeding strategies. Ultimately, this approach may accelerate the development of salt-tolerant soybean cultivars to ensure food security under climate change and soil degradation.

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## 1. Introduction

Soybean (*Glycine max* L.) is one of the most important oilseed and protein-rich crops globally, playing a vital role in food security and sustainable agricultural development. According to the Food and Agriculture Organization (FAO), soybean seeds contain approximately 36–40% protein and 18–20% oil, making them a strategic resource for human nutrition, animal feed, and various industrial applications. Soybean accounts for nearly 48% of global vegetable oil consumption, solidifying its position as the leading oilseed crop. In Iran, soybean is cultivated on more than 100,000 hectares, primarily in the northern provinces (Golestan, Mazandaran, Gilan, and Ardabil), with an average yield of 2.2 tons per hectare. However, the country's heavy reliance on imported soybean oil and meal—over 90%—underscores the urgent need to enhance domestic productivity and expand cultivation into suitable new regions. A major limitation to achieving this goal is soybean's high sensitivity to environmental stresses, particularly salinity, which severely constrains its yield in arid and semi-arid regions of Iran (Majidian *et al.*, 2024).

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Soil salinity is among the most damaging abiotic stresses, affecting over 830 million hectares of agricultural land worldwide (Shrivastava and Kumar, 2015). In Iran alone, approximately 25 million hectares are at risk of salinization, resulting in annual losses exceeding $1.2 billion in the agricultural sector. Salinity impacts plants primarily through two mechanisms: osmotic stress, which reduces water uptake by lowering soil water potential, and ionic toxicity, caused by the accumulation of toxic ions such as $Na^+$ and $Cl^-$ in plant tissues. These factors disrupt ionic homeostasis, trigger oxidative stress responses, and inhibit essential enzymes like Rubisco (RuBisCO), ultimately reducing shoot and root growth, leaf development, and grain yield (Acosta-Motos *et al*., 2017). In soybean, salinity stress has been reported to reduce stem length by 30–50%, dry weight by 40–60%, and leaf number by 20–30% (Han *et al*., 2016), primarily due to impaired photosynthesis, disrupted assimilate transport, membrane instability, and chlorophyll degradation (Hussain *et al*., 2013).

Despite its overall sensitivity to salinity, soybean exhibits genetic variation among cultivars, offering the opportunity to identify tolerant genotypes and uncover underlying resistance mechanisms. For example, field studies on cultivars such as DPX and Williams 82 have shown that certain genotypes can mitigate salinity damage by enhancing antioxidant enzyme activity (e.g., superoxide dismutase and catalase), accumulating proline, and maintaining osmotic balance (Arzani, 2008). However, most prior research has focused on morpho-physiological traits, while the molecular and proteomic responses underlying salinity tolerance remain poorly understood, limiting the success of marker-assisted selection in breeding programs.

Proteomics has recently emerged as a powerful tool for identifying key proteins involved in plant responses to environmental stresses. For instance, in durum wheat, two-dimensional electrophoresis (2-DE) revealed salinity-induced changes in 38 proteins associated with antioxidant defense, ion transport, and membrane stability (Caruso *et al*., 2008). In rapeseed, Dolatabadi et al. (2024) identified 44 differentially expressed proteins under salt stress, many linked to ionic regulation and DNA repair. In soybean, while some studies (Shafiei *et al*., 2023) have explored seed storage protein responses to salinity, leaf proteomic analyses—which are particularly relevant given the leaf's central role in photosynthesis and metabolism—remain limited. Leaf proteins such as chitinase (pathogen defense), ferritin (iron storage and oxidative stress reduction), and glutamine synthetase (nitrogen metabolism) may play crucial roles in conferring salt tolerance (Zhao *et al*., 2019). Furthermore, studies in maize hybrids, such as SC704, suggest that salinity-induced changes in Calvin cycle enzymes like Rubisco and phosphoribulokinase may contribute to sustained photosynthetic activity under stress (Shafiei *et al*., 2024). Yet, these relationships have not been systematically examined in soybean.

Accordingly, the present study aimed to evaluate the effects of different salinity levels (0, 3, 6, and 9 $dS \cdot m^{-1}$) on the growth parameters of seven soybean cultivars, focusing on stem length, root length, leaf number, and dry weight under controlled greenhouse conditions. Additionally, leaf proteomic responses were analyzed to determine whether changes in protein expression correlated with morphological tolerance. The study also investigated the potential of differentially expressed proteins to serve as biomarkers for identifying salt-tolerant genotypes in molecular breeding programs.

## 2. Materials and methods

This study was conducted as a factorial experiment in a completely randomized design (CRD) with three replications during the 2019–2020 cropping season in the greenhouse of the Faculty of Agriculture, Mohaghegh Ardabili University. The experimental factors included four salinity levels (0, 3, 6, and 9 $dS \cdot m^{-1}$) and seven soybean cultivars (Rubin, Parsa, Saba, Williams, Arian, Saman, and DPX), which were obtained from the Agricultural and Natural Resources Research Center of Ardabil, Iran. Based on the results of the current study, DPX was identified as the most salt-tolerant cultivar and Arian as the most salt-sensitive one; therefore, these two contrasting genotypes were examined in greater detail in the proteomic analysis.

Soybean seeds were obtained from the Agricultural and Natural Resources Research Center of Ardabil. To induce salinity stress, appropriate amounts of soil were filled into each pot. After determining the soil saturation percentage, the amount of salt (NaCl) required to reach each salinity level was calculated

based on charts provided by the U.S. Salinity Laboratory (Munns and Tester, 2008), dissolved in water, and applied to the soil. The pots were irrigated regularly for one week to ensure uniform salt distribution. Soil electrical conductivity (EC) was measured after salinity treatment and re-evaluated at the end of the experiment to confirm salinity stability.

Initial irrigation was applied based on the field capacity (FC) of the soil and later adjusted according to greenhouse temperature and soil moisture conditions. Throughout the growth period, greenness indices were recorded weekly. Seedling growth traits—including stem length, root length, leaf number, and dry biomass—were measured at six sampling points (Sampling 1 to Sampling 6), corresponding to weeks 2, 3, 4, 5, 6, and 9 after planting. To determine dry weight, the seedlings were carefully removed from the soil, and their aerial and underground parts were separated. The tissues were oven-dried at 70°C for 48 hours and weighed using a precision digital scale (accuracy ± 0.0001 g).

For proteomic analysis, leaf samples were collected at the ninth week of growth (fruiting stage). Fresh green leaves were immediately wrapped in aluminum foil, frozen in liquid nitrogen, and stored at –70°C until protein extraction. The extraction procedure was performed with slight modifications based on the method of Shafiei et al. (2024). Briefly, approximately 0.1 g of frozen leaf tissue was ground in liquid nitrogen and transferred into microtubes. A cold 10% TCA-acetone solution was added, and the samples were vortexed and incubated at –20°C for one hour. After centrifugation at 13,500 rpm at 4°C, the resulting protein pellets were washed five times with a cold washing solutionProtein pellets were re-suspended using the method of Shafiei et al. (2024), and protein concentrations were determined using the Bradford method (Bradford, 1976). Two-dimensional electrophoresis (2-DE) was carried out using linear IPG strips with a pH range of 4–7 (Bio-Rad) and an IPGphor3 apparatus at 20°C and 75 mA for 10.5 hours. The second-dimension separation was performed by SDS-PAGE on 11% acrylamide gels using an electrophoresis system (initially at 50 V for 30 minutes, followed by 165 V for 3 hours). 2-DE was used to identify differentially expressed leaf proteins under salinity stress and link them to salt tolerance mechanisms. Gels were stained with Coomassie Brilliant Blue and scanned with a GS-800 scanner (Bio-Rad) at 300 dpi resolution. Gel images were analyzed using ImageMaster 6.0 software to identify and quantify protein spots, and the relative volume of each spot was considered as an index of protein expression. Finally, all statistical analyses were performed using SAS software version 9.1. The significance of treatment effects was assessed using the F-test, and mean comparisons were conducted using the LSD test at the 5% significance level.

## 3. Results and discussion

### *3.1. Evaluation of salinity effects on morphological traits in different soybean cultivars*

The analysis of variance revealed that the three-way interaction between sampling time × salinity treatment × cultivar had a statistically significant effect on total seedling dry weight and leaf number at the 5% probability level, and on stem length and root length at the 1% probability level (Table 1).

**Table 1. Analysis of variance (mean squares) for stem length, root length, leaf number, and total seedling dry weight in soybean cultivars under various salinity levels and sampling times**

| SOV | df | Stem length | Root length | Leaf number | Total seedling dry weight |
|---|---|---|---|---|---|
| Sampling time (T) | 5 | 2.18 | $4.50^{**}$ | $5.60^{**}$ | $0.446^{**}$ |
| Error | 12 | 15.23 | 0.32 | 0.28 | 0.018 |
| Salinity (S) | 3 | $24.61^{**}$ | $0.18^{ns}$ | $0.53^{*}$ | $0.016^{ns}$ |
| Cultivar (C) | 6 | $2.38^{**}$ | $1.27^{**}$ | $1.48^{**}$ | $0.112^{**}$ |
| S × C | 18 | $2.03^{**}$ | $0.69^{**}$ | $0.371^{**}$ | $0.022^{ns}$ |
| T × S | 15 | $5.52^{**}$ | $0.49^{**}$ | $0.219^{ns}$ | $0.030^{ns}$ |
| T × C | 30 | $1.86^{**}$ | $0.53^{**}$ | $0.844^{**}$ | $0.0978^{**}$ |
| T × S × C | 90 | $0.915^{**}$ | $0.35^{**}$ | $0.221^{*}$ | $0.031^{*}$ |
| Error | 324 | 2.18 | 0.226 | 0.157 | 0.023 |
| CV (%) | - | 17.05 | 23.58 | 18.83 | 31.41 |

*ns, * and ** indicate non-significant, significant at 5%, and significant at 1% probability levels, respectively.

#### *3.1.1. Stem length*

As shown in Table 2, stem length decreased with increasing salinity levels in all studied cultivars except for DPX. Moreover, stem length was generally lower in the first sampling compared to the sixth sampling. The highest and lowest stem lengths were observed in the salt-tolerant cultivar DPX (71.67 cm) under 6 $dS \cdot m^{-1}$ salinity in the sixth sampling, and in the cultivar Saba (7.33 cm) under the same salinity level during the first sampling, respectively.

Researchers have indicated that salinity stress disrupts water uptake by plants, leads to the accumulation of toxic salts such as sodium in plant

tissues, and disturbs ion balance in both soil and plant systems. These effects result in reduced germination, impaired vegetative growth, and ultimately a decrease in crop yield (Zhao *et al*., 2019). Many studies have reported that salinity stress reduces chlorophyll content in leaves, thereby negatively impacting photosynthesis (Hameed *et al*., 2021). In addition, drought reduces plant height by limiting cell division and elongation (Quamruzzaman *et al*., 2021). Salinity stress also decreases shoot height due to the ionic toxicity of harmful elements and disruption of biological and metabolic processes, and leads to reduced biomass in both shoot and root systems due to the loss of osmotic and ionic balance. In the present study, salinity negatively affected stem length in all cultivars, consistent with the findings of other researchers (Arzani, 2008).

**Table 2. Mean comparison of the interaction effects of sampling time × salinity level × cultivar on stem length in soybean**

| Cultivar | Salinity (dS·m$^{-1}$) | Sampling 1 | Sampling 2 | Sampling 3 | Sampling 4 | Sampling 5 | Sampling 6 |
|---|---|---|---|---|---|---|---|
| Rubin | 1 | 32.67 | 35.67 | 39.00 | 41.00 | 48.17 | 44.83 |
| Parsa | 1 | 35.00 | 35.33 | 39.33 | 43.67 | 47.00 | 47.33 |
| Saba | 1 | 18.83 | 30.50 | 34.33 | 37.33 | 41.83 | 47.33 |
| Williams | 1 | 22.83 | 32.67 | 38.83 | 40.67 | 50.67 | 56.33 |
| Arian | 1 | 21.33 | 22.67 | 25.67 | 28.13 | 32.50 | 43.17 |
| Saman | 1 | 27.00 | 32.20 | 36.33 | 44.00 | 46.00 | 68.00 |
| DPX | 1 | 18.67 | 30.06 | 28.33 | 39.33 | 40.33 | 42.17 |
| Rubin | 3 | 30.67 | 35.67 | 39.00 | 40.17 | 48.17 | 48.67 |
| Parsa | 3 | 32.33 | 32.33 | 37.33 | 42.67 | 43.67 | 45.00 |
| Saba | 3 | 11.17 | 24.67 | 24.93 | 36.00 | 39.00 | 45.17 |
| Williams | 3 | 20.33 | 25.67 | 37.33 | 40.33 | 50.00 | 53.33 |
| Arian | 3 | 18.17 | 22.33 | 24.67 | 25.33 | 29.17 | 35.83 |
| Saman | 3 | 25.43 | 26.67 | 34.67 | 41.33 | 43.33 | 64.17 |
| DPX | 3 | 23.00 | 27.00 | 33.00 | 38.67 | 40.67 | 57.67 |
| Rubin | 6 | 27.73 | 31.17 | 31.60 | 31.83 | 33.33 | 40.33 |
| Parsa | 6 | 27.33 | 29.33 | 32.83 | 33.33 | 39.00 | 44.67 |
| Saba | 6 | 7.33 | 16.33 | 24.57 | 29.67 | 31.33 | 37.67 |
| Williams | 6 | 19.83 | 22.83 | 32.67 | 39.00 | 45.33 | 52.17 |
| Arian | 6 | 13.00 | 22.17 | 23.33 | 23.67 | 26.67 | 29.13 |
| Saman | 6 | 23.60 | 25.33 | 34.33 | 35.00 | 38.67 | 63.83 |
| DPX | 6 | 24.87 | 32.67 | 38.33 | 42.67 | 43.33 | 71.67 |
| LSD (0.05) | | | | 3.34 | | | |

### *3.1.2. Root length*

The mean comparison results showed that sampling time had a positive effect on root length. Specifically, root length increased in the sixth sampling compared to the first. Conversely, salinity stress had a negative effect on root length, with increasing salinity concentrations leading to shorter roots. The highest root length (10.33 cm) was observed in the salt-tolerant DPX cultivar under non-saline conditions during the sixth sampling, while the lowest value (1.83 cm) belonged to the Saman cultivar under severe salinity stress (6 dS·m$^{-1}$) during the first sampling (Table 3).

During the initial phase of salinity stress, reduced water uptake and transport capacity limit leaf, shoot, and root growth. If stress persists, plants enter a secondary phase in which excessive salt uptake leads to its accumulation in vacuoles of older leaf cells. Continued stress results in salt buildup in the cytoplasm, causing cell death and eventually senescence of older leaves. This process indirectly reduces growth more severely in salt-sensitive genotypes than in tolerant ones (Zhao *et al*., 2019).

Naturally, a reduction in shoot length leads to lower shoot biomass and consequently reduced total dry matter. In the present study, salinity had a negative impact on both root and shoot length, consistent with findings from previous researchers (Arzani, 2008). Salinity can interfere with transporter activity and ion channels in roots—such as potassium-selective channels—by enabling sodium to compete with potassium, or by osmotic effects that inhibit root growth. It can also disrupt soil structure and reduce the uptake of water and nutrients (Quamruzzaman *et al*., 2021; Khan *et al*., 2014).

**Table 3. Mean comparison of the interaction effects of sampling time × salinity level × cultivar on root length (cm) in soybean**

| Cultivar | Salinity (dS·m$^{-1}$) | Sampling 1 | Sampling 2 | Sampling 3 | Sampling 4 | Sampling 5 | Sampling 6 |
|---|---|---|---|---|---|---|---|
| Rubin | 1 | 4.17 | 5.00 | 6.83 | 7.00 | 7.83 | 9.33 |
| Parsa | 1 | 3.00 | 4.67 | 5.67 | 6.00 | 6.00 | 7.17 |
| Saba | 1 | 2.67 | 3.17 | 5.17 | 6.00 | 6.50 | 9.00 |
| Williams | 1 | 3.33 | 3.83 | 4.33 | 5.00 | 5.33 | 7.66 |
| Arian | 1 | 2.43 | 3.00 | 3.80 | 4.00 | 5.00 | 8.17 |
| Saman | 1 | 3.67 | 3.83 | 4.23 | 5.33 | 6.00 | 10.00 |
| DPX | 1 | 3.00 | 3.00 | 3.00 | 4.00 | 6.33 | 10.33 |
| Rubin | 3 | 3.83 | 4.00 | 4.50 | 5.17 | 6.33 | 7.67 |
| Parsa | 3 | 2.50 | 3.53 | 3.66 | 3.67 | 5.00 | 5.67 |
| Saba | 3 | 2.33 | 2.33 | 5.00 | 5.66 | 6.00 | 8.17 |
| Williams | 3 | 2.50 | 2.67 | 3.33 | 4.67 | 4.67 | 6.17 |
| Arian | 3 | 2.33 | 3.00 | 3.50 | 3.83 | 4.50 | 5.00 |
| Saman | 3 | 3.33 | 3.33 | 4.00 | 4.83 | 5.67 | 10.00 |
| DPX | 3 | 2.30 | 3.00 | 3.00 | 3.33 | 3.67 | 8.67 |
| Rubin | 6 | 3.06 | 3.33 | 3.66 | 4.17 | 4.67 | 6.67 |
| Parsa | 6 | 2.33 | 2.67 | 3.03 | 3.50 | 4.00 | 5.00 |
| Saba | 6 | 2.17 | 2.67 | 3.33 | 3.33 | 4.33 | 5.33 |
| Williams | 6 | 2.17 | 2.33 | 3.00 | 3.33 | 3.33 | 5.67 |
| Arian | 6 | 2.26 | 2.83 | 3.50 | 3.67 | 4.00 | 4.67 |
| Saman | 6 | 1.83 | 2.33 | 2.67 | 3.67 | 5.33 | 9.33 |
| DPX | 6 | 2.26 | 2.67 | 3.00 | 3.27 | 3.33 | 6.33 |
| LSD (0.05) | | | | 3.14 | | | |

In Iran's agricultural soils, sodium chloride-induced salinity is the most common type, leading to higher salt concentration around the root zone compared to inside the roots. This ultimately causes wilting, reduced vigor, and inhibited growth (Zhao *et al*., 2019). The immediate plant response to elevated salinity is a decrease in leaf area and leaf number. However, other parts of the plant, particularly root and shoot systems, also experience growth inhibition due to reduced turgor pressure in plant cells. Another typical plant response is a change in the root-to-shoot ratio, which is often more pronounced than the effect of salinity on yield itself (Arzani, 2008)

*3.1.3. Leaf number*

The mean comparison results showed that sampling time had a positive effect on leaf number, with the highest values observed during the sixth sampling compared to the first. Conversely, salinity stress negatively affected leaf number, such that increasing salinity levels resulted in a gradual reduction in leaf count. The highest and lowest leaf numbers were recorded in the salt-tolerant DPX cultivar (10.67 leaves) under non-saline conditions during the sixth sampling, and in the Williams cultivar (1.67 leaves) under severe salinity stress (6 $dS \cdot m^{-1}$) during the first sampling, respectively (Table 4). Typically, most crop varieties have 20 to 30 leaves, though some cultivars can produce more than 50 leaves. Leaf number is relatively less influenced by environmental factors. However, in the current study, the reduction in both leaf number and leaf area under increased salinity likely led to decreased light interception, lower net photosynthesis, and reduced dry matter accumulation. Consequently, the shoot dry weight—comprising both stem and leaf biomass—was negatively affected (Zhao *et al*., 2019).

*3.1.4. Total seedling dry weight*

The mean comparison results revealed that salinity stress significantly reduced the total seedling dry weight of soybean compared to the control. Additionally, in the present study, an increase in sampling time was associated with an increase in total dry weight. The highest dry weight (0.977 g per plant) was observed in the DPX cultivar under non-saline conditions during the sixth sampling, while the lowest value (0.107 g per plant) was recorded in the Arian cultivar under severe salinity stress during the first sampling (Table 5). Total dry weight is considered one of the key indicators of salinity tolerance, and in some studies, it has been used as a defining criterion for evaluating salinity resistance. Increased solar radiation use efficiency is directly linked to enhanced photosynthesis, leading to greater biomass accumulation and biological yield (Arzani, 2008). Given the decrease in both leaf area and number under salinity, it can be inferred that light interception, net photosynthesis, and dry matter accumulation are reduced, ultimately resulting in lower shoot dry weight, which includes the dry mass of stems and leaves.

Salinity stress significantly reduces both fresh and dry weight of leaves, shoots, and roots. In fact, shoot dry weight is affected by both reduced vegetative growth and a decline in photosynthetic activity. Zhao et al. (2019) reported that salinity-induced damage can lead to chlorophyll degradation, leaf discoloration, and chlorosis. These changes, along with leaf area reduction and defoliation, lower the photosynthetic potential of the plant, reducing growth and dry matter accumulation. In a hydroponic study on tobacco under controlled environmental conditions, a significant reduction in shoot dry weight was observed at 200 $mmol \cdot m^{-3}$ NaCl (Quamruzzaman *et al*., 2021).

**Table 4. Mean comparison of the interaction effects of sampling time × salinity level × cultivar on leaf number in soybean**

| Cultivar | Salinity ($dS \cdot m^{-1}$) | Sampling 1 | Sampling 2 | Sampling 3 | Sampling 4 | Sampling 5 | Sampling 6 |
|---|---|---|---|---|---|---|---|
| Rubin | 1 | 3.00 | 3.67 | 4.33 | 4.67 | 5.00 | 5.00 |
| Parsa | 1 | 4.00 | 4.00 | 5.00 | 5.67 | 7.00 | 9.67 |
| Saba | 1 | 2.33 | 4.00 | 4.00 | 4.33 | 4.67 | 5.00 |
| Williams | 1 | 2.67 | 4.00 | 5.33 | 6.00 | 7.00 | 9.00 |
| Arian | 1 | 3.67 | 3.67 | 4.00 | 5.00 | 5.33 | 6.33 |
| Saman | 1 | 2.67 | 3.33 | 5.33 | 5.67 | 7.00 | 10.00 |
| DPX | 1 | 4.33 | 4.33 | 6.00 | 7.33 | 8.00 | 10.67 |
| Rubin | 3 | 2.67 | 3.33 | 4.00 | 4.33 | 5.00 | 5.00 |
| Parsa | 3 | 3.67 | 4.00 | 4.67 | 5.33 | 6.33 | 8.67 |
| Saba | 3 | 2.00 | 2.67 | 4.00 | 4.33 | 4.67 | 5.00 |
| Williams | 3 | 2.33 | 3.00 | 4.67 | 5.67 | 7.00 | 8.67 |
| Arian | 3 | 3.00 | 3.67 | 4.00 | 4.67 | 5.33 | 5.33 |
| Saman | 3 | 2.33 | 3.33 | 5.33 | 5.33 | 6.33 | 7.33 |
| DPX | 3 | 3.33 | 3.67 | 5.67 | 6.00 | 7.00 | 9.33 |
| Rubin | 6 | 2.67 | 3.33 | 4.00 | 4.33 | 4.33 | 5.00 |
| Parsa | 6 | 3.33 | 3.67 | 4.33 | 5.00 | 5.67 | 7.33 |
| Saba | 6 | 2.00 | 2.33 | 3.67 | 4.00 | 4.67 | 5.00 |
| Williams | 6 | 1.67 | 2.83 | 4.00 | 5.67 | 6.00 | 7.00 |
| Arian | 6 | 3.00 | 3.33 | 4.00 | 4.00 | 4.00 | 5.00 |
| Saman | 6 | 2.00 | 2.67 | 4.33 | 4.67 | 5.00 | 5.33 |
| DPX | 6 | 3.00 | 3.00 | 5.00 | 5.67 | 6.00 | 9.00 |
| LSD (0.05) | | | | 2.87 | | | |

**Table 5. Mean comparison of the interaction effects of sampling time × salinity level × cultivar on total seedling dry weight in soybean**

| Cultivar | Salinity ($dS \cdot m^{-1}$) | Sampling 1 | Sampling 2 | Sampling 3 | Sampling 4 | Sampling 5 | Sampling 6 |
|---|---|---|---|---|---|---|---|
| Rubin | 1 | 0.207 | 0.210 | 0.220 | 0.240 | 0.393 | 0.480 |
| Parsa | 1 | 0.150 | 0.163 | 0.210 | 0.253 | 0.410 | 0.753 |
| Saba | 1 | 0.150 | 0.185 | 0.217 | 0.250 | 0.283 | 0.453 |
| Williams | 1 | 0.133 | 0.160 | 0.223 | 0.287 | 0.320 | 0.450 |
| Arian | 1 | 0.143 | 0.203 | 0.340 | 0.357 | 0.370 | 0.587 |
| Saman | 1 | 0.170 | 0.180 | 0.230 | 0.263 | 0.317 | 0.820 |
| DPX | 1 | 0.173 | 0.207 | 0.227 | 0.270 | 0.687 | 0.977 |
| Rubin | 3 | 0.147 | 0.197 | 0.200 | 0.213 | 0.373 | 0.413 |
| Parsa | 3 | 0.133 | 0.143 | 0.193 | 0.243 | 0.373 | 0.550 |
| Saba | 3 | 0.108 | 0.153 | 0.190 | 0.210 | 0.270 | 0.350 |
| Williams | 3 | 0.130 | 0.150 | 0.193 | 0.233 | 0.293 | 0.373 |
| Arian | 3 | 0.123 | 0.130 | 0.157 | 0.162 | 0.342 | 0.377 |
| Saman | 3 | 0.167 | 0.180 | 0.217 | 0.253 | 0.307 | 0.760 |
| DPX | 3 | 0.157 | 0.183 | 0.223 | 0.266 | 0.387 | 0.730 |
| Rubin | 6 | 0.133 | 0.167 | 0.190 | 0.210 | 0.226 | 0.410 |
| Parsa | 6 | 0.130 | 0.137 | 0.177 | 0.217 | 0.303 | 0.437 |
| Saba | 6 | 0.073 | 0.143 | 0.180 | 0.190 | 0.233 | 0.345 |
| Williams | 6 | 0.130 | 0.130 | 0.163 | 0.233 | 0.273 | 0.368 |
| Arian | 6 | 0.107 | 0.123 | 0.133 | 0.143 | 0.148 | 0.167 |
| Saman | 6 | 0.160 | 0.163 | 0.200 | 0.207 | 0.253 | 0.267 |
| DPX | 6 | 0.150 | 0.180 | 0.217 | 0.237 | 0.330 | 0.697 |
| LSD (0.05) | | | | 0.275 | | | |

### *3.2. Proteomics*

After scanning the two-dimensional electrophoresis (2-DE) gels, the resulting images were analyzed using ImageMaster 6.0 software. Leaf protein profiles of soybean under different salinity levels were examined and compared between two cultivars: DPX (salt-tolerant) and Arian (salt-sensitive). The molecular weights of the proteins were estimated based on standard protein markers that were co-electrophoresed on the gels. The isoelectric points (pI) of the proteins were determined according to the positions of the spots on the 18 cm linear pH 4–7 IPG strips. At this stage, preliminary and tentative protein identifications were made using the location, shape, and characteristics of the protein spots on the gels, and by comparison with published data from relevant studies. A total of 25 significant protein spots were identified. To quantify changes in protein expression, the relative volume of each spot was used as a normalized index. The resulting data were statistically analyzed using SAS software. F-tests were conducted at the 5% significance level to compare treatments. For each treatment stage (control and stress levels), two replicates were included, and a total of 8 gels were analyzed (Table 6).

**Table 6. Functional classification and expression changes of identified proteins in soybean leaves under different salinity levels in DPX and Arian cultivars**

| Protein Name | pI | MW (kD) | Expression change | Genotype | Functional |
|---|---|---|---|---|---|
| Heat shock protein 7 | 6.8 | 73 | Upregulated | DPX | Protein folding, stress tolerance |
| Alanine aminotransferase | 4.6 | 93.5 | Upregulated | DPX | Amino acid metabolism, nitrogen balance |
| $H^+$-transporting two-sector ATPase | 7 | 62.5 | Upregulated | DPX | Ion transport, osmotic balance |
| Chitinase | 6.9 | 32.7 | Upregulated | DPX | Defense against pathogens, cell wall modification |
| DEP1 | 6.16 | 32 | Upregulated | DPX | Developmental regulation, panicle architecture |
| Peptide methionine sulfoxide reductase (cPSMR) | 5.67 | 32 | Upregulated | DPX | ROS detoxification, protein repair |
| Lipocalin | 5.2 | 22.1 | Upregulated | DPX | Membrane protection, ROS buffering |
| Methionine Synthase | 4.84 | 26.05 | Upregulated | DPX | Methionine biosynthesis, methyl group metabolism |
| High-affinity phosphate transporter PT1 | 6.72 | 62 | Upregulated | DPX | Phosphate uptake under stress |
| Ferritin | 5.3 | 29.2 | Upregulated | DPX | Iron storage, oxidative stress protection |
| Rubisco large subunit | 6.88 | 47.1 | Downregulated | Arian | $CO_2$ fixation, Calvin cycle |
| ATPase α-subunit | 6.24 | 55.32 | Upregulated | DPX | Ion homeostasis, $Na^+$ exclusion |
| Glutathione S-transferase (GST) | 5.9 | 30.4 | Upregulated | DPX | Antioxidant defense, xenobiotic detox |
| Ferredoxin-NADP(H)-oxidoreductase | 6.22 | 39 | Upregulated | DPX | Electron transport, redox balance |
| Adenine phosphoribosyltransferase 4 | 5.8 | 24 | Upregulated | Arian | Purine salvage pathway |
| Protein translocase subunit SECA1 (chloroplast) | 5.6 | 98 | Upregulated | Arian | Protein import into chloroplasts |
| Vacuolar protein sorting-associated 35B | 6.2 | 90 | Upregulated | Arian | Vesicle-mediated protein sorting |
| Cathepsin B-like protease 1 | 5.5 | 38 | Upregulated | Arian | Protein turnover under stress |
| Isoform 3 of Protein-L-isoaspartate O-methyltransferase 2 | 5.7 | 25 | Upregulated | Arian | Protein repair, stress adaptation |
| Transcription factor ILI6 | 6.0 | 42 | Upregulated | Arian | Transcriptional reprogramming |
| SAM synthetase (SAMS) | 6.72 | 62 | Upregulated | Arian | Polyamine & ethylene biosynthesis |
| GAPDH | 5.3 | 29.2 | Upregulated | Arian | Glycolysis, ATP/NADH production |
| Phosphoribulokinase (PRK) | 6.0 | 40 | Downregulated | Arian | Calvin cycle, RuBP regeneration |
| Glutamine synthetase | 5.8 | 39 | Upregulated | Arian | Nitrogen assimilation |
| Heat shock Protein 7 (duplicated) | 6.8 | 73 | Upregulated | Arian | Protein protection under stress |

*3.2.1. Proteomic responses of soybean to salinity stress: identification of tolerance mechanisms and key biomarkers*

Investigating proteomic responses in plants under abiotic stress conditions is a key strategy for identifying stress tolerance mechanisms and discovering effective biomarkers for breeding programs. In this study, two-dimensional electrophoresis (2-DE) was employed to examine the leaf protein profiles of soybean (Glycine max) under control and salinity stress conditions. The results revealed that the expression of several proteins was significantly altered in response to salinity, with these changes being genotype-dependent, highlighting the critical role of genetic variation in regulating molecular responses to salt stress.

One of the most prominent protein groups identified was antioxidant enzymes, such as Glutathione S-transferase (GST), which showed increased expression in salt-tolerant genotypes. GST contributes to neutralizing oxidative stress in plant cells by conjugating glutathione to reactive oxygen species and toxic compounds. These findings are consistent with those of Shafiei et al. (2023), who also reported increased GST expression in salt-tolerant soybean genotypes. Proteins such as Glutathione S-transferase, Ferritin, ATPase, Chitinase, and Glutamine synthetase were upregulated in DPX, contributing to stress defense.

Another key protein identified was Ferritin, which exhibited significantly higher expression in salt-tolerant genotypes. Ferritin is an iron-storage protein involved in maintaining iron homeostasis and scavenging reactive oxygen species. This function helps prevent lipid peroxidation and protects cellular membranes. Similarly, Moharramnejad et al. (2021) reported that elevated Ferritin levels contribute to salinity tolerance in maize.

Photosynthesis-related proteins were also among the groups affected by salinity. Reduced expression of Rubisco large subunit and Phosphoribulokinase (PRK) in sensitive genotypes indicated photosynthetic impairment under salt stress. These two enzymes are central to the Calvin cycle, and their reduction may lead to decreased carbon fixation and plant growth. Our findings align with those of Shafiei et al. (2020), who reported a decline in Rubisco under salt stress in wheat. Rubisco and PRK were downregulated in Arian, indicating reduced photosynthesis, while DPX maintained better expression and photosynthetic function.

In terms of ion homeostasis regulation, the upregulation of ATPase α-subunit in tolerant genotypes was notable. This enzyme plays a role in ion pumping activity, helping to expel sodium ions from cells and maintain electrochemical gradients. Activation of ATPase is a major strategy for mitigating ionic toxicity and sustaining osmotic balance under saline conditions. Moharramnejad et al. (2021) also identified increased ATPase activity as a marker of salt resistance in maize leaves.

Among the defense-related proteins, increased expression of Chitinase was observed in certain genotypes. In addition to its role in pathogen defense, Chitinase contributes to abiotic stress responses by modifying cell wall structure and enhancing mechanical strength. Furthermore, the upregulation of Lipocalin, a carrier protein for hydrophobic compounds, suggests its involvement in membrane protection against salt-induced damage.

Regarding nitrogen metabolism, both Glutamine synthetase and S-adenosyl methionine synthetase (SAMS) played important roles. Glutamine synthetase catalyzes the conversion of ammonia to glutamine, contributing to nitrogen recycling and amino acid synthesis. Its increased expression in tolerant genotypes reflects the plant's adaptive efforts under stress. SAMS, on the other hand, produces S-adenosyl methionine, which is involved in the synthesis of polyamines, ethylene, and methylated biomolecules, playing a key role in plant growth regulation and adaptation (Caruso *et al*., 2008).

In the realm of energy metabolism, increased expression of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected in specific genotypes. As a central enzyme in glycolysis, its upregulation suggests heightened cellular demand for ATP and NADH under stress conditions. These observations are consistent with those of Shafiei et al. (2020), who reported elevated GAPDH levels under salt stress in wheat.

Correlation analysis between proteomic data and morphological traits such as root length, dry weight, and leaf number revealed that genotypes with elevated expression of proteins like GST, ATPase, and Ferritin also demonstrated superior growth performance. This linkage between molecular and physiological

responses underscores the value of proteomic data in plant breeding applications.

Accordingly, proteins such as Glutathione S-transferase, Ferritin, ATPase, Glutamine synthetase, and Rubisco may serve as key biomarkers for screening salt-tolerant genotypes. These proteins also hold potential for use in molecular marker development, genetic engineering, and plant biotechnology. Their application at early growth stages could facilitate and accelerate the selection of stress-tolerant genotypes.

Ultimately, this study provides new insights into the proteomic responses of soybean under salinity stress and lays the foundation for leveraging this knowledge to improve sustainable production and enhance crop resilience under adverse environmental conditions.

### *3.2.2. Functional distribution of identified proteins*

The functional classification analysis of the proteins identified in soybean leaves under salinity stress revealed that these proteins are involved in diverse biological pathways. However, the highest proportions belonged to the categories of "protein folding and stress response" (20%) and "antioxidant defense" (16%). These results suggest that salinity stress activates cellular pathways associated with structural protein stability, oxidative damage repair, and stress signaling regulation.

Following these dominant categories, the groups "ion homeostasis regulation," "nitrogen and amino acid metabolism," and "photosynthetic processes and the Calvin cycle" each accounted for 12% of the total, placing them in the next highest ranks. This finding highlights that, alongside defense mechanisms, plants require the regulation of ionic balance, nitrogen metabolism maintenance, and energy restoration. The observed reduction of photosynthesis-related proteins in the sensitive cultivar, and conversely, their increased expression in the tolerant cultivar DPX, may reflect differences in the ability to sustain photosynthetic processes under stress between the two genotypes.

Additionally, although categories such as "transcriptional and cell signaling roles," "protein transport and organization," and "cell wall structural defense" had smaller shares, their roles in orchestrating secondary stress responses should not be underestimated. Altogether, these findings suggest that the plant's response to salinity stress is not confined to a single pathway but rather involves a coordinated interaction of multiple biochemical and physiological networks, which are differentially activated in various soybean genotypes (Fig. 1).

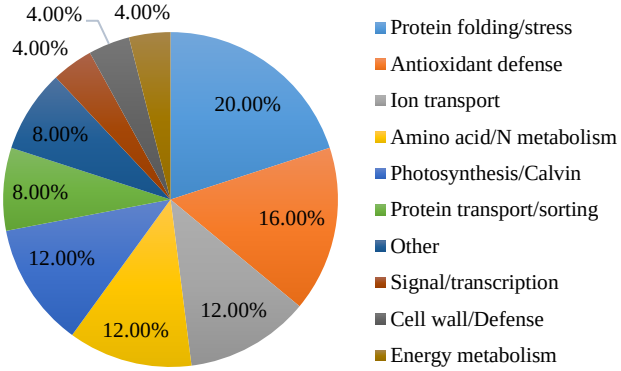


**Figure 1. Percentage distribution of identified proteins in soybean leaves under salinity stress based on functional biological categories**

The results of this study clearly demonstrated that salinity stress had significant negative impacts on the morphological traits of soybean. Reductions in stem length, dry weight of shoots and roots, and leaf number in both cultivars indicated the direct effect of salinity stress on plant growth and development. These reductions are likely due to limited access to water and essential minerals caused by increased osmotic pressure and the accumulation of toxic ions such as $Na^+$ and $Cl^-$ in plant cells (Munns and Tester, 2008; Gupta and Huang, 2014). Additionally, such changes result in reduced photosynthesis, disrupted energy metabolism, and impaired overall plant growth (Ashraf and Harris, 2013).

The comparison between the two cultivars, DPX and Arian, revealed that DPX showed better performance under salinity stress in maintaining growth and physiological traits. Specifically, shoot and root dry weights were less reduced in DPX, possibly due to its superior ability to regulate ionic balance and preserve cellular structure (Mittler, 2017). In contrast, Arian showed greater reductions in these traits, indicating its higher sensitivity to salt stress. One of the key findings of this study was the contrasting physiological responses between the two genotypes. DPX likely employs more effective mechanisms for coping with salinity, including enhanced antioxidant system activity and maintenance of photosynthetic function (Cakmak, 2008). These findings suggest that identifying and enhancing such mechanisms could play a crucial role in breeding salt-tolerant cultivars.

The results showed that salinity stress significantly affected plant dry weight. This reduction could be attributed to impaired photosynthetic activity due to damage to chloroplasts and reduced chlorophyll content (Younis *et al*., 2024). Salinity may also induce the production of reactive oxygen species (ROS), which damage proteins, lipids, and plant DNA (Atta *et al*., 2023). In the DPX cultivar, less reduction in the measured parameters was observed, suggesting a greater capacity to mitigate ROS effects through increased antioxidant enzyme activity. Such responses may help preserve cellular function and prevent salt-induced damage. Similar findings in other crops have shown that salt-tolerant genotypes typically possess stronger antioxidant systems and a better ability to maintain ionic balance. For instance, in wheat, salt-tolerant cultivars were shown to regulate potassium uptake more efficiently and reduce sodium accumulation (Gupta and Huang, 2014), in agreement with the findings for DPX in this study.

In addition to physiological observations, the analysis of protein expression patterns in leaf tissue provided more precise insights into the molecular mechanisms involved in the salt stress response. Proteomic analysis revealed that several key proteins were upregulated in DPX under stress, likely contributing to cellular and physiological resilience. Enhanced expression of enzymes such as Glutathione S-transferase (GST) and Ferritin in DPX was identified as part of an effective antioxidant response, involved in ROS scavenging and membrane protection (Moharramnejad *et al*., 2021). Furthermore, the increased expression of ATPase α-subunit in DPX was associated with ion homeostasis regulation and osmotic potential maintenance in leaf cells, supporting root growth and leaf vitality (Shafiei *et al*., 2023). Defensive and structural proteins such as Chitinase and Lipocalin also showed increased expression in DPX, indicating a role in enhancing cellular tolerance to mechanical and oxidative stress. On the other hand, photosynthesis-related proteins such as Rubisco large subunit and Phosphoribulokinase were downregulated in Arian, which may explain its greater growth reduction under salinity, as salt stress directly impairs chloroplast function and the Calvin cycle.

Additionally, enzymes such as Glutamine synthetase and S-adenosyl methionine synthetase (SAMS), which play vital roles in nitrogen metabolism and growth-related pathways, were upregulated in DPX. These enzymes contribute to the synthesis of amino acids and regulatory molecules under stress conditions (Caruso *et al*., 2008). These findings are not only consistent with the morphological results of this study but also indicate that DPX's salinity tolerance is a result of synergistic biochemical mechanisms, including energy metabolism regulation and structural preservation at the proteomic level.

In conclusion, the alignment between morphological and proteomic results in this study confirms the multilayered nature of plant responses to abiotic stress such as salinity. The DPX cultivar was able to mitigate the negative effects of salt stress by modulating defense pathways and maintaining metabolic equilibrium at the cellular level. These results highlight the importance of integrative analysis approaches (morpho-physiological + proteomics) in understanding salinity tolerance and identifying resilient genotypes. Therefore, the findings of this study can serve as a foundation for developing biomarkers, advancing genetic improvement programs, and designing agronomic strategies for managing saline environments.

## 4. Conclusion

This study clearly demonstrates that salinity stress imposes substantial adverse effects on soybean growth and physiology by disrupting water balance, inducing ionic toxicity due to $Na^+$ and $Cl^-$ accumulation, and increasing the production of reactive oxygen species (ROS). These changes collectively impair cellular integrity, reduce photosynthetic efficiency, and hinder energy metabolism, thereby compromising plant development. Comparative analysis of the two cultivars revealed that DPX exhibited greater tolerance to salinity stress than Arian. Morphologically, DPX showed less reduction in shoot and root growth and maintained higher biomass accumulation. At the proteomic level, the enhanced expression of key stress-related proteins such as Glutathione S-transferase (GST), Ferritin, ATPase α-subunit, and Glutamine synthetase in DPX highlighted its ability to activate antioxidant defense, ion homeostasis, and metabolic resilience under stress conditions. In contrast, Arian displayed downregulation of vital photosynthetic and metabolic proteins, including Rubisco, which corresponded with leaf senescence and severe growth inhibition.

These findings confirm that salt tolerance in soybean is governed by a complex network of physiological and molecular responses. The integration of morphological and proteomic data provides a robust framework for understanding stress adaptation mechanisms and identifying key biomarker proteins associated with salinity resilience. Proteins such as GST and ATPase may serve as promising markers for use in marker-assisted selection (MAS) and genetic engineering strategies aimed at improving salt tolerance in soybean breeding programs. From an applied perspective, the DPX cultivar represents a valuable genetic resource for enhancing soybean productivity in saline environments. Future research should build upon these insights by employing multi-omics approaches—including transcriptomics, metabolomics, and epigenomics—to further unravel the regulatory networks and cross-talk among pathways that contribute to salinity tolerance in legumes and other crop species.

## Conflict of interests

All authors declare no conflict of interest.

## Ethics approval and consent to participate

No humans or animals were used in the present research. The authors have adhered to ethical standards, including avoiding plagiarism, data fabrication, and double publication.

## Consent for publications

All authors read and approved the final manuscript for publication.

## Availability of data and material

All the data are embedded in the manuscript.

## Authors' contributions

All authors had an equal role in study design, work, statistical analysis and manuscript writing.

## Informed consent

The authors declare not to use any patients in this research.

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