



PROTEOMIC EVALUATION OF TRITICUM BOEOTICUM RESPONSE TO SALINITY STRESS

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ABSTRACT

Wild wheat (*Triticum boeoticum*) has been an essential agricultural crop and primary food source since ancient times. With the growing population and increasing demand for agricultural products, particularly wheat, boosting wheat production is crucial. Due to the limited arable land, enhancing wheat production requires improving yield per unit area, which can be achieved through advanced agricultural practices and developing varieties resistant to diseases and environmental stresses such as salinity. Salinity stress is a significant non-biotic stress that adversely affects the quantity and quality of agricultural products, with 20% of the world's irrigated agricultural lands affected. Understanding the molecular mechanisms of salinity stress tolerance in wheat is therefore vital. In 2017, an experimental study was conducted in a controlled growth chamber at the Faculty of Agriculture, University of Tehran. Five populations of wild wheat (*Triticum boeoticum*) were evaluated using a pot culture. The study was designed as a factorial experiment in a completely randomized design with three replications in a greenhouse. Factors included genotype (5 genotypes) and salinity (0 and 150 mM). Seeds were sown in plastic pots, and after two weeks, at the beginning of the three-leaf stage, salinity stress was applied using 150 mM NaCl. Two weeks after salinity stress application, seedlings were harvested, and traits such as shoot and root length, fresh and dry weight of shoots, total protein, and chlorophyll and carotenoid content in leaf tissues were measured. Results showed that population B4 had the highest values for all traits except shoot height. The mean effect of population on traits such as shoot length, root length, chlorophyll a, and b indicated that population A1, followed by B4, had the highest values for shoot height. The lowest values for shoot length, root length, chlorophyll a, and b were observed in populations B3, C5, B3, and A10, respectively. The interaction effect of population and salinity on traits such as chlorophyll a, b, and total carotenoids was not significant. This might be due to the higher tolerance of wild wheat populations to salinity stress over the two-week stress application period. The mean interaction effect of population and salinity on total protein showed that population B4 had the highest value under salinity stress, while population A1 had the lowest value under salinity stress. This suggests that population B4 responds to salinity stress by altering protein expression to tolerate these conditions. Proteomic analysis of genotype B4 using two-dimensional electrophoresis identified 10 protein spots with significant

expression changes, with 9 spots showing increased expression and 1 spot showing decreased expression. The identified proteins were categorized into various biological function groups, including energy metabolism, translation, protein processing and degradation, photosynthesis, defense, and resistance mechanisms.

Keywords: Wild Wheat, Salinity Stress, Proteomics, Genotype, Chlorophyll, Resistance.

INTRODUCTION

Wild wheat (*Triticum boeoticum*) has been an essential agricultural plant and a major food source for humans long before other plants were utilized for consumption purposes (Khoda Bendeh, 2003). Considering the population growth and increasing demand for agricultural products, especially wheat, it is crucial to enhance wheat production in the country. Due to the limited arable land, increasing wheat yield per unit area is necessary, achievable through advanced agricultural practices and developing high-yielding, disease-resistant, and stress-tolerant varieties (Ahdaei, 2009). Plant stress refers to deviations in physiology, growth, and natural performance, often causing irreversible damage. Biotic stresses are caused by living organisms like pathogens, while abiotic stresses result from non-living factors such as salinity, temperature, and soil properties (Nagarajan, 2010). Environmental stresses, including drought, salinity, and cold, significantly impact agricultural plant performance and survival (Latini et al., 2007). Salinity stress is a major abiotic factor affecting agricultural products' quantity and quality, with 20% of irrigated agricultural lands globally affected (Zhao et al., 2007). In Iran's arid and semi-arid regions, nearly 50% of cultivated areas face salinity and alkalinity issues (Mirmahdi Meybodi & Qara Yazdi, 2003). Proteomics technology, with advancements in protein extraction, separation, and identification techniques, plays a key role in developing protein sets associated with biological activity (Wang et al., 2011). Proteomics, based on two-dimensional electrophoresis, identifies proteins by isoelectric points and molecular weights, aiding in identifying stress resistance genes (Chen & Harmon, 2006). Numerous studies have used proteomics to identify salinity-responsive proteins, revealing their roles in energy metabolism, ROS inhibition, detoxification, protein translation, and more (Jiang et al., 2007). Wild relatives of crop plants are valuable sources for salinity tolerance genes. This study examined ten populations of *Triticum boeoticum* under 0- and 150-mM salinity stress in a greenhouse. Sodium and potassium ions were measured, and the most tolerant population was identified based on Na⁺ concentration and K⁺/Na⁺ ratio. Two-dimensional electrophoresis identified 177 reproducible spots, with 13 showing differential expression under salinity stress, indicating adaptive responses to high salinity (Alavi et al., 2016). Significant differences were found among 20 wheat genotypes for all indices and grain yield under both salinity stress and non-stress conditions. Correlation analysis identified MP, GMP, STI, and HM as effective for selecting high-yielding genotypes, with the HM index particularly important for identifying salt-tolerant genotypes. Genotypes 7 and 8 were the most tolerant, with the highest yields under salinity stress (Makarian et al., 2021). Soil salinity adversely affects agricultural production and food security. Tritipyrum, an amphiploid wheat (2n=6x=42, AABBEBeb), derived from *Triticum durum* (2n=4x=28, AABB) and *Thinopyrum bessarabicum* (2n=2x=14, EbEb), serves as a dual-purpose crop. In a 2019 hydroponic study at Shahrekord University, various physiological traits of primary Tritipyrum lines were evaluated under 0, 100, and 200 mM NaCl salinity. Significant differences were found among genotypes for all studied traits. Under 200 mM salinity, the promising Tritipyrum line (Ka/b) (Cr/b) (F6) had the highest biological and grain yields with the lowest shoot sodium concentration, indicating a salinity tolerance mechanism by limiting sodium translocation to shoots (Razavi Jahromi et al., 2022). This study investigates the proteomic changes in *Triticum boeoticum* (a wild wheat species) under salinity stress conditions. Researchers used advanced proteomic techniques to identify key proteins involved in the plant's salinity tolerance mechanisms. The results of this research indicate that certain proteins are activated to help the plant cope with harsh salinity conditions, for example, by limiting sodium translocation to the shoots and maintaining osmotic balance within the cells

Razavi Jahromi et al. (2022). This article examines the adaptive mechanisms of *Triticum boeoticum* (a wild wheat species) in coping with salinity stress through proteomic analysis. Researchers used advanced proteomic techniques to identify key proteins that help the plant adapt to high salinity conditions. The results indicate that certain proteins activate specific biological pathways that assist the plant in limiting sodium translocation to the shoots and maintaining osmotic balance within the cells, ultimately enhancing the plant's tolerance to salinity Liu H et al. (2015).

MATERIALS AND METHODS

This study was conducted as a factorial experiment in a completely randomized design with three replications in a research greenhouse. Five populations of wild wheat (*Triticum boeoticum*) were evaluated in pot cultivation. The factors included genotype (5 genotypes) and salinity (two levels: 0 and 150 mM NaCl). Seeds were sown in plastic pots, and two weeks after growth at the beginning of the three-leaf stage, stress was applied using 150 mM NaCl. Seedlings were harvested two weeks after the application of salinity stress, and the traits of shoot and root length, fresh and dry weight of aerial parts, total protein, and chlorophyll and carotenoid content in leaf tissue were measure.

Table1: Here is a table of the diploid wheat masses used in this research

Code	Collection Location	Row
A1	Lorestan - Dorud	1
A10	Lorestan - Lorestan	2
B3	Kermanshah - Kamyaran	3
B4	Kermanshah - Paveh	4

Experimental Site Specifications:

The experiment was conducted under laboratory conditions in the greenhouse of the Faculty of Agriculture at Karaj University in the year 2017. Growth conditions were as follows:

- Temperature: 24-25°C during the day and 20-21°C at night.
- Light: 15 hours of daylight and 9 hours of darkness.
- Humidity: 50-60%.

EXPERIMENTAL METHOD

For seed planting, 16 cm diameter pots were selected, and four small holes were made at the bottom of each pot to drain excess water and prevent root suffocation. The pots were filled with fine sand (to eliminate nutrients). After moistening the sand, seeds were evenly distributed to ensure uniform density. A small amount of distilled water was sprinkled over the pots. The experiment was conducted in a completely randomized design with three pots for each treatment as three replications. The treatment combination included 5 *Triticum boeoticum* genotypes and two stress levels, forming the experimental treatments.



Figure1: Harvesting Roots and Shoots

The length of aerial parts and roots was measured using a ruler in centimeters. The fresh weight of aerial parts was measured using a digital scale. The dry weight of aerial parts was measured after drying the samples in an oven at 72°C for 48 hours, and the data were recorded in grams using a digital scale.



Figure 2: Plants after NaCl Stress Application

The Arnon method was used to measure photosynthetic pigments. Half a gram of fresh plant material was ground and homogenized with liquid nitrogen. Twenty milliliters of 80% acetone was added to the sample, which was then centrifuged at 6000 rpm for 10 minutes. The supernatant was transferred to a glass flask, and absorbance was read at 663 nm for chlorophyll a, 645 nm for chlorophyll b, and 470 nm for carotenoids using a spectrophotometer. Pigment concentrations were calculated using specific formulas.

Protein Concentration Determination

Protein concentration was determined using the Bradford method (1976). The stock dye solution was prepared by dissolving 0.1 grams of Coomassie Brilliant Blue G-250 in 50 milliliters of ethanol, filtering, and then adding 100 milliliters of 85% phosphoric acid. The volume was then adjusted to 200 milliliters with distilled water. The prepared solution was stored in a dark bottle at 4°C indefinitely. For the assay solution, one volume of the stock dye solution was diluted with four volumes of distilled water. The solution was freshly prepared and slightly warmed before use. The BSA protein stock solution was prepared by dissolving 0.1 grams of bovine serum albumin in 50 milliliters of 0.15 M NaCl to reach a concentration of 2000 micrograms per milliliter. The standard curve was prepared according to the equation $C1V1=C2V2$.

Table 2: Required Components for Standard Preparation

Total Volume (µl)	Water Volume (µl)	Lysis Buffer Volume (µl)	Protein Volume (µl)	Concentration (µg/ml)
60	55	5	0	0
60	52	5	3	100
60	46	5	9	300
60	40	5	15	500
60	34	5	21	700
60	25	5	30	1000

To plot the standard curve, 60 microliters of each concentration were added to 3 milliliters of assay solution and placed in a quartz cell (cuvette). Absorbance of the prepared samples was measured at 595 nm, and a linear increasing trend graph was plotted by the spectrophotometer. For measuring the protein concentration of samples, 5 microliters of protein solution in lysis buffer were added to 55 microliters of deionized water and then to 3 milliliters of assay solution. After measuring the absorbance against the blank (55 microliters of deionized water plus 5 microliters of lysis buffer and 3 milliliters of assay solution), the absorbance values were applied to the standard curve formula to determine the protein concentration in micrograms per milliliter. Since the samples were diluted 12 times, the obtained concentrations were multiplied by 12 to get the actual concentrations.

Table 3: Absorbance Values of Standards

Concentration (Y axes)	Absorbance (X axes)
0	0
100	141/0
300	314/0
500	544/0
700	774/0
1000	974/0

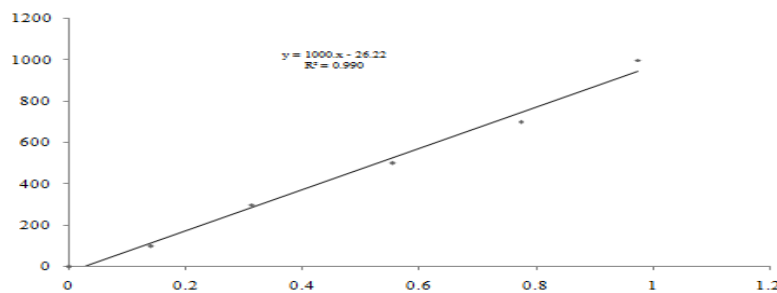


Chart (1): Standard Curve for Protein Concentration Determination

In this experiment, proteins were dissolved in stock rehydration solution and simultaneously loaded into the gel during the rehydration process. The required amount of protein varies depending on the staining method, the length of the gel, the pH range, and whether the gel is used for analysis or spot separation. Table 4-5 shows the amount of protein needed based on the staining system and the type of gel usage. In this experiment, 320 microliters of rehydration solution (8M Urea, 2% CHAPS, 0.002% Bromophenol Blue, 2% IPG Buffer, 0.018M DTT) containing 120 micrograms of protein were pipetted into the rehydration tray. The IPG Strip (17cm) was placed on the slot with the gel edge facing down. Rehydration took 14 to 16 hours. For the first-dimension electrophoresis, the MultiPhor II system was used for IEF. The power supply was set to a gradient program. For a 17cm gel with pH 4-7, 42KVh was applied in four stages: 150 Vh to 300 V, 300 Vh to 500 V, 2000 Vh to 3500 V, and 39500 Vh at 3500 V. A constant current of 1mA and power of 5W were maintained. The process took approximately 15 to 16 hours. After rehydration, the gels were either stored at -20°C or prepared for the second dimension.

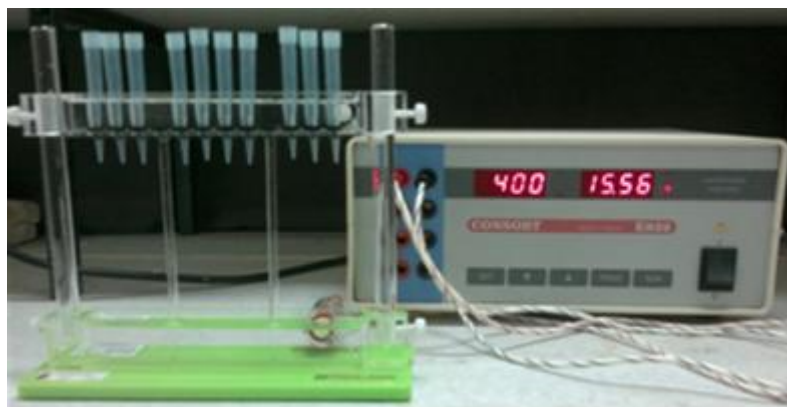


Figure 3: Protean II xi Cell Electrophoresis System

First Dimension Electrophoresis Device for Protein Separation Based on Isoelectric Point (IEF) To perform the second dimension, the Protein II Xi Cell from Bio-Rad was used. The electrophoresis device was thoroughly cleaned and prepared, then the gel cassette was assembled and filled with 12.5% acrylamide solution, covered with n-butanol. The first-dimension gel strip was equilibrated in a specific solution and placed on the second-dimension gel, covered with 0.5% molten agarose. The electrophoresis was run in three stages: 5 mA for 2 hours, 10 mA for 6 hours, and 20 mA for 3 hours, at 4°C.

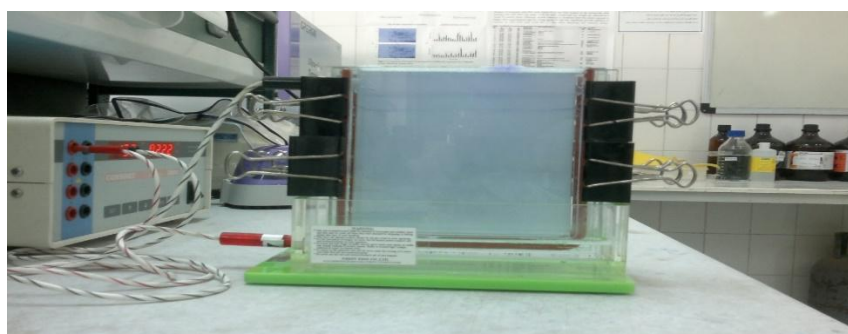


Figure 4: Bio-Rad Protein II Xi Cell electrophoresis device

RESULTS AND DISCUSSION

Physiological Analysis

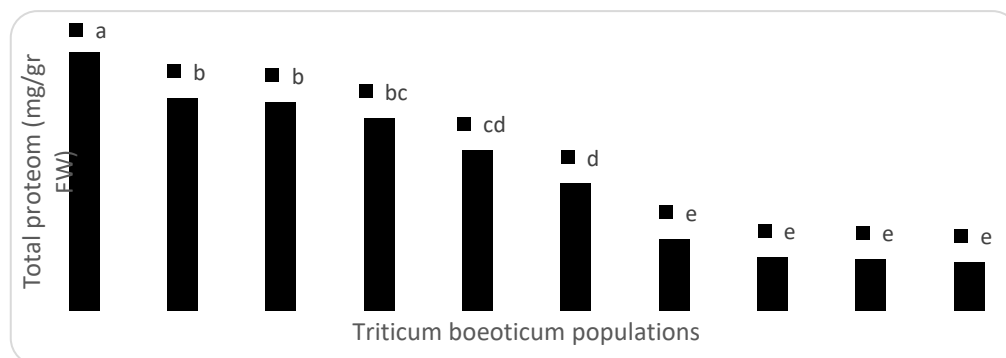
In this study, traits such as shoot and root length, fresh and dry weight of shoots, total protein, chlorophyll a, b, and total carotenoid were statistically analyzed. Analysis of variance results indicated that the population effect was significant for all traits except for total carotenoid. Additionally, the effect of salinity stress was significant for all traits except for chlorophyll a. The interaction effect of population and salinity stress was significant only for the traits of fresh and dry weight of shoots and total protein (Table 1).

Carotenoid	Total Chlorophyll	Chlorophyll b	Chlorophyll a	Total Protein (mg/g)	Dry Shoot Weight	Fresh Shoot Weight	Root Length	Shoot Length	DF	Source of Variation
361.9	159.2**	5.27*	137.4**	57218.4**	0.0012**	0.023*	4.16**	45.12**	4	Population
3490.7**	118.1**	45.4**	17.03	14830.7**	0.019**	0.21**	15.95**	151.33**	1	Salinity
442.9	9.18	0.78	4.97	4831.9**	0.0043**	0.011**	0.73	3.39	4	Population * Salinity
260.8	8.79	1.52	6.97	227.1	0.000029	0.00076	1.03	3.61	20	Error

The mean comparison results of populatin effects on traits such as shoot length, root length, and chlorophyll a and b showed that population B4 had the highest values for all traits except shoot height (Table 2). For the shoot height trait, population A1 and then B4 had the highest values. The lowest values for shoot length, root length, chlorophyll a, and b were attributed to B3, C5, B3, and A10 respectively (Table 2). A reduction in photosynthetic pigment content under prolonged salinity stress conditions has been reported for many salinity-sensitive plants (Weisany et al. 2011; Askari et al. 2015, Abedini 2016). However, in this study, the interaction effect of population and salinity was not significant for chlorophyll a, b, and total carotenoid traits. One possible reason could be the higher tolerance of wild populations of wheat to salinity stress during the 2-week salinity stress application period.

T. boeoticum populations	Shoot height (cm)	Root height (cm)	Chlorophyll a (mg/gr FW)	Chlorophyll b (mg/gr FW)	Total Chlorophyll
A1	43.05 A	15.15 AB	21.49 B	6.49 AB	27.98 BC
B4	39.37 B	15.7 A	24.95 A	7.13 A	32.08 A
C5	38.15 BC	13.7 C	23.35 AB	7.98 A	31.34 AB
A10	37.27 BC	13.9 BC	21.05 B	5.42 B	26.48 C
B3	35.81 C	14.2 BC	12.59 C	6.59 AB	19.18 D

Under normal conditions, population B4 had the highest fresh weight and performed best under salinity stress. For dry weight, A1 excelled in normal conditions, while B4 and C5 were superior under salinity. B4 exhibited the highest total protein under salinity stress, suggesting its adaptive response through altered protein expression.



Mean comparison of the interaction effects for the trait of total protein (Chart 3)

After obtaining the images, protein spots on the gels were detected using Melanie 7 software. Analysis revealed 205 reproducible protein spots, which were statistically examined. Seventeen protein spots showed a 1.5-fold change in expression when comparing salinity treatment with control; ten spots increased in expression, and seven disappeared under stress. In population B4, ten protein spots showed a 1.5-fold change under salinity, with nine increasing and one decreasing in expression (Table 3).

Landmark	Match ID	Name	IF	M S Score	% Covera ge	Theoreti cal MW/PI	Experimen tal MW/PI
147	142	predicted protein [Hordeum vulgare subsp. vulgare]	+2.62	95	34	26.696/4 .78	24/4.31
119	114	2-Cys peroxiredoxin BAS1,	+1.3	79	33	23.398/5	24/4.97

		chloroplast				.48	
40	39	cytosolic malate dehydrogenase, partial [Triticum aestivum]	+1.48	12 7	81	24.56/6. 60	44/5.99
98	93	Thylakoid luminal 29 kDa protein, chloroplast [Triticum Urartu]	+3.14	17 4	71	38.454/7 .59	27/5.34
113	108	thioredoxin-like protein CDSP32, chloroplast [Aegilops tauschii subsp. tauschii]	+2.13	11 9	62	33.179/6 .16	35/5.54
85	80	PREDICTED: importin-5-like [Nicotiana tabacum]	+2.35	92	34	131.264/ 5.04	29/5.06
Landmark	Match ID	Name	IF	M S Score	% Covera ge	Theoreti cal MW/PI	Experiment al MW/PI
147	142	predicted protein [Hordeum vulgare subsp. vulgare]	+2.62	95	34	26.696/4 .78	24/4.31
119	114	2-Cys peroxiredoxin BAS1, chloroplast	+1.3	79	33	23.398/5 .48	24/4.97

The results indicated that wheat genotypes with a higher potassium-to-sodium ratio had higher grain yield and better salinity response (Khan et al. 2009). In sensitive rice varieties, PSII performance dropped to zero under salinity stress, potassium levels decreased, and the potassium-to-sodium ratio minimized, unlike the tolerant varieties (Morales et al. 2012). Maintaining a higher potassium-to-sodium ratio is crucial for increased stress tolerance, as observed in tolerant genotypes.

CONCLUSION

The comparison results showed that population B4 had the highest values for all traits except shoot height. For shoot height, population A1 and B4 had the highest values, while the lowest values for shoot length, root length, chlorophyll a, and b were attributed to B3, C5, B3, and A10 respectively. The interaction effect of population and salinity on chlorophyll a, b, and total carotenoids was not significant. This may be due to the higher tolerance of wild populations of *Triticum boeoticum* to salinity stress over the 2-week period. The mean comparison of population effects under salinity stress for total protein showed that population B4 had the highest value under salinity stress, indicating its adaptive response through protein expression changes. Proteomic analysis of population B4 using two-dimensional electrophoresis identified 10 protein spots with significant expression changes, with nine increasing and one decreasing. The proteins were classified into key functional groups such as energy metabolism, translation, protein processing and degradation, photosynthesis, defense, antioxidant activity, and resistance mechanisms.

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