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Salinity stress responsive leaf proteins in alfalfa (*Medicago sativa* L.)

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Abstract
Salinity stress is one of the most harmful abiotic stresses that affect agronomical, physiological and biochemical processes in crop plants. In order to evaluate the effect of salinity stress on alfalfa (Bami ecotype) leaf proteins based on two-dimensional gel electrophoresis, an experiment was conducted with two salinity treatments (0 and 200 mM NaCl) and six replications under hydroponic cultural system. Salinity stress decreased fresh and dry weight of alfalfa about 24.5 and 39.5 percent, respectively. Proteome analysis was carried out on the leaf tissue using three replications. Fourteen repeatable protein spots had significant change in expression under salt stress. Probabilistic identification of proteins from the data bank was performed by isoelectric point and molecular weight. Nine proteins showed significant up-regulation and five proteins had down regulation. Candidate proteins were among the proteins involved in the defense system, regulation, metabolic pathways, nitrogen fixation and canalization. Higher abundance of trehalose-phosphate phosphatase 2, involved in regulation, and also uracil phosphoribosyl transferase lastic and β-hydroxyisobutyryl-CoA hydrolase 1, involved in the energy metabolism, demonstrated the important function of these proteins under salinity stress. It seems that the alfalfa plant manages to reduce the adverse effects of salinity through the candidate proteins identified in this investigation.

Keywords: Abiotic stress; Defense system; Metabolism; Proteomics; Two-DE gel.

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Introduction

Salinity stress is one of the most important environmental limiting factors that reduces crop production, especially in arid and semi-arid areas (Eilers *et al.* 1997). In Iran, this event is going to occur widely in the north-west provinces due to drying of Urmia Lake, where 15% of Iran's agricultural lands are located (Karbassi *et al.* 2010).

Alfalfa (*Medicago sativa* L.) is one of the major forage crops growing in the temperate areas. It is cultivated in 34 million hectares worldwide (Michaud *et al*. 1988). Alfalfa production area in Iran is more than 620000 hectares (Anonymous 2019). North-western provinces are considered as the major alfalfa growing areas in of Iran. Cultivated alfalfa in Iran mostly consisted of various adaptive landraces, including Ghara-Yonjeh, Hamadani and Bami as well as some foreign cultivars such as Ranger and Maopa (Valizadeh *et al.* 2011).

Improving salt resistant crops will greatly increase the world's food supply. To effectively grow more salt tolerant crops, the genetic mechanisms of salt tolerance have to be determined (Valizadeh *et al.* 2013). Salinity influences the growth and production of alfalfa negatively by osmotic stress and toxic Na^+ and Cl^- ions (Munns 1992). Furthermore, salt stress triggers high accumulation of reactive oxygen species (ROS)

which may damage the biological membranes (Ashraf 2009). For example, different types of cellular ROS (superoxide radical, hydrogen peroxide, singlet oxygen and the hydroxyl radical) are frequently generated in cells under salt stress (Mittler 2002). These oxidants are highly reactive and damage proteins, lipids, carbohydrates and DNA (Gill and Tuteja 2010). However, to counteract with the oxidative damage, most plants have efficient enzymatic and non-enzymatic defense systems to protect the cells by scavenging of ROS (Gill and Tuteja 2010).

Plants adapts themselves to salinity through biochemical and molecular pathways (Bohnert *et al*. 1995; Munns and Tester 2008). Changes in gene expression due to the impact of different environments are a typical reaction in plant cell metabolism (Seki 2003). Activation of genes by environmental stimuli has a vital role in adaptation of plants to adverse conditions by regulating the production of different proteins (Sachs and Ho 1986). Exploring new strategies conferring salinity stress to plants will be useful in developing of salt-tolerant cultivars (Ashraf 2009).

Proteomics is an effective approach for studying the molecular response of alfalfa plants to salt stress. Gao *et al*. (2011) identified various proteins such as those associated with transport, detoxification, carbon metabolism and protein folding. A wide range of proteomic experiments on different alfalfa tissues, such as roots and shoots (Xiong *et al.* 2017), leaves (Zeng *et al.* 2019) and seeds (Yacoubi *et al.* 2013) have been reported.

There are insufficient details regarding the protein networks in alfalfa under salinity stress (Ma *et al*. 2016). In this investigation we studied the possible change in the expression of leaf proteins of the Bami ecotype under salt stress using 2-DE gel.

Materials and Methods

Plant and experimental conditions

Alfalfa seeds of the Bami ecotype was obtained from the Research Station of the Agricultural Research, Education and Extension Organization, Tabriz, Iran. This ecotype is regarded as of the popular alfalfa ecotypes in Iran. Seeds were surface sterilized with 2.5% sodium hypochlorite solution for three minutes, followed by washing several times with distilled water. The seeds were germinated in Petrie dishes containing of filter paper dampened with the Thiram solution for a week and then moved to the Hoagland solution (Hoagland and Arnon 1950). The greenhouse conditions were as follows: relative humidity (60%), temperature (25±2 °C day/night), light (14 h daily) and pH of Hoagland solution. Salt stress (NaCl concentration at 200 mM), was imposed by incubation of the seedlings in Hoagland solution for three weeks. The plants were also grown on Hoagland medium without NaCl as the control.

Protein extraction and two-dimensional electrophoresis

The protein were extracted in TCA-acetone as explained by Pavoković *et al.* (2012).To obtain the leaf sample, newly well-developed third leaves were collected. The fresh leaves (300 mg) were ground to powder in the liquid nitrogen with a mortar and pestle. The powdered material was added to a solution containing 10% trichloroacetic acid and 0.07% 2-mercaptoethanol in acetone and

shaked/vortexed. The suspension was incubated at -20 °C for 45 min and, then centrifuged at 15000 × g for 25 min at 4 °C. The supernatant was removed and the pellet was returned to the solution of 2-mercaptoethanol (0.07%) in acetone for 60 min. The resulting suspension was centrifuged (20,000 × g at 4 °C for 5 min) and washed twice at with 2-mercaptoethanol (0.07%) in acetone. The resulting pellet was dried, and then suspended in lysis buffer with the following composition: thiourea (2 M), urea (7 M), tributylphosphine (2 mM) and CHAPS (5%). The concentration of protein was estimated using BSA as standard (Bradford 1976). The extracted proteins were loaded on the first dimension/Isoelectric Focusing (IEF). The proteins were separated over a pH range of 3 to 10, based on their isoelectric point. The second electrophoretic dimension was by SDS-PAGE. Coomassie Brilliant Blue (CBB) was used to visualize the spots. The gel scan was performed using Bio-Rad GS-800 densitometer.

Protein identification

The proteins involved in modification were surveyed by PDQuest software. To identify protein spots with significant changes in expression, proteins with the quantitative ratio of more than 2 and less than 0.5 were considered as up-regulated and down-regulated, respectively ($p \leq 0.05$). Proteins were identified by Expasy-TagIdent (http://web.expasy.org/tagident/) online tool in UniProtKB/Swiss-Prot data base, using their isoelectric point (pI) and molecular weight.

Statistical analysis

The statistical significance of the two groups was determined by t-test ($p \leq 0.05$) with three replications for gels and six replications for fresh and dry weight. Data were analyzed using SPSS (version: 23.0) for Windows (SPSS, Inc., Chicago, USA).

Results and Discussion

Salinity had a negative effect on growth of alfalfa (Figure 1) and significantly reduced the fresh and dry weight of the Bami ecotype (Figure 2). Reduction in plant growth caused by salt stress could be attributed to the negative effect of salinity on some key physio-biochemical processes regulating plant growth, e.g. photosynthesis, nutrient uptake/accumulation, osmolyte accumulation and enzyme activities, which eventually leads to the reduction of crop growth (Ashraf 2009; Ashraf and Harris 2013). In several studies on alfalfa, salt stress has affected biomass production. Valizadeh *et al.* (2013) found 33.5% growth reduction in alfalfa when salinity was imposed at the seedling stage. In a study by Farissi *et al*. (2014), the increase in concentration of NaCl steadily decreased plant biomass in alfalfa.

Study of proteome pattern in the alfalfa leaf, identified 112 reproducible protein spots under salt stress, from which 14 spots had significant change in in abundance (Figure 3). These protein spots were identified by their isoelectric point (pI) and molecular weight (Mw). Differentially expressed proteins were categorized into five functional groups (Figure 4), including defense (36%), regulation (29%), energy metabolism (21%), nitrogen fixation (7%) and canalization (7%). Of these 14 proteins, nine proteins had up-regulation and five proteins had down-regulation (Figure 5).

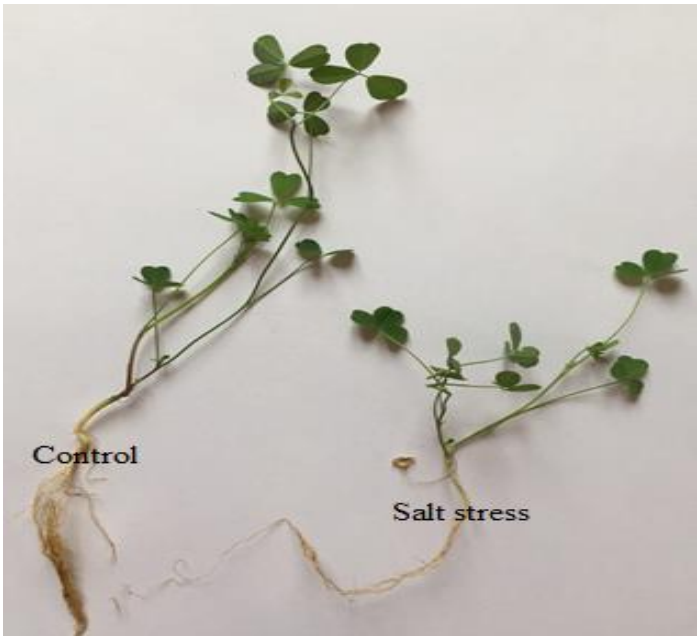

Figure 1. Effect of salt stress on growth of alfalfa (Bami ecotype).

Ma *et al*. (2016) detected 800 protein spots on 2-DE gels, out of which 35 proteins showed significant changes under salinity stress. They classified the detected spots into several functional groups such as carbohydrate metabolism, photosynthetic metabolism, folding and assembly, stress and defense, nucleic acid metabolism and protein biosynthesis. Xiong *et al*. (2017) found 27 and 36 spots in the shoots and roots of alfalfa under salinity by MALDI-TOF-TOF MS, respectively. These spots had significant change in abundance and were involved in the functions including cell wall and cytoskeleton metabolism, energy and protein metabolism, secondary metabolism, ion transport, photosynthesis, signal transduction, transcriptional regulation and stress and defense.

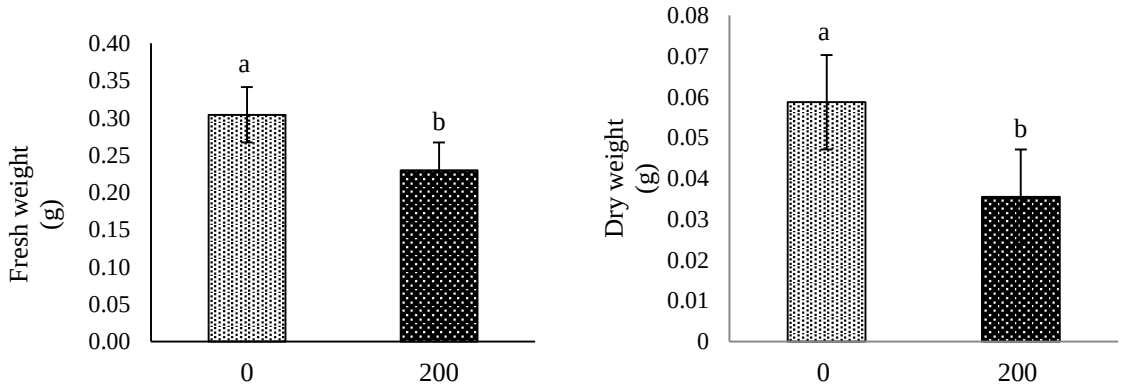

Figure 2. Fresh and dry weight of the alfalfa ecotype of Bami under salinity stress.

The protein spots 2305, 4501, 5102, 8401 and 9402 were classified in the defense system group (Table 1), including cinnamyl alcohol dehydrogenase, receptor-like protein 42, LIM domain-containing protein PLIM2a, UDP-glycosyltransferase 73B4 and glutamyl-tRNA reductase 2, chloroplastic, respectively. Cinnamyl alcohol dehydrogenase, receptor-like protein 42 and glutamyl-tRNA reductase 2, chloroplastic showed significant decrease in abundance, and LIM domain-containing protein PLIM2a and UDP-glycosyltransferase 73B4 had significantly increased abundance under salt stress (Figure 5).

Cinnamyl alcohol dehydrogenase and receptor-like protein 42 are related to lignin biosynthesis and pattern recognition receptors, respectively (Brill *et al.* 1999; Zhang *et al.* 2014). The LIM family is an eukaryotic-specific protein family, and has LIM domain including cysteine-rich zinc-binding domain (Srivastava and Verma 2017). Park *et al.* (2014) stated that LIM genes may be involved in resistance against biotic and abiotic stresses in *Brassica*. These proteins may reduce the effect of salinity in alfalfa.

The protein spots 3403, 4109, 5105 and 7102 were in the regulator protein group (Table 1),

Table 1. Identification of differentially expressed protein spots in alfalfa (Bami ecotype) under salinity stress using isoelectric point (pI) and molecular weight (MW) of spots in UniprotKB/Swissprot database.

Spot No.	Identified protein	Accession[a] No.	O.pI[b]	T.pI[c]	O.MW[d] (KD)	T.MW[e] (KD)	Plant
2006	Leghemoglobin	P28010	5.38	5.34	14.61	15.93	Alfalfa
2305	Cinnamyl alcohol dehydrogenase	P31656	5.51	5.56	37.32	38.95	Alfalfa
3403	Trehalose-phosphate phosphatase 2	Q09WE7	5.64	5.69	65.34	66.12	Soybean
4109	Cell number regulator 1	B6TZ45	5.94	5.91	20.84	20.46	Maize
4501	Receptor-like protein 42	Q9LJS0	5.7	5.69	96.08	96.11	*Arabidopsis*
5102	LIM domain-containing protein PLIM2a	O80839	6.41	6.44	24.37	24.94	*Arabidopsis*
5105	DNA-directed RNA polymerase IV subunit 7	Q8LE42	6.14	6.10	20.51	19.85	*Arabidopsis*
5111	Uracil phosphoribosyltrachloropnsferase, lastic	Q9M336	6.35	6.30	25.89	25.16	*Arabidopsis*
6503	Triacylglycerol lipase SDP1	Q9LZA6	6.55	6.55	92.09	93.09	*Arabidopsis*
7102	NAC domain containing protein 52	Q9SQY0	7.01	7.03	23.19	26.95	*Arabidopsis*
7302	Voltage-gated potassium channel subunit beta	O23016	6.96	6.93	36.98	36.53	*Arabidopsis*
8115	β-hydroxyisobutyryl-CoA hydrolase 1	Q9LKJ1	7.62	7.66	23.17	23.25	*Arabidopsis*
8401	UDP-glycosyltransferase 73B4	Q7Y232	7.16	7.13	52.28	53.96	*Arabidopsis*
9402	Glutamyl-tRNA reductase 2, chloroplastic	P49294	7.72	7.75	50.56	51.33	*Arabidopsis*

[a]UniProtKB-SWISS-Prot
[b]Observed isoelectric point
[c]Theoretical isoelectric point
[d]Observed molecular weight
[e]Theoretical molecular weight

including trehalose-phosphate phosphatase 2, cell number regulator 1, DNA-directed RNA polymerase IV subunit 7 and NAC domain containing protein 52, respectively. All these regulator proteins were significantly up-regulated. Trehalose-phosphate phosphatase 2 (3403) and cell number regulator 1 (4109) showed the highest abundance in this study (Figure 5). The results indicated that the regulator protein group could be important in the alfalfa ecotype of Bami under salt stress. According to Höper *et al.* (2006), large amount of energy is required for the growth and development of *Kandelia candel* under salt stress and this energy is mainly provided by metabolism of carbohydrates. NAC domain proteins are involved in response to biotic and abiotic stresses (Nakashima *et al.* 2012).

The protein spots 5111, 6503 and 8115 were classified in the energy metabolism protein group (Table 1). This group includes uracil phosphoribosyltrachloropnsferase, lastic, triacylglycerol lipase SDP1 and β-hydroxyisobutyryl-CoA hydrolase 1, respectively. Uracil phosphoribosyltrachloropnsferase, lastic (5111) and β-hydroxyisobutyryl-CoA hydrolase 1 (8115) showed significant increase in abundance, and triacylglycerol lipase SDP1 (6503) had a decrease in abundance (Figure 5). Uridine monophosphate is the precursor of pyrimidine nucleotide in the biosynthesis of pyrimidine. Some ubiquitous metabolic processes such as RNA and DNA metabolism are linked via their utilization of nucleotides (Mainguet *et al.* 2009). B-hydroxyisobutyryl-CoA hydrolase plays a role in valine metabolism (Zolman *et al.* 2001).

The protein spot 2006 (leghemoglobin) is a nitrogen fixation factor and 7302 (voltage-gated potassium channel subunit beta) is a canalization

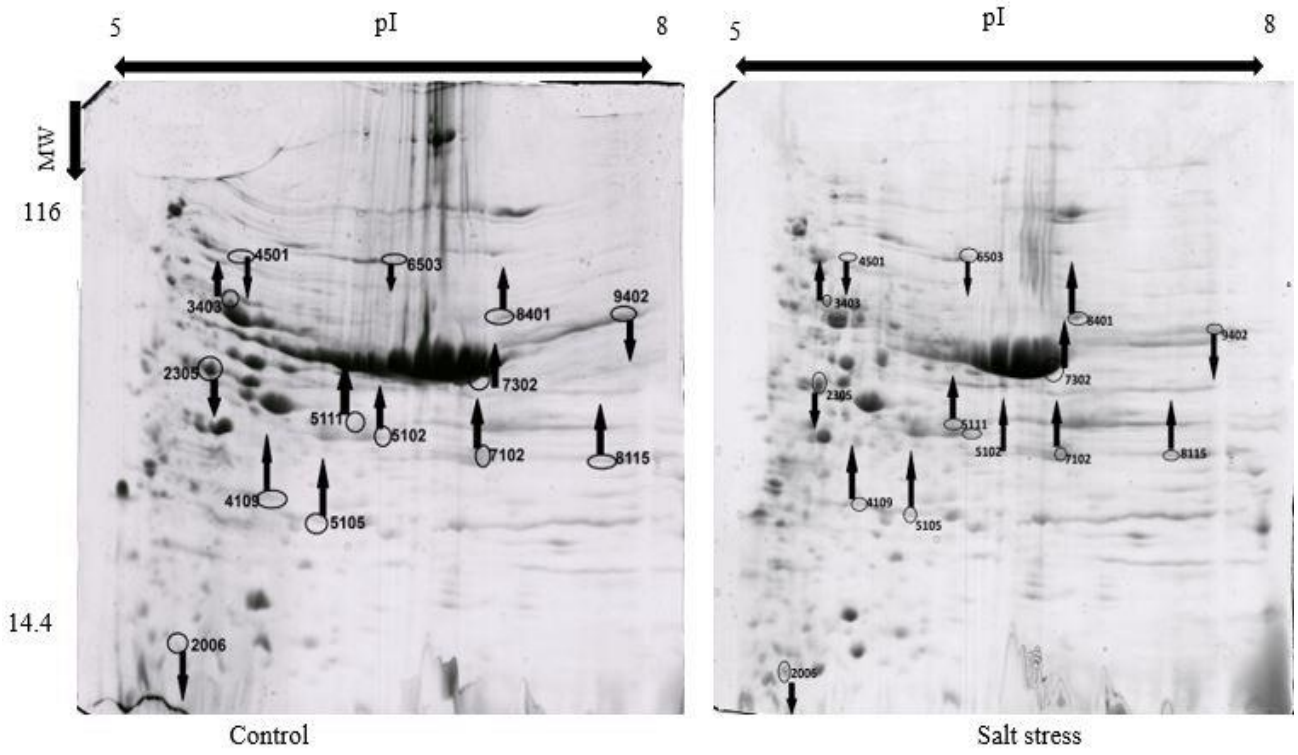

Figure 3. Two-dimensional protein patterns of alfalfa (Bami ecotype) leaf under salinity stress.

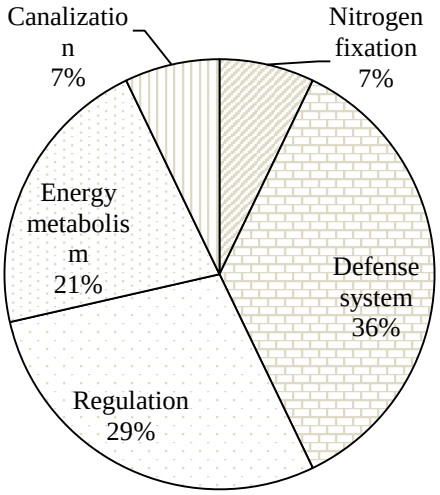

Figure 4. Functional classification of differentially identified proteins under salt stress in alfalfa (Bami ecotype) leaves.

factor (Table 1). Leghemoglobin and voltage-gated potassium channel subunit beta were significantly down and up-regulated, respectively (Figure 5). Leguminous biological nitrogen fixation is sensitive to environmental fluctuation, and abiotic stresses reduces it (Marino *et al.* 2013). Potassium ion is important for crop yield and quality. After nitrogen, potassium is needed by plants in the largest amount (Marschner 1995). K+ and its accompanying anions have a great role in the osmotic potential of cells and tissues of glycophytic plants. Potassium neutralizes the soluble and insoluble macromolecular anions, which results in the stability of the pH in the cytosol. It also participate in other activities such as cell extension, stomatal opening and adjustment of turgor pressure, protein synthesis, photosynthesis, solute transport, cation-anion balance, activation of enzymes and stress response (Marschner 1995). Percey *et al.* (2016) indicated the role of potassium in photosynthesis and growth in glycophyte species, and as element of salinity tissue tolerance in halophytes.

Conclusions

In this study, salinity limited plant growth and decreased fresh and dry weight of the Bami ecotype of alfalfa. Proteomic analysis in alfalfa leaves identified a total of 112 reproducible proteins under salt stress and 14 proteins were significantly regulated by salinity. The identified proteins were classified into five functional groups: defense system, regulator, energy metabolism, nitrogen fixation and canalization. Our results showed that a major fraction of the detected proteins are involved in regulatory processes. Thus, our results indicate that proteins linked to defense systems and regulatory-related proteins have vital role in the adaptation of alfalfa to salt stress. Energy metabolism-related proteins are the third functional group that were affected by salinity. This study is a step towards establishing, at the proteomic level, an unambiguous role for certain salinity-stress responsive proteins in alfalfa.

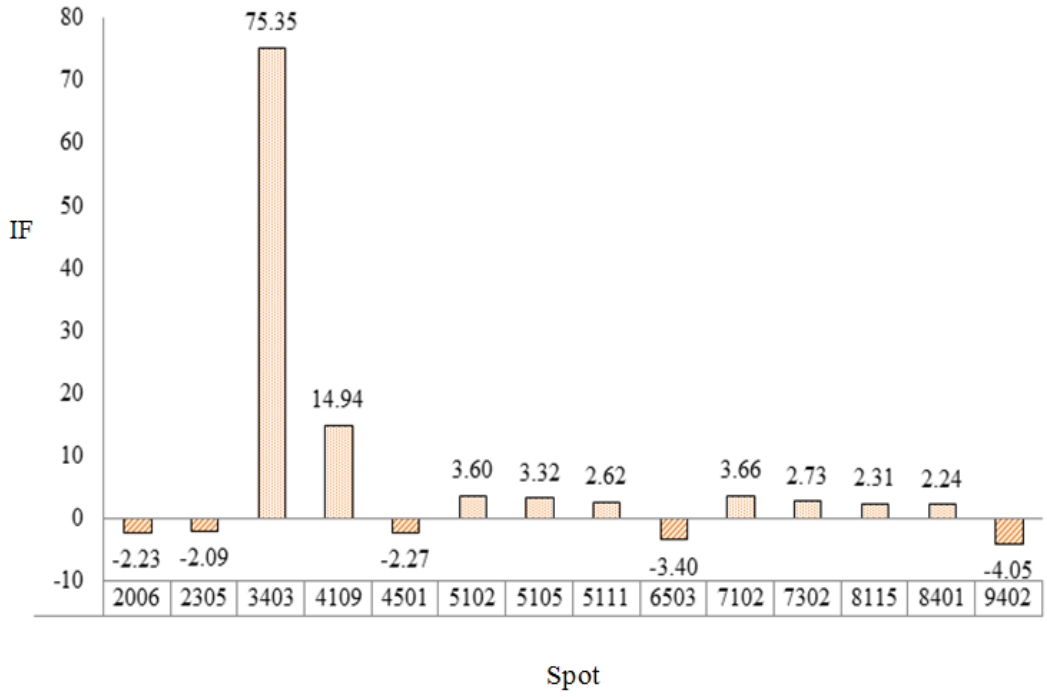

Figure 5. Changes in expression of proteins identified in alfalfa (Bami ecotype) leaf under salt stress. Positive and negative signs indicate up and down-regulation.

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پروتئین‌های پاسخ دهنده به تنش شوری در یونجه (*Medicago sativa* L.)

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چکیده

تنش شوری یکی از مهم‌ترین تنش‌های محیطی است که روی صفات زراعی، فیزیولوژیکی و بیوشیمیایی گیاه تاثیر می‌گذارد. به منظور ارزیابی اثر تنش شوری (صفر و ۲۰۰ میلی‌مولار کلرید سدیم) روی پروتئین‌های برگ یونجه به روش الکتروفورز دوبُعدی، آزمایشی با دو تیمار شوری (سطح صفر و ۲۰۰ میلی‌مولار کلرید سدیم) با شش تکرار تحت شرایط هیدروپونیک اجرا شد. تنش شوری باعث کاهش ۲۴/۵ و ۳۹/۵ درصد وزن تر و وزن خشک گیاهچه‌ها در اکوتیپ بمی یونجه شد. شناسایی احتمالی پروتئین‌ها توسط pI و وزن ملکولی تقریبی انجام شد. نتایج حاصل از تجزیه پروتئوم برگ یونجه نشان داد که ۱۴ لکه تکرار پذیر تحت تنش شوری تغییر بیان داشتند به طوری که ۹ لکه پروتئینی افزایش بیان و پنج لکه پروتئینی کاهش بیان نشان دادند. پروتئین‌های کاندید جزء پروتئین‌های دخیل در سیستم دفاعی، تنظیم، مسیرهای متابولیسم، تثبیت نیتروژن و کانالی بودند. بیان بالاتر trehalose-phosphate phosphatase 2، پروتئین درگیر در عمل تنظیم، و نیز uracil phosphoribosyl transferase, lastic و β-hydroxyisobutyryl-CoA hydrolase 1، پروتئین‌های درگیر در متابولیسم انرژی، عملکرد مهم این پروتئین‌ها را تحت تنش شوری نشان داد. چنین به نظر می‌رسد که گیاه یونجه از طریق پروتئین‌های کاندید مشخص شده در این پژوهش موجب کاهش اثر تنش شوری می‌شود.

واژه‌های کلیدی: پروتئوم؛ تنش غیرزیستی؛ ژل دوبُعدی؛ سیستم دفاعی؛ متابولیسم.