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Salt-Stress Induced Protein Pattern Related to Seed Germination Indices in Lemon Balm (*Melissa officinalis* L.)

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Abstract

Salinity is one of the major environmental stress factors that cause many adverse effects on growth and productivity of plants. The objective of the present study was to investigate the effects of salinity levels on seed germination indices and protein patterns in *Melissa officinalis* seedlings. An experiment was carried out based on randomized complete block design with five concentrations of NaCl and four replicates with sand medium. The results indicated that salt stress had negative effects on the seed germination percentage (GP), mean germination time (MGT) and germination rate (GR), primary shoot and root length, as well as the protein content. After two days of salt exposure, the lowest (0%) GP was obtained for seeds treated with 12 dSm^{-1} salinity, as compared to the control (48.5%). Although, high salinity levels delayed seed germination, but during the experimental period GP increased and no significant differences were observed among the treatments in terms of GP after two weeks. The highest MGT (4.97 days) was obtained for plants stressed with 12 dSm^{-1} salinity level, as compared to the control (1.89 days). Analysis of the protein bands revealed that salinity suppressed the expression of two proteins with the size of 45 (Y1), and 40 kDa (Y2) and protein of 60 kDa (D1) was down-regulated. Also, the synthesis of four proteins of 70 (X1), 30 (X2), 25 (X3), and 20 kDa (X4) was induced in the seedlings under salinity stress. The changes in protein pattern under salinity stress indicated that the synthesis of new proteins may be associated to the stress specific proteins.

Keywords: germination rate, mean germination time, *Melissa officinalis*, protein pattern, salinity

1. Introduction

Salinity is one of the most important environmental factors limiting crop production of marginal agricultural soils in many parts of the world (Abbasian & Moemeni, 2013). More than 6% of the total land area in the world and approximately 20% of the total agricultural lands are affected by salt. Sodium chloride is the predominant salt causing salinization, and it is unsurprising that plants have evolved mechanisms to regulate its accumulation (Keshavarzi et al., 2011). Approximately 15.2% of surface area of Iran is affected by salinity (Rezaie et al., 2013). Seed germination and early seedling growth are critical stages for the establishment of the plant populations under saline conditions (Gulzar & Khan, 2003; Zhang et al., 2010).

A high salt level interferes with the germination of seeds. Salinity and drought stresses have similar effects on plants, preventing roots from performing their osmotic activity where water and nutrients move from an area of higher concentration (the soil) into the roots. Therefore, because of the increased salt level in the soil, water and nutrients cannot move into the plant roots (Bojović et al., 2010). Salinity can affect growth and yield of most crops, high salinity is known to cause both hyper ionic and hyper osmotic effects in plants, leading to membrane disorganization, increase in reactive oxygen species production and metabolic toxicity (Abbasian & Moemeni, 2013). The effects of salinity on all stages of plant growth have been mentioned. Soil salinity affects various physiological and biochemical processes which result in reduced biomass production. This adverse effect of salt stress appears on the entire plant at almost every stage of growth including germination, seedling development,

vegetative and reproductive stages. However, tolerance to salt stress at each stage varies from species to species (Ratnakar & Rai, 2014).

Anbumalarmathi and Mehta (2013) studied the response of eight *indica* rice (*Oryza sativa* var. *indica*) varieties to six salinity levels (0, 4, 8, 12, 16 and 20 dSm^{-1}) at germination and early seedling growth stage and reported that salinity decreased the total germination percentage (TGP), speed of germination (SG) and germination energy percentage (GEP) and led to reduction in shoot and root length and dry weight in all varieties and the magnitude of reduction increased with increasing salinity stress.

Lemon balm (*Melissa officinalis* L.), is one of the important medicinal plant species in the family Lamiaceae that is blessed with bioactive compounds (Kim et al., 2010). Today, it is used in different branches of industry (such as medicine, perfume, cosmetic and food) in many countries of the world (Bagdat & Cosge, 2012). The main components of the essential oil are citronellal (2-40%) and citral (neral and geranial) 10-30%, along with β-caryophyllene, germacrene D, ocimene and citronellol (Vaverková et al., 2012). It's essential oil is currently used in medicine and pharmacology as anti-tumor, anti-bacterial, anti-microbial, anti-histaminic, anti-spasmodic and anti-oxidant, by means of its antiviral effect curing the symptoms of herpes, anti-ulcerogenic, it helps to moderate Alzheimer's disease, acting modulation of mood and enhancing cognitive performance, stimulating the immune system (against HIV-1) and the heart. Essential oil obtained from aerial parts of plant is used to alleviate insect bites, painful menstruation, colds, headaches, mumps, insomnia, as a mild sedative and anti-depressant. In food industry, lemon balm essential oil is used for food spoilage yeasts to extend the storage periods; in soft drinks industry and herbal tea industries because of its fresh lemon taste and in cosmetic industry (for its hydrosol content) to cure some dermatological problems (Bagdat & Cosge, 2012; Moradkhani et al., 2010).

The protein profiling study is an important alternative measurement of gene expression in plants; when this expression occurs in response to various abiotic stress conditions. Therefore, these results are necessary to examine the salt stress at the molecular level and to investigate the relationship between biomarkers (such as protein bands) and germination indices changes. Many proteins undergo post-translational modifications, which play an important role in their activity and subcellular allocation (Graves & Haystead, 2002; Kettman et al., 2002). In this research, we demonstrate the impact of salinity on some germination indices in *M. officinalis*.

2. Materials and Methods

2.1 Plant Materials

Lemon balm seeds were provided from Medicinal Plants Research Center, Shahed University, Tehran, Iran.

2.2 Experimental Design

The experiment was carried out as a randomized complete block design (RCBD) with five salinity levels (0 (as control), 3, 6, 9 and 12 dSm^{-1}) and four replicates. Seeds were soaked in separate petri dishes in each level of treatment. The seeds were surface sterilized by soaking them in 10% (v/v) sodium hypochlorite (NaOCl) solution for 3 min and thoroughly rinsed with distilled water. Treatments were randomly assigned to the experimental units, and were distributed evenly on a Whatman paper (No. 1) placed into a sterile petri dish. Fifty seeds for each of the five NaCl treatments were used and 6 mL of NaCl solution in water were added into each petri dish and sealed with parafilm and were located in a controlled growth chamber in the laboratory of Medicinal Plants Research Center. Seeds were allowed to germinate in and the number of germinant seeds was counted every day up to 14 days and the seeds were considered germinated when the radical emerged. The average temperature of the growth chamber was set between 28-30 ^{o}C with relative humidity between 60-75%. After two days, the germinated seeds were counted every day. And at the end of the experimental period of 14 days, the TGP (seeds germinated/total seeds × 100) and average seed germination percentage (AGP) were then calculated. The mean germination time (MGT) was calculated using the described formula of Ellis and Roberts (1981) as follow:

$$MGT = \sum nD/\sum n \quad (1)$$

Where, n = number of seeds newly germinated at time D; D = days from the beginning of the germination test; $\sum n$ = final germination.

The germination rate (GR) was calculated by dividing the germination percentage obtained at each counting to the actual number of that particular counting day. The effects of the treatments were evaluated based on the germination percentage and time. The primary root and shoot length of each seedling was measured after 14 days.

Analysis of variance of data was performed with SPSS version 22 and means were compared using Tukey's Multiple Range Test (TMRT).

2.3 Protein Extraction and Estimation of Total Soluble Protein Content

One gram of each the *M. officinalis* seedling samples was ground in liquid nitrogen using pre-cooled mortar and pestle to obtain a fine powder and then homogenized with extraction buffer (20 mM HEPES/KOH pH 7.5, 40 mM KCl, 1 mM EDTA, 10% (v/v) Glycerol and 1 mM PMSF) as described by Talei et al. (2013). The supernatants were collected, and the total protein concentration was determined using the Bradford method (1976). Bovine serum albumin (Sigma-Aldrich, USA) was employed as a standard at 595 nm, using a spectrophotometer (Perkin Elmer Lambda 25; UV/VIS, USA). The protein samples were run on SDS-PAGE electrophoresis separation following the method described in Laemmli (1970). Fifteen µg of the solubilized protein from each sample was loaded in each lane of the 12% concentrated separating gel. Electrophoresis was accomplished at 100 V over 90 minutes using a Bio-Rad, Mini Protein electrophoresis system (Bio-Rad, USA). The observed protein bands were scored using the UVIDoc Analyzer software (UVIDoc, Houston, Texas, USA).

3. Results and Discussion

From the results in Table 1 it can be seen that salinity levels had significant ($P \leq 0.01$) effects on the seed germination percentage (GP), mean germination time (MGT) and germination rate (GR). After two days of salt exposure, the highest (48.5%) and the lowest (0%) germination percentages were obtained for control seeds and seeds treated with 12 dSm^{-1} salinity level, respectively. Exposing the seeds to salinity showed that increasing the salinity levels led to significant decrease in the germination percentage and germination rate, as well as the primary shoot and root lengths. Although high salinity delayed initiation of seed germination, during the experimental period germination percentage increased and no significant differences were observed among the treatments in terms of GP after two weeks. The results of the data mean comparisons indicated that there were significant differences among salinity levels in terms of MGT, so that the lowest (1.89 days) and the highest (4.97 days) values for this index were obtained for control seeds and seeds treated with 12 dSm^{-1} salinity level, respectively. The primary shoot and root length significantly decreased with increasing salinity levels.

Table 1. Variance analysis of some germination and growth of seedling characteristics of *M. officinalis* under salinity stress

Source of variance	df	Mean Square						
		GP (%)	TGP (%)	MGT (min)	AGP (%)	GR	RL (cm)	SL (cm)
Replication	3	27.33^{ns}	30.067^{ns}	0.104^{ns}	22.707^{ns}	11.789^{ns}	0.023^{ns}	0.012^{ns}
Salinity levels	4	1759.70^{**}	20.8^{ns}	5.735^{**}	284.428^{**}	135.115^{**}	3.363^{**}	2.883^{**}
Test error	12	14.50	33.067	0.059	44.345	24.615	0.024	0.008

Note. ** and ns, refer to 1% and not significant, respectively. GP: germination percentage at 2 day, TGP: total germination percentage at 14 day, MGT: mean germination time at 14 day, AGP: average germination percentage at 14 day, GR: germination rate at 14 day, RL: root length, SL: shoot length.

Germination is a dynamic process in the life cycle of plants while the seed undergoes a rapid transition from a state of dormancy to a metabolically active seedling. Seed is one of the important organs of a plant which plays a crucial role in the continuation of the race. The sequence of germination follows simple events such as imbibition of water, enzyme activation, and hydrolysis of stored material, initiation of growth, rupture of seed coat and emergence of the seedling. According to Siti Aishah et al. (2010), salinity adversely affects germination by decreasing the osmotic potential of the soil solution to such a point that it prevents the intake of water. Salinity may even produce toxic effects on the embryo and the seedlings which results in delayed germination and or reduced percentage germination, which may be true even in the present study.

According to Begum et al. (2010), germination of seed depends on the utilization of reserved food materials of the seed. Salinity interferes with the process of water absorption by the seeds. This subsequently inhibits the hydrolysis of seed reserves which ultimately delays and decreases seed germination. The findings of this study indicated that salinity levels had significant effect on some germination indices, such as GP, MGT and GR. In agreement with the reports of Bagheri et al. (2012) on *Thymus* species and Siti Aishah et al. (2010) on forage sorghums, who showed that increasing of salinity led to decrease in GP, GR, plumule and radicle lengths, the findings of this study indicated that salinity level was an important factor affecting the GP and GR, as well as the

primary shoot and root lengths. This may be due to the negative effects of salt stress on water absorption, nutrient uptake and osmotic imbalance in the environment of plumules and radicles, ion toxicity and ultimately reduction in GP and GR, and finally led to a significant reduction in seedling emergence and plant establishment. Although, the high salinity caused delay on seed germination, but during the experimental period, germination percentage increased, and no significant differences were observed among the treatments in terms of GP after two weeks. The mean comparison of the treatments showed that the MGT was the lowest in control (1.89 days) and reached to a maximum value (4.97 days) at 12 dSm^{-1} salinity level (Figure 1).

The Root and shoot lengths are the most important parameters for studying salt stress, as roots have direct contact with soil. In the present investigation, the primary shoot and root length significantly decreased with increasing salinity levels, which matched up well with the findings of Jaleel et al. (2008), who showed that salt stress decreased root length in *Catharanthus roseus*. This may be due to the negative effects of salt stress on water absorption and disproportion of nutrient absorption by the seedlings, as suggested by Bybordi and Tabatabaei (2009). Similarly, Nyagah and Musyimi (2009) observed a reduction in growth in passion fruit (*Passiflora edulis*) seedlings with increasing concentrations of salt in the medium.

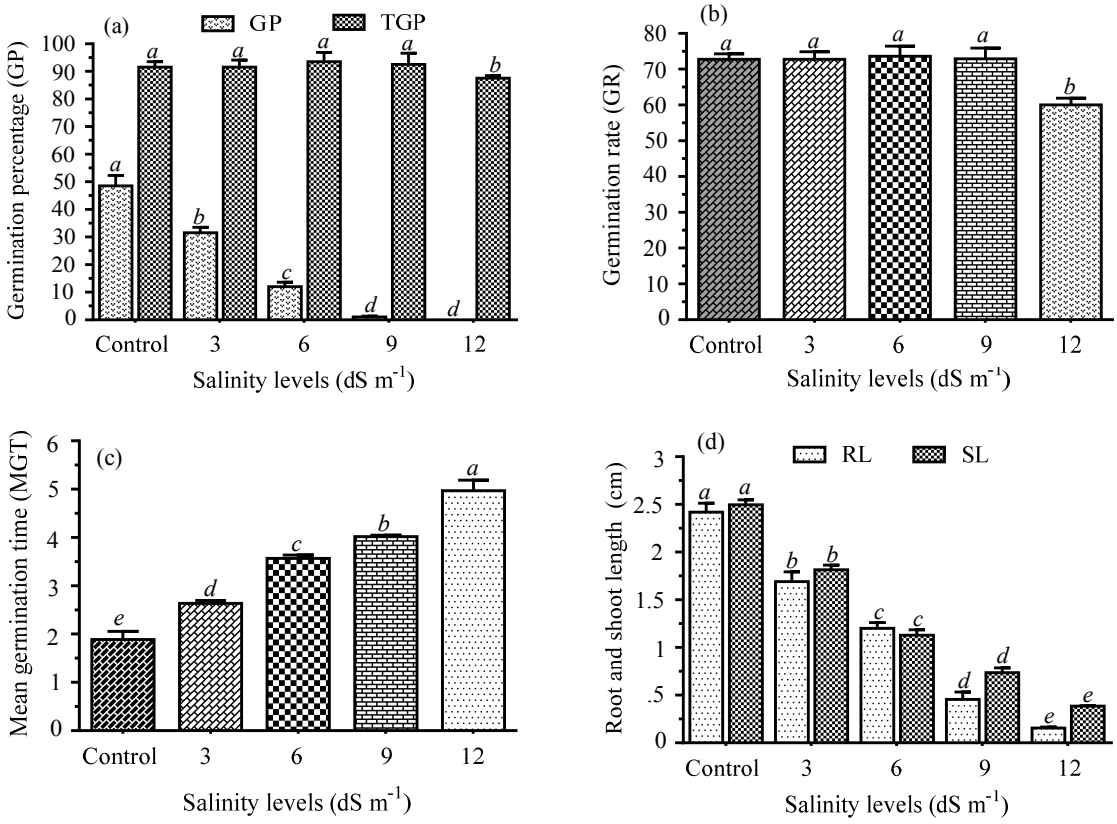

Figure 1. The effect of different salinity levels on germination percentage (GP) at day 2 and total germination percentage (TGP) at day 14 (a), germination rate (GR) at day 14 (b), mean germination time (MGT) at day 14 (c) and root (RL) and shoot (RL) length at day 14 (d). Mean values ± SE are from three independent replicates and values superscripted by different letters are significantly different by Tukey's multiple range test ($P \leq 0.01$)

3.1 Seedling Protein Pattern

In the present study, a significant salt-induced alteration in the protein expression profiles of primary roots and shoots of seedlings was noticed (Figure 2). The results of SDS-PAGE protein profile revealed that there were qualitative and quantitative differences in protein profiles between samples under different salinity levels. As shown in Figure 2, fifteen protein bands with a wide range of molecular masses from 10-120 kDa were detected. The results of SDS-PAGE protein profile indicated that there were obvious qualitative and quantitative differences in protein patterns between control and salt-stressed samples. Salinity inhibited the synthesis of two protein bands with the size of 45 kDa (protein Y_1), and 40 kDa (protein Y2). Interesting to note that, salt

treatment enhanced the synthesis of four new protein bands having MW of 70 kDa (protein X1), 30 kDa (protein X2), 25 kDa (protein X3), and 20 kDa (protein X4), which completely were absent in controlled condition. The results suggested that these four induced protein bands might be related to the seed germination indices. As protein samples were loaded in equal amounts of 15 µg in all cases, the results showed that protein bands of "Y1 and Y2" were not found in the highest salinity level (12 dSm^{-1}), while there were found the protein bands of "X1 to X4". Also, density the protein band of "D1" (60 kDa) was visibly reduced at the maximum salinity level.

Differential expression of polypeptides in the primary shoots and roots of salt-stressed *M. officinalis* seedlings in the present study, probably indicating the molecular adaptive mechanism of those to osmotic stress. Protein profiles in revealed that seeds grown under salinity conditions encountered with induction or repression in the synthesis of a few polypeptides.

During the experimental period GP increased but no significant differences were observed among the salinity levels in terms of GP at day 14. This could be related to the expression of new four induced proteins for salt tolerance of seedlings during the experimental period. In accordance with our results, the reports of Khedr et al. (2003) on *Pancratium maritimum*, Meratan et al. (2008) on *Vicia faba* seeds, and Mohsen et al. (2013) on *Acanthophyllum* species proved the increase of protein content and polypeptide bands under salt treatment. The observation of new low molecular weight proteins, specially a 25 kDa protein in the present study, which may be were specific to adaptation to salt stress was confirmed by the earlier report of Gomathi et al. (2013) on sugarcane. Analysis of the proteomes of many plant species under salinity conditions has revealed that they can respond to salt stress by high-regulating (especially osmotin-like proteins and heat shock proteins) or low-regulating (especially photosynthesis-related proteins) of specific proteins. Heat shock proteins and osmotin-like proteins are the most well-known stress-related proteins in plants and their expression is mostly observed in the salt-tolerant plants under salt stress (Sobhanian et al., 2011).

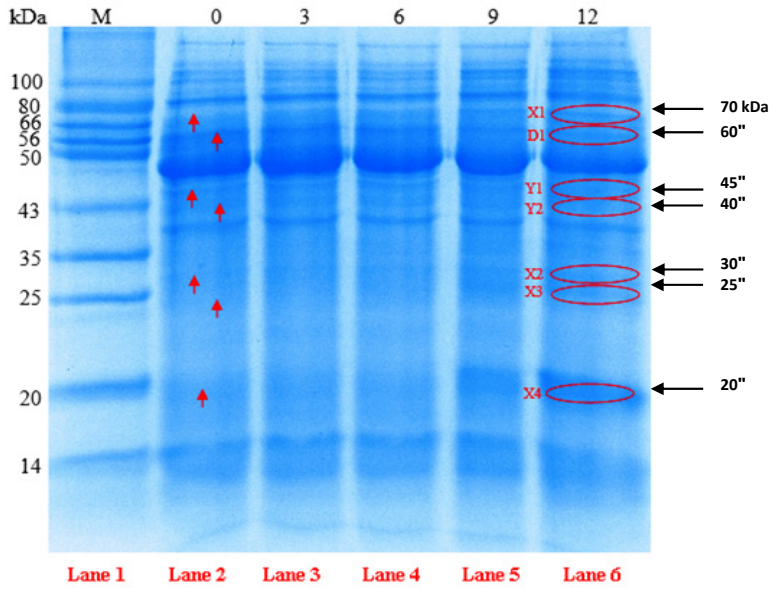

Figure 2. SDS-PAGE profile of total protein extracts from seedlings of *M. officinalis* on 12% polyacrylamide gel. The lane 1 represents protein molecular weight marker, and the lanes of 2-6 represent protein samples at different salinity levels (0, 3, 6, 9, and 12 dSm^{-1}). Protein samples were loaded with equal amount of 15 µg. The "Y1 and Y2" proteins were not found at high salinity level (12 dSm^{-1}), and four bands corresponding to "X1 to X4" proteins were detected at 12 dSm^{-1} salinity level, and the protein band "D1" was low-regulated at high salinity level

According to Zhang et al. (2012), abiotic stresses such as salinity have harmful effects on the structure of proteins and their function in plant cells and could potentially damage proteins. The disappearance of proteins in response to NaCl-based salinity has been observed in wheat (El-Shintinawy & El-Shourbagy, 2001) and *Bruguiera parviflora* (Parida et al., 2004). In agreement with the reports of and Talei et al. (2015), the findings

of this study indicated that the protein amounts of salt-treated plants were decreased, possibly due to the changes in the ratio of the lipoprotein of pigment protein complexes and/or chlorophyllase activity. Our results also matched up well with the findings of Nazarpour et al. (2017), who showed that salt stress decreased the germination percentage of *M. officinalis*. Electrophoresis information on the primary shoot and root proteins can be specifically matched with the seed protein data (Talei et al., 2014) of *M. officinalis* to make fruitful decisions on the breeding programs. Consequently the salinity level significantly affected the germination indices, primary shoot and root length in *Melissa officinalis*.

The findings of this study in the entire measured traits indicated that salt stress adversely affected the germination rate and mean germination time of the *M. officinalis* seeds, as well as shoot and root lengths; however no absolute inhibition was observed in total germination percentages of the seeds at different salinity levels**.** Furthermore, salt-stress induced alterations in protein profiles of primary roots and shoots of the seedlings. Present experiment proved that the specific enhanced expression of some polypeptides was associated with salt tolerance in *M. officinalis*. Protein sequencing and proteomic analysis can be conducted based on the present findings, in the future. It can be concluded that salinity stress significantly affected all the studied seed germination and growth parameters of *M. officinalis* and this information should be taken into consideration when a medicinal plant grown under saline condition.

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Abbreviation

AGP: average germination percentage; GEP: germination energy percentage; MGT: mean germination time; GP: germination percentage; GR: germination rate; RL: root length; SL: shoot length; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; SG: speed of germination; TGP: total germination percentage.