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RESEARCH ARTICLE

WILD MEDICS FROM DIFFERENT ORIGINAL HABITATS CAN BE USED AS FORAGE LEGUMES IN SALT AFFECTED SOIL

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ABSTRACT

Legumes are a key player in sustainable agriculture. They are a potential tool as forage for reclamation of saline soils. However, still there is a need to balance between tolerance of the forage during different developmental stages and its productivity. The present work aimed to study salinity tolerance of four wild *Medicago* species, as an initial step to select new species that can be grown in salt-affected soils or used as wild relatives to improve alfalfa. Seeds of *M. polymorpha*, *M. intertexta*, *M. truncatula* and *M. lupulina*, collected from different natural habitats, as well as alfalfa were germinated under different salinity levels to evaluate germination percentage and germination speed. Generally, seeds of *M. truncatula* collected from desert habitat showed the highest mean germination percentage followed by alfaalfa, while seeds of *M. intertexta* collected from salt-affected habitat exhibited the highest mean germination speed, followed by *M. lupulina*. Under severe salt stress, *M. intertexta* exhibited the highest aerial biomass index, followed by *M. truncatula* and *M. lupulina*, while *M. polymorpha* and alfalfa came as inferiors. Mineral contents and ion leakage of the studied species were determined and discussed. *M. intertexta, M. truncatula* and *M. lupulina*, collected from stressful habitats, tended to maintain osmotic and ionic homeostasis by relying on accumulation of the less energetic cost ions (Na^+) in roots and sugars and K^+ in shoots scoring the highest aerial biomass and tolerance index, orderly. Therefore, the results recommend cultivating these species in salt-affected lands or using them as wild relatives to improve alfalfa.

KEYWORDS

Legume, Salinity, Mineral content, Sugars, Tolerance index.

1. INTRODUCTION

Salinization of soils and groundwater is a serious land-degradation problem in arid and semi-arid areas, increasing steadily in many parts of the world and causing major problems for crop productivity. According to estimates, 20 percent of the total irrigated area is affected by soil salinity (Waldrona et. Al., 2020; FAO, 2015, Minhas et al. 2020 a,b). Egypt is among the countries with large amounts of salt-affected lands in irrigation districts (Squires and Glenn, 2011), and about 93 percent of the cultivated lands are affected by salinization and water logging in Egypt (FAO, 2015).

Interest in the use of saline land resources has escalated over the last 20 years, with a renewed focus on saline agriculture in a range of countries, including Pakistan (Qureshi and Barrett-Lennard, 1998), Australia (Rogers et al., 2006) and Egypt (Al Sherif, 2007; 2009). There are plants that grow under saline conditions, and historically, have been opportunistically used as fodder for grazing livestock or as components of mixed rations to replace roughage. The selection of such economic plants, with appropriate management, could result in the rehabilitation and re-vegetation of salt-affected lands.

Leguminosae is one of the largest families of flowering plants with more than 18,000 species classified into 650 genera (Sprent, 2001), just under one-twelfth of all known flowering plants. Despite examples of success in legume breeding and adoption, it is of some concern that there are perhaps only 50 species of forage legumes and less than 15 species of grain legumes in global commercial trade (Kelley et al., 2000). It is documented that wild forage legumes are the most potential forage legumes grown in salt-affected soils (Al Sherif, 2009; Ashraf and Bashir 2003; Zahran et al., 2007) The economic importance of legumes is related with their capacity to fix atmospheric nitrogen, thereby play an essential role in the structure of ecosystems and sustainable agriculture, worldwide. *Medicago* a leguminous genus contains more than 80 species and distributed mainly around the Mediterranean basin (Prosperi et al., 2001). The best known member of the genus is alfalfa (*M. sativa*), an important forage crop. In the USA alone, alfalfa (*Medicago sativa*) is estimated to be the third or fourth most valuable crop (Graham and Vance, 2003). Many *Medicago* species such as *M. sativa*, *M. polymorpha* and *M. intertexta* (Al Sherif et al., 2004; Nichols et al. 2009; Zahran et al., 2007, Bhandaria et al, 2020) were regarded as promising legumes for forage in arid and semi-arid areas for being relatively salt tolerant. However, a balance between the tolerance of the forage during different developmental stages and its productivity still been needed. Additionally, for plants grown as forage under salt stress, germination and early seedling growth is very important processes, as they bottleneck plants establishment and development under the stressful condition. For facing this, several strategies for genetic improvement are available, including classical breeding, physiological and genetic enhancement. All necessitate screening legumes for salt tolerance variability and understanding the mechanisms underlying this variable tolerance at different developmental stages in relation to the original or

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parental habitats. Moreover, studying differences between closely related plants got much interest as a way of probable identification of factors influencing salt tolerance (Tester and Davenport, 2003; Vicente et al., 2004).

The objective of the present study was to investigate the morphological and physiological responses of four wild different *Medicago* species to salt stress in comparison with *M. sativa* (alfalfa), as an initial step for selecting new species can be cultivated in salt affected lands. A further objective of the study was to assess how the diversity in the original habitat will exert a differential tolerance and adaptive responses to salinity.

2. Materials and Methods

Seeds of four wild species were collected from their original habitats. *M. polymorpha* L were collected from plants grown on roadsides, while those of *M. intertexta* (L.) Mill were collected from salt affected soil (29°16'16.48"N, 31°11'15.26"E). Seeds of *M. truncatula* Gaertn were collected from plants grown in sandy soil in the desert (31° 7'26.33"N, 27°49'34.70"E) and those of *M. lupulina* L collected from plants associated with *Cynodon dactylon* growing in a garden with a loosely soil (29° 1'40.15"N, 31° 6'54.88"E). For comparison of wild species with cultivated one, *M. sativa* seeds were provided by Sids Institute of Agricultural Research Center, Beni-Suef, Egypt.

2.1 Soil sampling and analysis

Five soil samples were collected from rhizosphere of different *Medicago* species, then pooled together to form one composite sample, air dried, and mixed. Textures were estimated by the hydrometer method, giving quantitative data on the percentage of sand, silt, and clay. Soil water extracts (1:5) were prepared for the detection of electrical conductivity (EC) and pH by a digital pH-meter (pH/ORP/Ion/Conductivity meter SG78). Walkley–Black method further modified by Yeomans&Bremner(1998) was used for detection of the organic carbon content, and the total nitrogen was analyzed by Kjeldahl method (Bremner, 1996). The concentrations of soil minerals Na^+, K^+, Ca^{2+}, and Mg^{2+} in soil were determined using a Perkin 403 atomic absorption spectrophotometer (Perkin-Elmer Corp., Norwalk, CT, USA) according to the Analytical Methods for Atomic Absorption Spectrophotometry(1983).

2.2 Seed Germination

For the germination tests, the seeds were sown in sterilized Petri dishes on a double layer of filter paper moistened with 5 ml of the treatment solution. The treatment solutions were 0.0 (control), 50, 100, 150 and 200 mM NaCl. Three replicates, each was a Petri-dish with 25 seeds, were used in each treatment; germination was under conditions of natural light and room temperature (during April, with average temperature 18.1°C. Seeds were considered to be germinated after the radicle reach one cm. Germination speed calculated using the following formula (Bradbeer 1988): V= (a/1 + b/2 + c/3 +d/4 +…….+ x/n) x 100/S, where a, b, c..... respectively, represent the number of seeds which germinated after 1, 2, 3 days of imbibitions, x is the number after n days, and S is the total number of germinated seeds. The experiment was repeated two times each extended 15 days. Data were the mean of the two experiments.

2.3 Pot experiment

Seedlings were transplanted into pots (15 cm diameter) filled with sterilized clay loamy soil, and watered by the mineral nutrient solution twice a week. The nutrient solution contained the following, in mmolar/liter, $CaCl_2.2H_2O$; 1.0; $MgSO_4.7H_2O$, KNO_3 1.65; K_2SO_4, 0.50 and $NaH_2PO4.2H_2O$ 0.65; and micronutrient (in micromolar/liter) $FeSO_4.7H_2O$, 27.0; $MnCl_2.4H_2O$, 1.13; $CuSO_4.5H_2O$ 0.08; $ZnSO_4.7H_2O$, 0.19; $NaMoO_4.2H_2O$, 0.05 and H_3BO_3, 5.77 and the pH of the nutrient solution was adjusted to 7. Each pot contained four seedlings. Pots were arranged in a randomized blocks in the greenhouse. The average mean temperature was 18.1oC. Photoperiod was about 12 h; no artificial illumination (light) was supplied. The treatment solutions of NaCl (0, 100 and 200 mM) were applied two weeks after transplanting, while plants at vegetative growth. The salt treatments continued for two weeks and pots were flushed thoroughly with distilled water once a week to avoid salt accumulation in root zone. As the plant grew in size, the volume of liquid was increased and the differences between salt concentrations were kept constant.

2.4 Growth measurement

After harvest the lengths of root and shoot were measured. Fresh weights of root and shoot were estimated. Samples were dried at 70°C for 24 h and the dry weight was determined. Water content was estimated as the difference between fresh and dry weights. The dried material was powdered and kept for the biochemical analysis.

2.5 Sugars and amino acids

About 0.5 g of the powdered dry material was extracted with hot 80% aqueous ethanol, boiled on water bath for 2 h, and then centrifugation was carried out at 4000 rpm for 15 min. The clear supernatant was used for determination of soluble sugars as well as free amino acids. Reducing sugars (RS) were determined (Nelson, 1944) using glucose standard curve and then calculated as mg g-1 dry weight. For estimation of sucrose, one ml of ethanol extract was evaporated till dryness and then hydrolyzed with invertase in citrate buffer (pH 4.6). The content of RS in the hydrolyzed sample was determined as mentioned above. Sucrose was estimated by determining the difference between RS before and after hydrolysis. Estimation of free amino acids was adopted using ninhydrin reagent (Lee and Takahashi, 1966). The value of free amino acids was calculated using a standard curve of glycine, and expressed as mg g-1 dry weight.

2.6 Mineral content

A known weight of powdered dry tissue was wet-acid digested in a mixture of nitric and perchloric acids for complete oxidation. The clear digested samples were used for estimation of sodium and potassium using the atomic absorption spectrophotometer (GBC 932 AA).

2.7 Ion leakage

Root tissue was cut into segments, rinsed three times in de-ionized water and placed in test tubes containing 10 ml of de-ionized water. The tubes were kept on a shaker (80 rpm) at 25°C for 3 h. Electrical conductivity of the solution in the tube was determined using a conductance meter (C1). The tubes were autoclaved, and then cooled to 24°C. The electrical conductivity was measured (C2). Leakage percentage of ions was expressed as C1/C2×100 (Jambunathan 2010).

2.8 Aerial biomass index and tolerance index

Aerial biomass index and tolerance index were generated to study the differential tolerance of the investigated *Medicago* spp. These indices estimate the capacity of the plant cells or tissues to continue growth without injury, with a high internal Na^+ concentration, in response to a higher salt stress in the root zone (Munns et al. 2016). Aerial biomass index and tolerance index were calculated as following:

Aerial biomass index = (SDWs/SDWc)×100

Tolerance index = (TDWs×TNas)/(TDWc×TNac)

Where SDWs is shoot dry weight of stressed plant, SDWc is shoot dry weight of control plant, TDWs is total dry biomass of stressed plant, TDWc is total dry biomass of control plant, TNas is total concentration of sodium in the stressed plant and TNac is total concentration of sodium in control plant.

2.9 Statistical analysis

One-way analysis of variance (ANOVA) was applied to assess the differences between treatments for each parameter and species. Data were shown as means ± standard errors (n=3), unless stated otherwise. Differences among means were established using a Duncan test ($P < 0.05$). Partial correlation with NaCl as controlling factor of variables was performed applying the SPSS 10.0 program for Windows (SPSS, Chicago, IL, USA).

3. Results

3.1 Physicochemical characterization of the original soil

Among the original soil samples of the five investigated *Medicago* species, the soil of *M. truncatula* had the highest proportion of sand combined with the lowest silt and clay ones (Table 1). Chemical analysis of soils revealed a low organic matter (OM) content in *M. truncatula* soil, while there were no differences among the rest of the soil samples regarding this content. Soil of *M. intertexta* showed the highest EC and demonstrated the superiority in Na^+ content, while that of *M. truncatula* had the lowest value of EC, N, P and K^+, and the highest of Ca^{+2} and Mg^{+2} (Table 1).

Table 1: Physicochemical properties of the original soil collected from the vegetation of different *Medicago* spp. Values are means of 3 replicates ± SE.

	M. intertexta	*M. polymorpha*	*M. lupulina*	*M. truncatula*	*M. sativa*
Sand	35.7 ± 0.1	36.1± 0.1	37.5 ±0.0	87.4 ± 2.1	35.1± 0.1
Silt	21.9 ± 0.2	21.2± 0.3	25.8 ±1.3	1.9 ±0.0	20.2± 0.3
Clay	40.9 ± 1.2	42.6 ± 1.2	41.1 ±2.4	10.8 ± 1.1	44.6 ± 1.2
OM	5.6 ± 0.1	6.7 ± 0.1	5.7 ±0.1	1.0 ± 0.0	5.7 ± 0.1
pH	7.8 ± 0.3	7.9 ± 0.1	7.9 ±0.1	8.2 ± 1.5	7.8 ± 0.1
EC (mS/cm)	5.4 ± 0.1	3.5 ± 0.0	2.6 ± 0.0	1.8 ±0.0	3.5 ± 0.0
N	113 ± 1.8	127.7 ± 1.1	102 ± 1.6	37.2 1.6	3.7 ± 1.1
P	116 ± 2.3	143 ± 1.3	112 ± 3.4	35.5 ± 2.1	1453 ± 1.3
Na	2230 ± 6.8	242.5 ± 9.7	256 ± 5.8	115 ± 0.1	243 ± 9.7
K	140± 1.7	120 ± 2.1	130 ± 4.7	8.4 ± 2.7	125 ± 2.1
Ca	1168 ± 9.9	890 ± 6.7	950 ± 7.6	13658 ±8.5	890 ± 6.7
Mg	267 ± 1.5	300 ± 5.8	247 ± 5.4	742 ± 2.4	305 ± 5.8

OM; organic matter, EC; electric conductivity. Sand, silt, clay and OM were expressed as % of soil. N, P, Na, K, Ca and Mg were expressed as mg/100 gm soil.

3.2 Germination percentage and speed

As % of control, germination percentage of *M. sativa* and *M. lupulina* non-significantly changed in response to most salinity levels (Table 2), while that of *M. polymorpha* was significantly declined under the high levels of NaCl (150 and 200 mM NaCl). The harmful effect of salinity on germination percentage was the most obvious in case of *M. intertexta*. In contrast, the lower levels of salinity (50 and 100 mM NaCl) significantly increased the germination of *M. truncatula* over control and the higher ones (150 and 200 mM NaCl) non-significantly changed the same criterion. Although germination speed of *M. polymorpha*, *M. sativa* and *M. truncatula* decreased markedly as affected by salt-stress, particularly the high levels, that of *M. intertexta* and *M. lupulina* non-significantly changed in most of the investigated concentrations when compared with respective control (Table 2).

Table 2: Effect of various levels of salinity on germination of different *Medicago* spp. Values are means of 3 replicates ± SE. Each replicate was a Petri-dish containing 25 seeds. Values with at least one similar letter for the same species are non-significantly different at p=0.05.

Medicago spp.	NaCl	Germination percentage				Germination speed				Mean
	(mM)	as % of control				as % of control				
polymorpha	0	100.0	±	4.0	a	100.0	±	17.5	a	
	50	95.2	±	5.5	a	64.9	±	8.4	b	
	100	90.5	±	3.6	a	69.2	±	3.6	b	
	150	83.3	±	12.1	b	55.3	±	12.3	b	
	200	71.4	±	7.4	b	48.1	±	2.6	c	
Mean		88.1				67.5				77.8
intertexta	0	100.0	±	8.7	a	100.0	±	22.9	a	
	50	62.4	±	8.1	b	94.2	±	23.2	a	
	100	53.8	±	12.1	b	75.7	±	21.0	b	
	150	47.4	±	13.3	c	137.0	±	41.4	a	
	200	38.2	±	8.1	c	82.6	±	5.5	b	
Mean		60.3				97.9				79.1
sativa	0	100.0	±	1.5	a	100.0	±	6.5	a	
	50	100.6	±	6.8	a	83.5	±	11.9	b	
	100	100.3	±	3.0	a	94.2	±	5.2	a	
	150	98.3	±	1.5	a	80.9	±	3.2	b	
	200	91.9	±	2.4	a	79.2	±	4.5	b	
Mean		98.2				87.6				92.9
truncatula	0	100.0	±	17.0	b	100.0	±	21.1	a	
	50	153.4	±	18.2	a	53.5	±	7.8	b	
	100	126.8	±	37.5	a	49.2	±	6.5	b	
	150	71.6	±	40.9	b	46.3	±	5.2	b	
	200	79.5	±	19.3	b	47.6	±	13.2	b	
Mean		106.3				59.3				82.8
lupulina	0	100.0	±	1.5	a	100.0	±	18.4	a	
	50	99.6	±	5.2	a	110.8	±	16.0	a	
	100	96.1	±	1.6	a	107.4	±	2.2	a	
	150	94.9	±	10.6	a	89.5	±	6.0	a	
	200	37.5	±	8.0	b	77.4	±	3.8	b	
Mean		85.6				97.0				91.3

3.3 Plant Growth

In most cases, shoot and root lengths of all the investigated *Medicago* spp. non-significantly affected by salinity. Nonetheless, root length of *M. sativa* significantly decreased while that of *M. lupulina* exhibited its highest value at 200 mM NaCl (Table 3). Only *M. polymorpha* and *M. lupulina* recorded a significant increase in root/shoot length under severe stress (200 mM NaCl). *Medicago polymorpha* and *M. sativa* showed a significant loss in their biomass in response to salt stress, particularly in their total fresh weight and root dry weight. In contrast, *M. intertexta* increased its biomass, and *M. truncatula* and *M. lupulina* both kept their own non-significantly changed. All the investigated species maintained their water content, and superiority was for *M. lupulina* for increasing its water content under NaCl stress (Table 3). Calculations of aerial biomass index revealed that *M. intertexta* exhibited the highest score under severe salt stress, followed by *M. truncatula* and then *M. lupulina*, while *M. polymorpha* and *M. sativa* came as inferiors (Table 4).

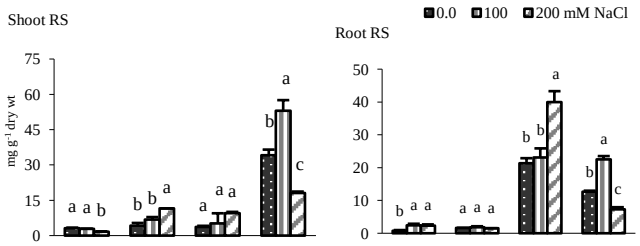
Table 3: Effect of various levels of salinity on growth criteria of different *Medicago* spp. Values are means of 5 replicates ± SE. Values with at least one similar letter for the same species are non-significantly different at p=0.05.

Growth criteria	NaCl (mM)	*Medicago* spp.																			
		polymorpha				*intertexta*				*sativa*				*truncatula*				*lupulina*			
Shoot length	0	10.23	±	0.73	a	6.47	±	1.23	a	11.13	±	1.85	a	3.77	±	1.05	a	6.70	±	0.65	a
	100	9.97	±	0.79	a	8.53	±	0.50	a	9.33	±	0.44	a	2.50	±	0.17	a	6.40	±	0.44	a
	200	7.87	±	0.43	a	7.83	±	0.57	a	9.27	±	0.93	a	5.43	±	0.98	a	6.33	±	0.18	a
Root length	0	6.17	±	0.94	a	8.30	±	0.61	a	20.93	±	2.00	a	8.47	±	0.58	a	8.63	±	1.35	b
	100	7.60	±	0.25	a	10.20	±	1.27	a	14.67	±	1.59	b	8.67	±	1.69	a	9.57	±	1.09	b
	200	7.43	±	0.68	a	10.07	±	1.71	a	12.77	±	1.50	b	8.20	±	0.53	a	15.97	±	0.58	a
Root/Shoot length	0	0.60	±	0.06	b	1.28	±	0.19	a	1.88	±	0.47	a	2.25	±	0.96	a	1.29	±	0.29	b
	100	0.76	±	0.04	b	1.20	±	0.08	a	1.57	±	0.22	a	3.47	±	0.95	a	1.49	±	0.23	b
	200	0.94	±	0.12	a	1.29	±	0.24	a	1.38	±	0.17	a	1.51	±	0.26	a	2.52	±	0.15	a
Shoot fresh wt	0	0.628	±	0.07	a	0.452	±	0.01	b	0.338	±	0.06	a	0.057	±	0.02	b	0.182	±	0.02	a
	100	0.270	±	0.03	b	0.618	±	0.15	a	0.273	±	0.03	a	0.063	±	0.02	b	0.208	±	0.03	a
	200	0.284	±	0.02	b	0.488	±	0.09	b	0.187	±	0.04	a	0.169	±	0.04	a	0.220	±	0.04	a
Root fresh wt	0	0.079	±	0.03	a	0.122	±	0.02	b	0.136	±	0.00	a	0.015	±	0.01	a	0.059	±	0.01	a
	100	0.061	±	0.01	a	0.340	±	0.04	a	0.096	±	0.02	a	0.017	±	0.00	a	0.042	±	0.01	a
	200	0.045	±	0.01	a	0.205	±	0.06	ab	0.077	±	0.01	b	0.013	±	0.00	a	0.052	±	0.01	a
Total fresh wt	0	0.71	±	0.09	a	0.57	±	0.03	b	0.47	±	0.07	a	0.07	±	0.02	b	0.24	±	0.03	a
	100	0.33	±	0.03	b	0.96	±	0.17	a	0.37	±	0.01	b	0.08	±	0.02	b	0.25	±	0.04	a
	200	0.33	±	0.02	b	0.69	±	0.09	a	0.26	±	0.05	b	0.18	±	0.05	a	0.27	±	0.05	a
Shoot dry wt	0	0.079	±	0.03	a	0.067	±	0.03	a	0.055	±	0.00	a	0.014	±	0.01	a	0.035	±	0.01	a
	100	0.052	±	0.00	a	0.103	±	0.03	a	0.053	±	0.00	a	0.010	±	0.00	b	0.038	±	0.01	a
	200	0.051	±	0.00	a	0.087	±	0.02	a	0.038	±	0.00	b	0.015	±	0.01	a	0.035	±	0.00	a
Root dry wt	0	0.017	±	0.00	a	0.013	±	0.00	a	0.029	±	0.00	a	0.003	±	0.00	a	0.009	±	0.00	a
	100	0.011	±	0.00	b	0.023	±	0.01	a	0.019	±	0.00	b	0.004	±	0.00	a	0.010	±	0.00	a
	200	0.012	±	0.00	ab	0.025	±	0.00	a	0.013	±	0.00	b	0.004	±	0.00	a	0.008	±	0.00	a
Total dry wt	0	0.10	±	0.03	a	0.08	±	0.04	a	0.08	±	0.00	a	0.02	±	0.01	a	0.04	±	0.01	a
	100	0.06	±	0.00	a	0.13	±	0.03	a	0.07	±	0.00	b	0.01	±	0.00	a	0.05	±	0.01	a
	200	0.06	±	0.01	a	0.11	±	0.02	a	0.05	±	0.00	c	0.02	±	0.01	a	0.04	±	0.00	a
Water content	0	6.3	±	0.71	a	6.3	±	1.50	a	4.6	±	0.76	a	3.4	±	0.88	a	4.4	±	0.13	ab
	100	4.2	±	0.42	a	6.6	±	1.05	a	4.1	±	0.35	a	5.1	±	1.31	a	4.2	±	0.23	b
	200	4.2	±	0.23	a	5.2	±	0.71	a	4.1	±	0.94	a	8.8	±	2.27	a	5.3	±	0.45	a

Table 4: Tolerance index of different *Medicago* spp. in response to salt stress.

Index	NaCl (mM)	*Medicago* spp.				
		polymorpha	*intertexta*	*sativa*	*truncatula*	*lupulina*
Aerial biomass index	100	65	155	95	69	109
	200	65	130	69	106	99
Tolerance index	100	0.72	1.58	1.00	1.33	1.46
	200	0.60	1.78	0.45	1.21	0.94

3.4 Sugars and amino acids



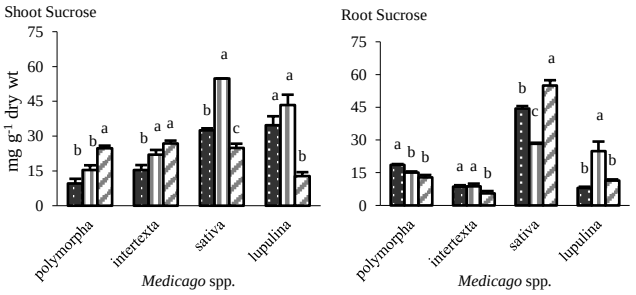


Figure 1

Most of the investigated *Medicago* spp. showed a shoot performance contrasting to that of root concerning sugars accumulation under salt-stress (Fig. 1). For example, *M. polymorpha* decreased RS and increased sucrose content in shoot, while increased the former and decreased the later significantly in root. A similar contrasting performance was noticed on *M. intertexta* as it significantly augmented both RS and sucrose of its shoot, but decreased those of root. *Medicago sativa* allocated most of its RS and sucrose to root, resulting in mounted levels of all sugars in root at 200 mM NaCl. Differently from the above mentioned *Medicago* spp., *M. lupulina* increased its sugars content with 100 mM, while decreased the same content under severe stress in both organs.

Most of the investigated species non-significantly changed their content of amino acids of both shoot and root in response to NaCl-stress (Fig. 2). Nevertheless, an obvious augmentation in the amino acids content was shown by the stressed *M. sativa* root relative to control.

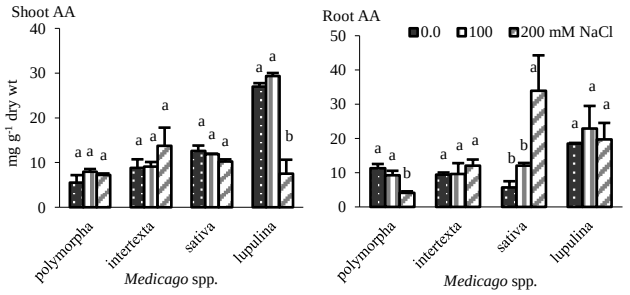
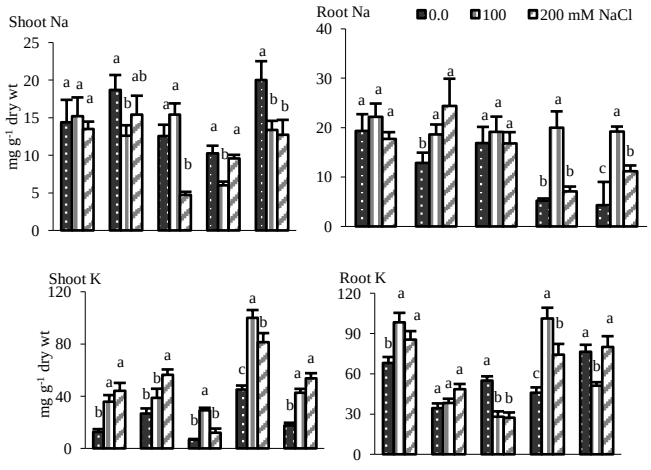


Figure 2.

3.5 Mineral content

Increasing salinity level seems to have no significant effect on Na^+ content in both shoot and root of *M. polymorpha*. While *M. sativa* non-obviously changed its Na^+ content in the root, *M. intertexta*, *M. truncatula* and *M. lupulina* accumulated this ion in their roots and decreased it in their shoots. Increasing the level of salt-stress elevated the content of K^+ in shoots of all the tested *Medicago* spp. (Fig. 3). Only *M. polymorpha* and *M. truncatula* increased their root K^+ content markedly, while *M. intertexta* and *M. lupulina* non-significantly changed their own and *M. sativa* declined this content under salt stress. A significant mounting in K^+/Na^+ ratio was revealed in shoots of all *Medicago* spp. On the other hand, roots of *M. sativa* and *M. lupulina* showed a significant reduction in their K^+/Na^+ ratio and the rest of species showed a non-significant change (Fig. 3).



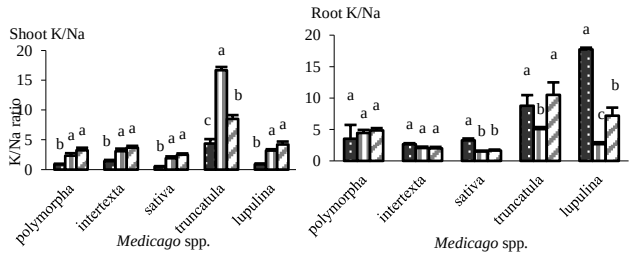


Figure 3.

3.6 Ion leakage and tolerance index

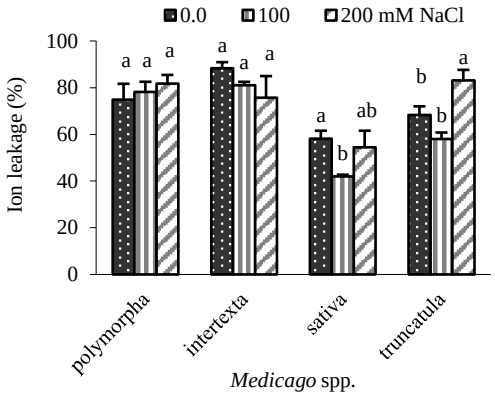


Figure 4.

No significant change was observed in ion leakage of root membranes of *M. polymorpha* and *M. intertexta*, while it decreased in those of *M. sativa* and increased in *M. truncatula*, when treated with NaCl (Fig. 4). However, *M. truncatula* showed a higher tolerance index than *M. sativa* that scored the lowest one in response to severe salinity (Table 4). Over the investigated *Medicago* spp., *M. intertexta* achieved the highest tolerance index.

4. Discussion

Both *M. polymorpha* and *M. intertexta* are widely distributed in Egyptian winter crops and occupy a wide range of habitats such as roadsides and waste ground. In these habitats in Egypt, one can notice that *M. intertexta* grows better than *M. polymorpha* in salt affected soil. *M. truncatula* grows mostly in sandy soil in the desert. Concerning *M. sativa*, it is cultivated as a fodder crop in Egypt in many different soils ranging from clay soil to sandy soil. The analysis of soil samples collected from the medices original habitats revealed that there were no significant differences among soils of *M. polymorpha*, *M. lupulina* and *M. sativa*. Soil of *M. intertexta* was characterized by high sodium content and EC, and that of *M. truncatula* was characterized by high sand content and low organic matter, indicating a presumable adaptability of these two plants to endure the severe conditions as salinity and drought.

Seed germination and early seedling growth are decisive phases in the life history of plants. Salinity stress may cause osmotic effects or ion toxicity to seeds, the former can delay germination and the latter may completely inhibit germination or render the seeds unviable (Guan et al., 2009; Khan and Gul, 2006). *Medicago* germination generally is reduced in saline soils with varying responses within and among species and genotypes (Castroluna et al., 2014; Soltani et al., 2012).

In the current study, data of germination percentage were not consistent with those of germination speed. Although *M. truncatula*, collected from sandy soil, showed the highest germination percentage, it was the slowest one among the investigated medices. In contrast to *M. truncatula*, *M. intertexta* that collected from saline soil was the least in germination percentage but the superior in germination speed. Evolving a higher germination percentage and a lower germination speed as in case of *M. sativa* and *M. truncatula* may indicate that this delay in germination occurred by osmotic effect rather than ion toxicity. In contrast, Farissi et al. (2011) found that the inhibition in alfalfa germination occurred by both an osmotic and an additional ion toxicity specific effect. The enhancement in germination percentage that scored by *M. truncatula* in response to the lower levels of salinity (50 and 100 mM NaCl) was recorded previously with other plants as *M. sativa* (Soltani et al. 2012), the halophyte quinoa

(Panuccio et al., 2014) and commercial cultivars of *Portulaca grandiflora* (Borsai et al. 2017). Generally, results in our study indicate that *M. sativa*, *M. truncatula* and *M. lupulina* exhibited the best performance in germination stage. Similarly, germination of *M. lupulina* non-significantly changed under salt stress ranged 0.0-250 mM NaCl (St-Arnaud and Vincent, 1988). The ability of seeds to germinate more rapidly or in a high percentage is important for the seeds of crop or forage to be established under stressful conditions (Ashraf and Foolad, 2005). However, performance in germination stage is not enough to consider these three species (*M. sativa, M. truncatula* and *M. lupulina*) as tolerant ones or to predict their growth in further developmental stages, when taking into consideration that salt sensitivity during the early stages of seed germination and seedling emergence is common even within halophytes (Debez et al., 2004; Vicente et al., 2004).

Over the investigated *Medicago* species in the current study, *M. intertexta* that collected from saline habitat was the most tolerant one in vegetative growth stage, though exhibiting the lowest germination percentage under salt stress, for enhancing its fresh weight significantly as well as the non-significant increase in its shoot and root dry weights and water content. In addition, this species scored the highest aerial biomass index followed by *M. truncatula* under sever salt stress. Being evolved in stressful original habitats, these two medics might experience a greater tolerance in the next generations, as stressful parental environments probably affected expression of traits in offspring, resulting in phenotypes that are adapted to the inducing stress (Herman et al., 2012; Moriuchi et al. 2016). In contrast to *M. intertexta*, the cultivated *M. sativa* was the least tolerant one to NaCl as it significantly decreased its length and biomass, and the decline was more apparent on root than shoot. Similar performance of root was reported on sensitive and moderately resistant commercial alfalfa cultivars (Castroluna et al., 2014). The observed contradictory performance of *M. intertexta* and *M. sativa* during their germination and vegetative growth supported the view about the unparalleled response to salt stress during germination, with its dominating simple water relations (Munns 2007), and later vegetative or reproductive stages.

In addition to *M. intertexta* and *M. truncatula*, maintenance of growth under salt stress was observed in *M. lupulina* indicating the ability to tolerate salinity. *M. lupulina* kept its growth criteria unchanged in response to salinity but increased its root length and root/shoot length. Due to evolving in an original loosely soil characterized with its deficiency in water and nutrients, at least at the soil surface layer, *M. lupulina* increased its root/shoot length as an evolved adaptive mechanism to enhance its water and nutrient uptake efficiency (Ben Salah et al., 2011).This enhanced root/shoot length is likely brought about the increased water content of *M. Lupulina* under severe stress. Similar to *M. lupulina*, *M. polymorpha* increased its root/shoot length, but this enhancement was not reflected on water uptake and could not secure fresh weight from the significant loss, besides it could be linked with the non-significant decrease in shoot length.

Tolerance of salt stress is very complex and includes many of the integrated physiological and molecular mechanisms utilized by the plant to endure this severe environmental condition. Among these are the mechanisms enabling the plant to decrease its water potential in order to maintain its water uptake and turgidity. Plant water potential may decrease passively by influx of soil solutes into plant, or by accumulating osmolytes that increase osmotic potential, helping the cells to keep its water homeostasis and preserve stability of cellular membranes and macromolecules, although coming on the account of growth (Munns and Gilliham, 2015). Reducing sugars (glucose and fructose), sucrose and amino acids, are among the osmolytes accumulated in plant under salt stress (Manchanda and Garg 2008; Singh et al. 2015) with varying levels within and between plant species. In the current study, the negative correlations (Table 5) that detected between shoot and root glucose (r= -0.794*), shoot and root sucrose (r= -0.813*) and shoot and root Total sugars (r= -0.954**), support the view about the dissimilar performance of shoot and root sugars in response to salt stress. This may indicate an adaptive response of the plant to fine tune its organ requirements of sugars and to regulate source and sink metabolism in relation to the defense responses. Notably, the species that collected from saline habitat and scored the highest biomass over the investigated medics under salt stress; *M. intertexta* accumulated much higher amount of RS as well as sucrose in its shoot in proportion to the root.

Under severe stress, (200 mM NaCl) ratio of *M. intertexta* shoot/root RS reached 7.6 compared with 2.6 under control conditions. While, *M. polymorpha* allocated most RS to root, and both of *M. sativa* and *M. lupulina* kept shoot/root RS ratio almost unchanged. A positive correlation was detected between tolerance index and shoot/root RS (r=0.714*) and shoot/root total sugars (r=0.962**), confirming the role of sugar accumulation in shoot as one of the adaptive mechanisms (Farooq et al. 2017; Manchanda and Garg, 2008). The non-significant change of amino acids in organs of the most investigated medics may indicate an unlikely role of amino acids in the osmotic adjustment in these plants under salt stress. The only species that significantly mounted its total free amino acids content in root by 2.1 and 5.9 fold of control under 100 and 200 mM NaCl respectively was *M. sativa*. However, this augmentation of amino acids was concomitant with a significant decrease in root length, fresh weight and dry weight in addition to the increase in sugar content. All indicated that the accumulation of these cost expensive osmolytes in *M. sativa* was not enough to confer tolerance at severe stress.

Table 5: Partial correlation among variables of *Medicago* spp. affect by various levels of salt stress (0.0, 100 and 200 mM NaCl), as controlling factor.

	SFwt	RFwt	SDwt	RDwt	SRL	SG	SS	SAA	RG	RS	RAA	SNa	SK	SKNa	RNa	RK	RKNa	EL	STS	RTS	SRTS	SRRS	ABI
RFwt	.976**																						
SDwt	.982**	.934**																					
RDwt	.954**	.888**	.935**																				
SRL	0.145	0.093	0.242	-0.011																			
SG	0.484	0.372	0.534	0.457	.796*																		
SS	-0.037	-0.012	-0.046	0.052	-.813*	-.785*																	
SAA	0.175	0.141	0.081	0.381	-.750*	-0.263	0.556																
RG	-0.61	-0.462	-.729*	-0.61	-0.643	-.794*	0.377	0.285															
RS	-0.014	0.064	-0.056	0.157	0.533	0.385	-.813*	-0.643	-0.053														
RAA	-0.272	-0.28	-0.216	-0.41	.864**	0.566	-.891**	-.801*	-0.256	.744*													
SNa	-0.278	-0.33	-0.233	-0.155	-0.527	-0.529	.796*	0.528	0.256	-.951**	-0.615												
SK	-	-	-	-	-	-	0.6	0.0	0.2	-	-	.85											

	0.401	0.393	0.333	0.428	0.215	0.521	17	9	99	.711*	0.275	4**											
SKNa	-0.619	-0.548	-0.553	-.806*	0.456	-0.132	-0.24	-0.696	0.247	0.246	0.63	0.016	0.493										
RNa	.835**	.734*	.827*	.902**	0.094	0.585	-0.024	0.486	-0.602	-0.304	-0.318	0.028	-0.241	-0.682									
RK	0.297	0.315	0.164	0.465	-.805*	-0.312	0.506	.925**	0.344	-0.4	-.811*	0.259	-0.163	-.795*	0.425								
RKNa	-0.272	-0.189	-0.395	-0.131	-.947**	-.778*	0.61	0.632	.761*	-0.252	-0.676	0.29	0.02	-0.381	-0.267	.754*							
EL	-0.327	-0.219	-0.463	-0.242	-0.669	-0.575	0.184	0.293	.716*	-0.252	-0.276	-0.182	-0.346	-0.254	-0.449	0.526	.869**						
STS	0.262	0.229	0.275	0.369	-0.656	-0.479	.919**	0.64	0.048	-.909**	-.911**	.794*	0.544	-0.428	0.349	0.539	0.38	-0.098					
RTS	-0.174	-0.094	-0.197	-0.341	0.611	0.353	-.831*	-.736*	0.003	.972**	.850**	-.863**	-0.542	0.459	-0.423	-0.552	-0.334	0.159	-.954**				
SRTS	0.217	0.131	0.254	0.378	-0.575	-0.333	.830*	0.674	-0.008	-.957**	-.841**	.834*	0.52	-0.469	0.426	0.503	0.289	-0.194	.963**	-.993**			
SRRS	0.654	0.49	.725*	.718*	0.517	.871**	-0.399	0.044	-.910**	-0.033	0.146	-0.198	-0.377	-0.451	-.815*	0.054	-0.63	-0.634	-0.018	-0.111	0.149		
ABI	.686*	.950**	.908**	.689*	-0.263	.641*	0.071	0.376	-0.311	-0.411	-0.156	0.176	0.474	.649*	0.28	-0.447	-0.541	0.176	0.204	-0.37	.760*	.701*	
TI	.698*	.806**	.920**	.642*	-0.082	0.56	-0.023	0.363	-0.551	-.623*	-0.357	0.46	.698*	.718*	0.537	-0.187	-0.388	0.314	0.097	-0.597	.924**	.870**	.916**

SFwt; Shoot fresh wt, RFwt; Root fresh wt, SDwt; Shoot dry wt, RDwt; Root dry wt, SRL; Shoot/root length, SG; Shoot glucose, SS; Shoot sucrose, SAA; Shoot amino acids, RG; Root glucose, RS; Root sucrose, RAA; Root amino acids, SNa; Shoot sodium, SK; Shoot potasium, SKNa; Shoot K/Na, RNa; Root sodium, , RK; Root potasium, RKNa; Root K/Na, EL; Ion leakage, STS; Shoot total sugars, RTS; Root total sugars, SRTS; Soot/Root total sugars, SRRS; Soot/Root reducing sugars, ABI; aerial biomass index, TI; Totlerance index. **. Correlation is significant at 0.01 level, *. Correlation is significant at 0.05 level.

The pressure selection exerted by the stressful environment is documented to induce adaptation on the genetic level in a process known as evolutionary adaptation (Bijlsma and Loeschcke 2005; Ahmed et al., 2010). Ion exclusion, retrieval of Na^+ from xylem and sequestration of Na^+ in vacuole are among the adaptation responses to the accumulated Na^+ in soil (Abiala et al., 2018; Manchanda and Garg, 2008; Sandhu et al., 2017). In the current study, none of the investigated *Medicago* species accumulated Na in its shoot; indicating a likely uploading in xylem and exclusion from the leaf surface or the retrieval to the root via xylem (Tester and Davenport, 2003). Moreover, all the investigated species increased K^+ accumulation in their shoots by increasing NaCl concentration in soil, besides enhancing shoot K^+/Na^+ ratio as one of the main tolerance mechanisms that maintain protein synthesis (Flowers and Dalmond, 1992). Sibole et al. (2005) reported that *M. citrina* was able to exclude Na^+ from the leaves more selectively, while *M. arborea* had a greater buildup of leaf blade Na^+. The better response of *M. citrina* was associated with its Na^+ exclusion capacity and the upregulation of plasma membrane H^+-ATPase. In contrast to the similar performance among the investigated *Medicago* spp shoots, variance in their original habitats renders the roots to undergo differential ion homeostasis adaptations. It seems that *M. intertexta*, followed by *M. truncatula* and *M. lupulina*, those collected from stressful original habitats and scored the highest tolerance index orderly, tended to accumulate Na in their roots and transport fewer amounts to the shoots. In contrast, *M. polymorpha* and *M. sativa*, the less tolerant, kept their roots Na^+ content unchanged under salt-stress. Zahran et al. (2007) reported that the low tolerance of *M. intertexta* in response to salt stress was related to the high accumulation of Na^+ in both roots and leaves. While in *M. ciliaris*, Na^+ accumulated in both shoots and roots of the tolerant and sensitive lines (Ben Salah et al., 2011). Although harmful effect of Na+ accumulation in plant cell is well documented (Tester and Davenport 2003), the plants (*M. intertexts, M. truncatula* and *M. lupulina*) that accumulated Na^+ in their roots showed a non-significant change, if not an increase, of root dry biomass. Additionally, positive correlations were found between the root Na^+ content and fresh weight of shoot (r=0.835**) and root (r=0.734*), and dry weight of shoot (r=0.827*) and root (r=0.902**). This indicated a good management of Na homeostasis in root organelles and a probable sequestration of Na^+ into vacuole to avoid the harmful effect of Na^+ on cytoplasm and to be utilized in osmotic adjustment and maintenance of water status of the plant (Liang et al. 2018; Sandhu et al. 2017). Stimulation of Na^+/H^+ tonoplast antiporter that is functioning in sequestration of Na into the vacuole was reported as one of legume responses to salt stress (Quan et al., 2016; Zahran et al., 2007).

Compartmentation of Na^+ in vacuole necessitates the accumulation of osmoticums like K^+ or organic solutes in cytoplasm to maintain water homeostasis and to keep the balance between Na^+ and K^+ contents in plant tissues (Munns et al., 2016). However, among the current three species that accumulated much Na^+ in their roots, *M. truncatula* was the only species that increased its potassium content in root, while *M. intertexta* and *M. lupulina* maintained this content unchanged. That resulted in a non-significantly changed K^+/ Na^+ ratio in *M. intertexta* and *M. trucatula* and a decreased ratio in *M. lupulina* under severe stress. Increasing both Na^+ and K^+ in root of *M. truncatula*, that collected from sandy soil with low EC value (Table 1), and that adaptive to only osmotic rather than salt stress, may be the reason of its high level of ion leakage. It seems that each of the three most tolerant species, *M. intertexta*, *M. truncatula* and *M. lupulina* relied on a specific adaptive mechanism. For more detailes, *M. intertexta* relied on high speed of germination and sequestration of Na^+ to its root cell vacules, *M. truncatula* relied on accumulation of k^+ and *M. lupulina* relied on enhancing root length, root/shoot ratio and the efficient water uptake. The variation in adaptive responses and mechanisms among the investigated species probably evolved as a result of the natural selection they experienced in their respective original habitats having various stressful levels.

A propensity to exclude Na^+ may be the reason of the non-significant change of Na content in roots of *M. polymorpha* and *M. sativa* as a result of increasing the exogenous NaCl concentration. The former species that collected from roadsides enhanced potassium accumulation in root and maintained K^+/ Na^+ ratio close to control level, while the later decreased K^+ accumulation as well as a K^+/ Na^+ ratio and increased the accumulation of root sugars and amino acids by increasing salinity level indicating that this species relied on accumulation of organic solutes as osmolytes and osmoprotectants over K^+, and hence exhibited the less tolerance index. There was no detected correlation between K^+ content and RS or sucrose. However, the positive correlation (r=0.744*) between root sucrose and amino acids in addition to the negative one (r=-0.811*) that detected between the later and root K^+ content may indicate that a coordinative or synergistic sugar-amino acids accumulation, although it was ineffective against stress, is managed by the plant only in case of a deficient accumulation of K^+ in root. Both species; *M. polymorpha* and *M. sativa* excluded sodium from their roots that accumulated high levels of RS. These sugars might be manipulated by those species in osmotic adjustment and/or as a substrate in respiration resulting in enhanced ATP

that is necessary for the H^+ pump and the associated sodium exclusion by Na^+/H^+ antiporter (Munns et al., 2016).

5. Conclusion

Generally, the results of the current study indicated that tolerance of the investigated *Medicago* species at germination was not analogous to that at vegetative stage. The current results recommend cultivating *M. intertexta, M. truncatula* and *M. lupulina* in salt-affected lands or using them as wild relatives to improve alfalfa. Results also indicate how the diversity in native habitat induces the evolutionary adaptation and the plasticity in responses to specific stressful condition.

Conflicts of Interest

The authors declare no conflicts of interest.

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