

Physiological and Morphological Responses of White Clover (*Trifolium repens*) and Red Clover (*Trifolium pratense*) Plants to Salinity Stress

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Trifolium spp. are native plants in Iran exhibiting good ground cover potential. Salinity is a major environmental stress and today, 20% of the world cultivated areas and nearly half of all irrigated lands are affected by salinity. The main aim of this research was to study morphological and physiological adaptations of these native species under different salinity levels for urban landscape usage. For this purpose a factorial experiment based on completely randomized design (CRD) with 4 replicates was conducted. Plants of *Trifolium repens* and two *Trifolium pratense* species (native and commercial) were submitted to salinity stress in a pot experiment with 4 different salinity levels (0, 75, 150, 225 mM NaCl) during the experiment period. Leaf area, LRWC (leaf relative water content), electrolyte leakage, root and shoot length and root and shoot fresh and dry weight were measured. Results showed significant differences within salinity treatments in all 3 species studied. Highest values measured for traits in cultivars were observed in *T. pratense* commercial followed by *T. pratense* native and *T. repens* except electrolyte leakage and root fresh weight. But salinity effect on these species increased along with severity, linearly. Leaf area was most affected and decreased to 40.21 mm² (225 mM) from 184.14 mm² in control. Generally *Trifolium* species showed sensitivity to salinity stress, especially in higher levels. This study should help understand some physiological and morphological responses of *Trifolium* species to salinity stress for urban landscape projects, parks and xeriscapes in Iran.

Abstract

Keywords: Environmental stress, Native species, Ornamental groundcovers, Plant response, Urban landscape.

INTRODUCTION

Iran's climate is mainly characterized by low rainfall, high temperatures in summer and low temperatures in winter in vast majority across the country. This, together with scarcity and low quality of the water resources, prevent a quick recovery of the soil plant covering (Save *et al.*, 1999). Because of these restrictions, it is important to consider the use of salt and drought tolerant species for new urban landscapes and to preserve soils with little plant cover (Morales *et al.*, 1998). Another great problem associated with low water resources and highly urbanized areas is salinization of soils. In the last decade these problems have increased the degraded areas, but at the same time society is demanding the reclamation of these areas. Activities such as landscaping and gardening are the answer for this reclamation but their costs are usually high, because of the resources used in their management (Luecke, 1993). In this sense, the use of native species may be of interest due to their characteristics and potential ability to adapt to adverse conditions in landscaping projects, parks, xeriscape gardening and public areas. Also, marketing studies have shown that nursery demand for wild species is growing continuously (Klougart, 1987).

Clover (*Trifolium*) as a member of Fabaceae family is a genus of about 300 species of plants native to some parts of Iran. The scientific name derives from the Latin tres, "three", and folium, "leaf", so called from the characteristic form of the leaf, which has three leaflets (trifoliate); hence the popular name trefoil. Highest diversity is found in the temperate Northern Hemisphere, but many species also occur in South America and Africa, including at high altitudes on mountains in the tropics.

Salinity is a major environmental stress and is a substantial constraint to crop production. Increased salinization of arable land is expected to have devastating global effects, resulting in 30% land loss within next 25 years and up to 50% by the middle of 21st century (Wang *et al.*, 2003). Many researches have been undertaken determining ornamental plant responses to environmental stresses.

Plant height was significantly inhibited by salinity in *Arbutus unedo* seedlings submitted to three irrigation treatments using solutions with an EC of 0.85 dS m⁻¹ (control treatment), 5.45 dS m⁻¹ and 9.45 dS m⁻¹ (Navaro *et al.*, 2008). Plants of *Argyranthemum coronopifolium* were submitted to salt stress (15 days of exposure to 140 mM NaCl followed by a recovery period of 11 days), independently. Water and salt stress promoted reductions in leaf biomass due to both senescence and death of leaves, what has been considered an avoidance mechanism that allows minimizing water losses (Herralde *et al.*, 1998). Three-week-old *Cakile maritima* plants were subjected to 0, 100, and 400 mM NaCl for 28 days under glasshouse conditions and growth indexes such as shoot biomass, leaf expansion was significantly restricted at 100 and 400 mM NaCl, compared to the control (Ksouri *et al.*, 2007). Four-month old potted *Cistus albidus* and *Cistus monspeliensis* plants were submitted to saline stress using irrigation water containing 0, 70 and 140 mM NaCl. Both species respond to saline stress by developing avoidance and tolerance mechanisms. The avoidance mechanism took place at a physiological and morphological level (Torrecillas *et al.*, 2003). *Crithmum maritimum* growth was significantly improved at moderate salt levels (50 mM NaCl), but drastically reduced at 200 mM NaCl (Nader *et al.*, 2005). *Asteriscus maritimus* plants under saline and water stress conditions, showed lower biomass and an early reduction in leaf expansion growth (Rodriguez *et al.*, 2005). A laboratory experiment was carried out to assess the physiological behavior of *Lotus creticus* in Tunisia. The presence of salt in the medium affects growth of the whole plant. Compared to root biomass, the shoot was more affected by salt (Rejili *et al.*, 2007). Eight herbaceous perennials and groundcovers (*Penstemon eatonii*, *P. pseudospectabilis*, *P. strictus*, *Ceratostigma plumbaginoides*, *Delosperma cooperi*, *Gazania rigens*, and *Lavandula angustifolia* and *Teucrium chamaedrys*) were subjected to 4 levels of saline water at electrical conductivity of 0.8, 3.2, 6.4 and 12 dS m⁻¹. Some of these plants did not survive at severe levels and shoot and root tissue at the end of the experiment on surviving plants were also affected by salinity levels and varied among species (Genhua and Denise, 2006).

The aim of the present work was to study the morphological and physiological adaptations of one native and two commercial species under different salinity conditions. Sensitiveness to salt stress was tested in order to know the response of this species to higher salt concentrations in soil solution.

MATERIALS AND METHODS

Plant material and growth conditions

Trifolium repens and *Trifolium pratense* seeds were supplied from Pakan Bazr Co. Esfahan, Iran and they were sown in a cold frame in October 2010. Four leaf plantlets were then transplanted into 30 cm diameter plastic pots filled with a mixture of sand and practical soil by mid March. The pots were placed under natural open conditions in spring 2011 of Mashhad. This experiment was conducted as a factorial experiment based on completely randomized design with salinity at (0, 75, 150 and 225 mM) and three *Trifolium* species with 4 replicates.

Salt stress experiment

Fully expanded plants were submitted to daily saline water irrigation stress. Six weeks after transplanting, the pots were left in four treatments corresponding to the different NaCl concentrations: 0, 75, 150 and 225 mM.

Leaf area

Leaf surface was determined using a Li-3100 area meter (LI, Lincoln, Nebraska, USA).

LRWC and EL

To measure LRWC, fresh weight (FW) of two excised leaves per plant were weighed and placed in plastic bags in the dark with their petioles plunged in distilled water overnight to allow them to reach full turgor and, hence, to determine their turgid weight (TW). These leaves were then dried at 70 °C for 24 h and their dry weight (DW) was recorded. Then LRWC was calculated using the following equation:

$$\% \text{ LRWC} = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100$$

Electrolyte leakage was calculated by following the standard method of Pinhero and Fletcher (1994).

SPAD

Leaf chlorophyll related SPAD units were determined using a SPAD-502 Chlorophyll Meter (Konica, Minolta, Tokyo) on 2 different leaves of each plant, and the mean value was calculated.

Root and shoot length

Root and shoot length was measured after picking up plants at the end of experiment period using a ruler.

Root and shoot fresh and dry weight

Roots, leaves, and stems of examined plants were weighted after picking up from experimental plots. They were then dried (70 °C for 48 h), and dry weights were recorded.

Sensitivity rate index (IS)

The effect of salt on growth can be appreciated by a sensitivity rate index (IS) calculated according to the following formula (Rejili *et al.*, 2007):

$$I_s = [(\text{DW}_{\text{NaCl}} - \text{DW}_{\text{Control}}) / \text{DW}_{\text{Control}}] \times 100$$

Statistical analysis

Data were analyzed as factorial ANOVA using JMP4. Where significant ($p \leq 0.05$) treatment effects were determined by ANOVA, data means were separated by the LSD test.

RESULTS AND DISCUSSION

Clover species

Analysis of variance showed significant effects of salinity within different growth characteristics of examined *Trifolium* species and salinity stress ($p \leq 0.01$ and $p \leq 0.05$) (Table 1). The cultivar effect was statistically significant in root length, shoot fresh weight, electrolyte leakage ($p \leq 0.001$) and shoot length, root fresh weight and leaf relative water content ($p \leq 0.05$). But no significant results was observed in leaf area, shoot dry weight, root dry weight, leaf fresh and dry weight, spad units and severity index. Growth characteristics of three *Trifolium* species studied are shown in Table 2. According to these results, there are noticeable differences among measured traits in them. Highest values are mainly recorded in *T. pratense* (F), except root fresh weight and electrolyte leakage (Table 1). *T. pratense* (N) and *T. repens* take the next scores, respectively. For landscape usage at a glance, it seems that between 3 *Trifolium* species studied, *T. pratense* (F) can be suggested the first candidate followed by *T. pratense* (N) and *T. repens* as alternatives. But on the other hand, growth habit is different among these species. In case of white clover, as the plant spreads its stems over the soil stolon make a carpet and protect the soil from erosion (Alcaraz *et al.*, 1997). *T. repens* bears also phytoremediation properties for contaminated soils (Wang and Oyazu, 2009). Therefore, in general considering all different aspects for a suitable green groundcover, all three species are suggested for this purpose. Interaction effects of treatments used were not significant at any statistical level.

Salt stress

Leaf relative water content

Results from leaf relative water content (%) measurement shows a significant difference in salinity treatment over control ($p \leq 0.001$) (Table 1). According to the results, salinity levels decreased LRWC of *Trifolium* species, significantly. The highest (56.52%) and lowest (38.86%) amount of this trait measured was observed in control and 225 mM most severe treatment which shows a 45% decrease (Fig. 1b). Leaf relative water content (LRWC) is an index representing the amount of water in the plant organs and shows the ability of a plant in maintaining water under stress conditions (Abbaszadeh *et al.*, 2008). So in a controlled environment for an experiment, the measured LRWC shows the response of a plant and the higher the measured amount, the greater the ability of a treatment for keeping water (Abbaszadeh *et al.*, 2008). Results in this experiment show a gradual linear decrease from 0 to 150 mM and a big collapse from then to 225 mM. These results explain optimum resistance levels in *Trifolium* species to a mild salinity stress and sensitivity to severe stress. Our findings agree with Genhua and Denise (2006) which LRWC of *Ceratostigma plumbagioides* decreased significantly from 78% to 64% as irrigation salinity increased and do not agree with Nader *et al.*, (2005) on *Crithmum maritimum* which shows a very small decrease in root and shoot LRWC with increasing salt concentration.

Leaf area

Salinity stress showed a significant effect in leaf area ($p \leq 0.01$) (Table 1). Highest and lowest values (184.147 and 40.21 cm²) were observed in control and 225 mM salinity treatment, respectively (Fig. 1c). As happens in many plant species, all three species examined had a lower total biomass and leaf area at the end of the stress period in comparison to control. However, each species act differently in response to salinity stress. In general, the first symptom of salt stress in ornamental plants is a reduction in leaf area (Rodriguez *et al.*, 2005). Our results agree with Navaro *et al.*, (2008) on *Arbutus unedo* seedlings which reduced leaf area from 5.63 to 3.22 cm² during salinity increase. However in case of *Cakile maritima* leaf area was salinity dependent and significantly stimulated in moderate levels of stress (Ksouri *et al.*, 2007). As a morphological point of view reduction in the canopy area can be considered as an avoidance mechanism (Torrecillas *et al.*, 2003). Leaf area of *A. maritimum* treated plants decreased earlier than other morphological pa-

rameters by salinity effect. This behavior confirms that, in general, the first symptoms of salt stress in the plants is a restriction in leaf expansion (Matsuda and Riazi, 1981; Alarcon *et al.*, 1993). Under the same conditions, the reduction in shoot dry weight was due to a more substantial effect in the stem than in leaf dry weight values (Sanchez-Blanco *et al.*, 1998). The reduction in leaf area under drought and saline stress can be considered as avoidance mechanisms, which minimize water losses when the stomata are closed, which happens to many species under osmotic stress (Blum, 1986; Save *et al.*, 1994; Ruiz-Sanchez *et al.*, 2000). Under saline conditions it is known that the reduction in total leaf area can be explained by a decrease in leaf turgor, changes in cell wall properties or a decreased photosynthesis rate (Franco *et al.*, 1997).

Leaf chlorophyll related SPAD units

Results from leaf chlorophyll related SPAD unit measurements show a significant difference between salinity treatments ($p \leq 0.01$) (Table 1). Compared to control (49.32), the degree of reduction reached (30.25) in 225 mM salinity stress (Fig. 1d). Collected results agrees with Forens *et al.*, (2007) results on pre-conditioning *Petunia*, *Calendula* and *Calceolaria* to drought by means of saline water irrigation as related to salinity tolerance. A reduction in canopy area as an avoidance mechanism along with chlorophyll deterioration which usually occur in salinity stress (Torrecillas *et al.*, 2003), may be the main reason for this slight decrease. Visual symptoms of salt injury, such as burning leaf margin were observed in plants.

Root and shoot length

Effect of salinity stress was highly significant in this trait ($p \leq 0.001$) (Table 1). At the end of the experimental period, salinity had led to a significant decrease in root and shoot length. Highest (26 and 21 cm) and lowest (18 and 11.55 cm) amounts measured for this trait were similarly observed in control and severest salinity stress (Fig. 1e and f). Abdul-Halim *et al.*, (1988) stated that an increase in irrigation water salinity reduces plant height. Also plant height was significantly inhibited by salinity and treated plants reached 70% of the height of control plants (Navaro *et al.*, 2008) which agrees with the results of this study.

Root and shoot fresh and dry weight

According to ANOVA in Table 1, applying salinity stress significantly affected shoot and root fresh weights of different organs in examined plants were significantly affected by salinity ($p \leq 0.001$ and $p \leq 0.05$, respectively), whereas dry weights were not affected (Table 1). 6.03, 3.62 and 3.8 grams, respectively shoot; root and leaf fresh weights were decreased to 1.8, 1.12 and 0.8 grams by 235, 250 and 375%, respectively. Salinity decreased shoot fresh weight in *Arbutus unedo* seedlings but root fresh weight was not affected although it was also reduced (Navaro *et al.*, 2008). Shoot and root dry weights were also reduced by salinity in all 4 transplanting times in *Cistus albidus* and *Cistus monspeliensis* (Torrecillas *et al.*, 2003) and in *Ceratostigma plumbaginoides* (Genhua and Denise, 2006). Several authors suggested that, under saline stress, the osmotic effect is responsible for the aerial organ growth reduction (Muuns and Termaat, 1986; Yeo *et al.*, 1991; Rengel, 1992). Root, shoot and leaf dry weight reduced with increased salinity water irrigation in *Asteriscus maritimus* plants (Rodriguez *et al.*, 2005). Under saline stress conditions the main growth limiting factor for *Cistus albidus* and *Cistus monspeliensis* was photosynthesis (Torrecillas *et al.*, 2003). It seems that a reduction in whole plant fresh and dry weight in different organs is due to the limiting factor of photosynthesis.

Sensitivity index (SI)

According to the results shown in Table 1, salinity treatments significantly increased severity index ($p \leq 0.01$) (Table 1). Whole plant sensitivity index (%), is shown in (Fig. 1i) plants did not

express their maximum potentialities. 25.2, 42.21 and finally 70.24% were calculated for this experiment which shows that nearly 70% of experimental plants are damaged or completely destructed by then. Increased severity goes on very sharp and spreading. This situation is observed in *Lotus creticus* with a little difference and not as this sharp (Rejili *et al.*, 2007). Other measured traits and interaction treatments were not significant in any statistical level.

Three *Trifolium* species in this study showed different levels of resistance and tolerance to salinity stress. Plant deterioration and death almost happened at the highest level of stress and in low and mild stress levels, plants showed a good appearance. All interaction effects were not significant in any statistical level which means that stress response of examined *Trifolium* species are the same. This study, in general, may be useful in landscape and revegetation projects by selecting suitable potential species.

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Tables

Table 1. Mean square analysis of studied traits in this experiment.

Mean square													
	RL	SL	SFW	SDW	RFW	RDW	LFW	LDW	LA	EL	LRWC	SPAD	SI
C	555.16***	229.31*	34.15***	1.04 ^{ns}	19.74*	0.53 ^{ns}	7.71 ^{ns}	0.52 ^{ns}	11864.82 ^{ns}	714.13***	371.98*	172.14 ^{ns}	209.87 ^{ns}
S	144.37***	231.8**	42.26***	0.47 ^{ns}	15.27*	0.78 ^{ns}	23.17***	0.73 ^{ns}	53000.61***	76.07 ^{ns}	760.15***	740.70***	988.63**
C×S	55.15 ^{ns}	14.05 ^{ns}	7.34 ^{ns}	0.22 ^{ns}	2.89 ^{ns}	0.25 ^{ns}	1.35 ^{ns}	0.27 ^{ns}	2203.11 ^{ns}	40.21 ^{ns}	97.18 ^{ns}	105.07 ^{ns}	748.48 ^{ns}
E	30.38	48.27	6.14	0.36	5.93	0.42	2.71	0.34	4702.9	135.09	91.06	116.55	688.24

C: Cultivar, S: Salinity, C×S: Cultivar × Salinity, E: Error. RL: Root length, SL: Shoot length, SFW: Shoot fresh weight, SDW: Shoot dry weight, RFW: Root fresh weight, RDW: Root dry weight, LFW: Leaf fresh weight, LDW: Leaf dry weight, LA: Leaf area, EL: Electrolyte leakage, LRWC: Leaf relative water content, SPAD: SPAD unit, SI: Severity index. ns, not significant; *, ** indicate significance at P < 0.05, 0.01, respectively

Table 2. Means comparison of investigated growth characteristics of three *Trifolium* species studied.

	RL	SL	SFW	SDW	RFW	RDW	LFW	LDW	LA	EL	LRWC	SI	SPAD
TR	17.9b	11.42b	3.77a	1.08a	3.56a	0.84a	2.01a	0.89a	87.72b	83.34a	49.77b	45.52a	37.18b
TP (N)	20.37b	16.63a	2.21b	0.7a	0.97b	0.49a	1.37b	0.56a	67.81c	69.88b	45.37b	52.36a	38.25b
TP (F)	29.18a	18a	5.23a	1.23a	3.19a	0.74a	2.8a	0.83a	122.91a	74.81b	54.83a	56.34a	43.32a

TR: *Trifolium repens*, TP (N): *Trifolium pratense* native, TP (F): *Trifolium pratense* commercial, RL: Root length, SL: Shoot length, SFW: Shoot fresh weight, SDW: Shoot dry weight, RFW: Root fresh weight, RDW: Root dry weight, LFW: Leaf fresh weight, LDW: Leaf dry weight, LA: Leaf area, EL: Electrolyte leakage, LRWC: Leaf relative water content, SPAD: SPAD unit, SI: Severity index. Figures with different letters in each column are significantly different at P≤5%.

Figures

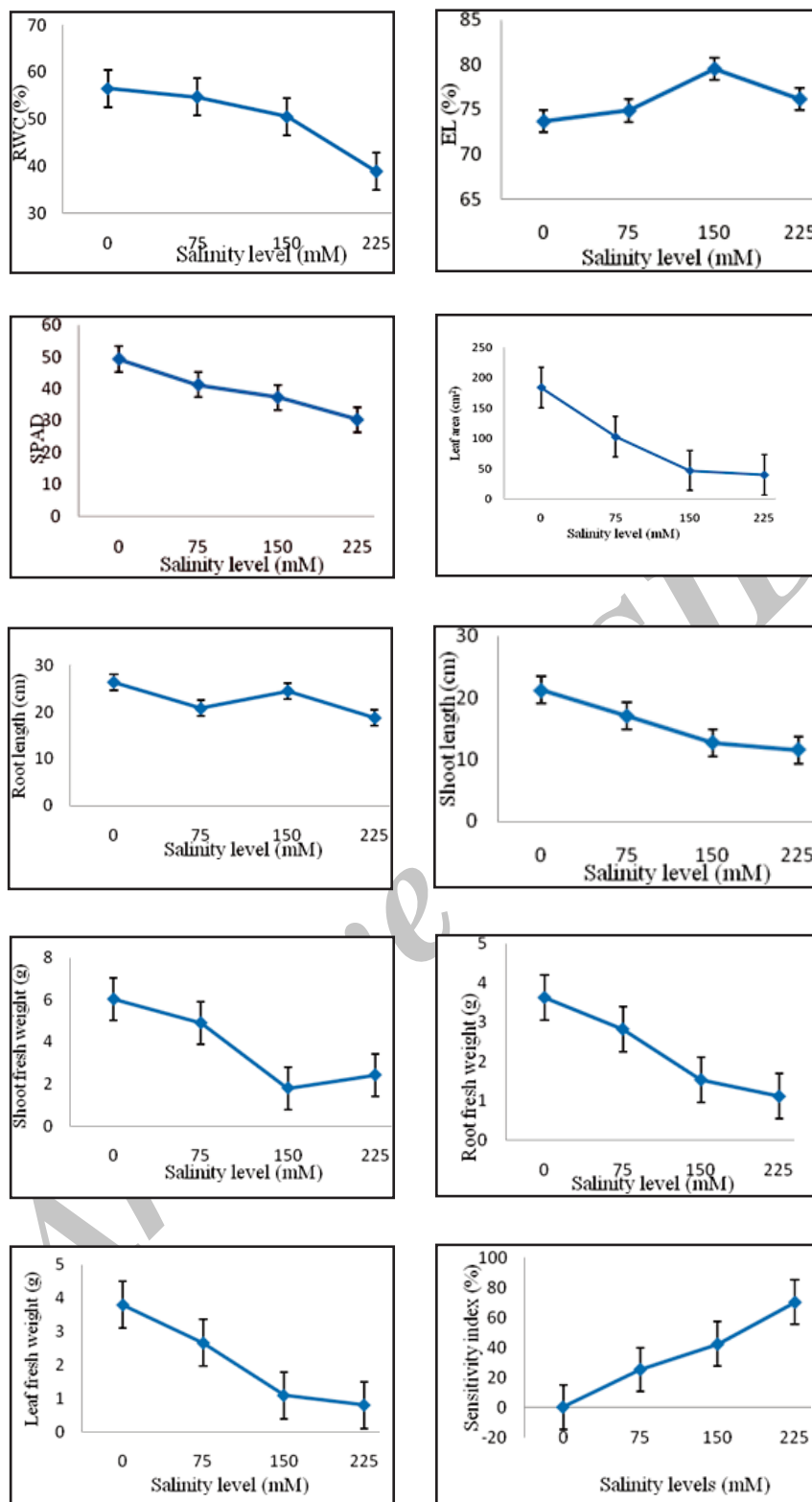


Fig. 1. Effects of different soil salinity solutions on measured indexes: a) electrolyte leakage, b) relative water content, c) leaf area, d) leaf chlorophyll related SPAD, e) shoot length, f) root length, g) root fresh weight, h) shoot fresh weight, i) sensitivity index, j) leaf fresh weight.