Effects of salinity and drought stress on germination, biomass and growth in three varieties of *Medicago sativa* L.

Efectos de la salinidad y sequía sobre la germinación, biomasa y crecimiento en tres variedades de *Medicago sativa* L.

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Abstract

To evaluate alfalfa (*Medicago sativa* L.) tolerance to osmotic stress induced by polyethylene glycol 6000 (PEG6000) and salinity (NaCl), germination and growth was investigated in early seedling stages in three genotypes: Dk166, Verdor and Salina varieties. This investigation was performed using a completely randomized design with five replications: energy and power of germination, length of leaves, fresh and dry weight from roots and leaves. Results showed that germination, in which both solutions were used, was delayed in all varieties, with differences between genotypes. Seedling growth under light stress showed an increase in aerial biomass, while in severe drought and salinity stress, the varieties died except var. Salina (tolerant). PEG caused death in Verdor variety that showed more sensibility to PEG than to NaCl. In roots, the more sensitive genotypes faced to light salt stress stop their growth and under light drought stress, they increased it. Our studies allowed us to define the commercial vari-

Resumen

En plántulas de alfalfa (*Medicago sativa* L.) se evaluó la germinación y el crecimiento y su tolerancia al estrés osmótico inducido por Polietilenglicol 6000 (PEG6000) y salinidad (NaCl) en tres genotipos distintos: Dk166, Verdor y Salina. Esta investigación se llevó a cabo con un diseño experimental completamente aleatorizado, con cinco réplicas y los parámetros que se determinaron fueron: energía y poder germinativo, largo de hojas y peso fresco y peso seco de raíces y hojas. Los resultados mostraron que la germinación fue retrasada en todas las variedades con ambas soluciones, con diferencias entre genotipos. El crecimiento de las plántulas, bajo un ligero estrés mostraron un incremento de la biomasa aérea y en un estrés hídrico y salino severo, muerte de las plantas excepto en Var. Salina (tolerante). PEG causó muerte en la variedad Verdor que mostró más sensibilidad a PEG que a NaCl. En raíces, los genotipos más sensibles frente a leves estrés salinos detuvieron su crecimiento y bajo leves

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eties of *Medicago sativa* as moderately tolerant to salinity and drought the Dk166 variety; as sensitive or intolerant to salinity and drought the Verdor variety and as highly tolerant to salinity and insensitive to drought the Salinity variety.

Keywords

Drought stress, germination, growth, *Medicago sativa*, salinity stress.

situaciones de estrés hídrico lo incrementaron. Nuestros estudios nos permiten definir las variedades comerciales de *Medicago sativa* como moderadamente tolerantes a salinidad y sequía la variedad DK166, sensible o intolerante a salinidad y sequía la variedad Verdor y con una alta tolerancia a salinidad e insensible a sequía la variedad Salina.

Palabras clave

Crecimiento, estrés hídrico, estrés salino, germinación, *Medicago sativa*.

Introduction

Abiotic stress conditions cause extensive losses to agricultural production worldwide (Bray *et al.*, 2000). Drought and salinity stress can significantly affect plant yield in arid and semi-arid regions. Productive lands represent the 53% of earth´s land surface, while the non-cultivated area a 12%, which are equivalent to 150 million ha (Massoud, 1981). Of these lands, around 23% of cultivated lands (340 million ha) are salinized or are becoming salinized. The saline- sodic soils represent approximately 10% of cultivated lands and are being spread around more than 100 countries.

This fact represents a challenge to keep the balance between agronomic productivity and salinization, taking care the quality of water resources. Other data estimate that a third part of the world surface under irrigation (230 million ha) are much or less affected by salinity (Tanji, 1990). The salinization problem is a slow process that responds to several causes; however irrigation practices were the principal activity that produced the dramatic effects on lands with agronomical behavior. This effect gains greater significance as the permanent requirement of improving food production responds to the rapid increase of population all over the world (Jurinak and Suarez, 1990), then agricultural activity growth and the agronomical frontier to semiarid regions are extending and increasing these effects (Jurinak and Suarez, 1990).

A decrease in plant growth in salinity soils is caused by the osmotic and water potential of soil, specific toxicity, and nutritional deficit. After these primaries effects, secondary stresses happen as the oxidative damage (Zhu, 2001). The physiological symptoms associated with the toxicity of the ions are: membrane perturbations and damage in organelles (Croughan *et al.*, 1978); decrease or inhibition of the enzymatic activity (Greenway and Munns, 1980), inhibition of the photosynthesis (Schwarz and Gale, 1981), (Walker *et al.*, 1981), derivation of the plant metabolic energy to defense processes (Yeo, 1983) and change in stress hormone as jasmonic acid (JA) (Pedranzani *et al.*, 2003). These metabolic changes determine a decrease in plant growth; and consequently, the plant dies (Munns, 1993).

When water stress occurs, plants react by slowing down or stopping their growth. This is a normal plant reaction to lack of water and it acts as a survival technique (Zhu, 2002). Water stress has become a worldwide problem, thus being a severe threat to sustainable agriculture. Due to an increase in population, water resources deficit and degrading environmental conditions on the globe, stress physiology in crops has become one of the central issueof plant biology. These conditions develop frequently in a semi-arid and arid climate. water-stress tolerance has been study in many plants, but it varies from species to species (Chaitanya *et al.*, 2003).

Alfalfa is the most commonly forage species used as bovine food in Argentina, because of its lower production costs, high quality (digestibility and protein content) and regular presence during the year. Regions with aridity and salinity in Argentina are increasing due to climate change and non-sustainable agricultural practices, which creates a need to study tolerant germplasm (Castro Luna, 2009).

The aim of our study was to evaluate salt stress and water stress response in three varieties of *Medicago sativa* commonly regarded as tolerant: DK166 (Dekalb); Verdor (Barenbrug-Palaversich) and Salina PV (Palo Verde).

Materials and methods

Experimental design

The experiment was carried out in a culture chamber under controlled conditions of temperature and light, in which the influence of salt and drought on the germination of seeds, growth and biomass of *Medicago sativa* plants were monitored. The model plants were of three varieties: DK166 (Dekalb); Verdor (Barenbrug-Palaversich) and Salina PV (Palo Verde).

Germination

The three varieties of seeds were germinated in a dish between wet filter paper towels in a dark chamber with 20ºC and 70% of relative humidity. The germination percentage was measured on the 3rd day as vigor or energy of germination (EG) and on the 7th day as final percentage of germination (FPG) performed on five replicates. We considered as germinated those seeds with a radicle length of one cm.

- Control variant: watering with three ml of distilled water in each dish two times a week.
- Stressed variant: watering with NaCl solutions (50 mM, 100 mM and 200 mM) and with PEG 6000 solutions (-0.5MPa, -1 MPa, -1.5 MPa) twice a week.

Plant growth

Plants were grown in pots with soil: perlite 4:1 (container diameter of 20 cm and volume of 1L).The conditions for growing was 16 h light, at 25ºC and 60% relative humidity, and 8 h dark, 20ºC and 70% relative humidity. Each variety of *Medicago sativa* was put under three treatments (control and stressed with salt and with PEG 6000). The stressed were started when plants had reached a length of 10 cm.

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- Control variant: watering was used to keep the water content in the substrate at the level of 70% MCC (Maximum capillary water capacity).
- Stressed variant: salinity stress, plants were watered with 100 ml of NaCl solutions (50 mM, 100 mM and 200 mM) and drought stress simulated by irrigations with 100 ml of PEG 6000 solutions, which generated an osmotic pressure of -0.5MPa, -1 MPa, -1.5 MPa respectively.

Both treatments were made twice in a week, for a period of seven days.

The samples were extracted and growth parameters were measured: fresh weight (FW), dry weight (DW), length of roots and leaves, seven days after the treatment began.

The statistical evaluation of the experiments was made using multifactor analysis of variance (ANOVA) and the obtained values were compared in more detail using the Tuckey test at the significance level ($\alpha = 0.05$). Statistical analysis was performed using the software Statistical 9.0 for MS Windows.

Results

Germination

In DK166, the energy of germination (EG) decreased significantly compared to control under all NaCl concentrations (39.66 and 91% respectively) (table 1). However, for the power germination (FPG) between the control and the lowest concentration of NaCl (50 mM) did not differ significantly (table 1) and in 100 and 200 mM treatments, the FPG decreased in 39 and 88 % respectively, showing significant differences from control (table 1). Under drought stress simulated by PEG 6000, EG diminished to 43% and 68% respectively in the treatments with -1 and -1.5 MPa of pressure. The FPG showed a significant decrease with the treatment of 1.5 MPa of pressure (table 1).

In Verdor, EG showed a significant decrease of 36 and 96% in the treatments with 100 and 200 mM NaCl respectively; however, FPG showed a significant decrease of 97% only with 200 mM NaCl. Under drought stress simulated by PEG 6000, EG diminished to 54 % and 94% respectively in the treatments with -1 and -1.5 MPa of pressure. The FPG decreased an 86% with the treatment of 1.5 MPa of pressure (table 1).

In Salina, the EG and the FPG decreased to 65% and 52% with regard to the control, respectively, with treatments of 200 mM NaCl (table 1). In other treatments, saline did not affect the germination. In drought stress, EG decreased to 23 and 83% with regard to the control respectively, with -1 and -1.5 MPa of pressure and FPG decreased to 68% only at -1.5 MPa (table 1).

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Table 1
Average percentage of germination in control and stressed conditions of *Medicago sativa* varieties (ANOVA results).

Germination in percentage (%)						
	Var. DK166		*Var. Verdor*		*Var. Salina*	
NaCl (mM)	*EG*	*FPG*	*EG*	*FPG*	*EG*	*FPG*
0	83 a	89 a	76 a	70 a	77 a	83 a
50	51 **b**	77 a	71 a	77 a	73 a	80 a
100	28 c	54 **b**	28 **b**	52 a**b**	77 a	79 a
200	7 **d**	10 **c**	2 **c**	2 **c**	27 **b**	40 **b**
PEG 6000 (MPa)						
0	69 a	72a	74 a	78 a	79 a	84 a
-0.5	55 a	71a	67 a	79 a	83 a	86 a
-1	19 **b**	41ab	33 **b**	66 a	61 **b**	76 a
-1.5	6 **b**	23**b**	4 **c**	11 **b**	13 **c**	27 **b**

a,b,c Statistically significant differences are observed between the control and stress variants (within each stress treatment?) ($P \leq 0.05$ according to ANOVA, Tukey test). The percentage of sowing after day 3 is energy germination (EG) and after day 7 is power germination (FPG).

Biomass

When DK166 had increased concentrations of NaCl, FW showed a different response in roots and foliage. The FW of foliage increased in all treatments and the roots kept similar to those of the control. The DW in leaves increased for all treatments but there were no differences in roots (table 2). All plants watered with a solution 200 mM NaCl, were alive for 15 days. In drought stress conditions (PEG 6000), foliage FW and DW increased when watered with solutions of -0.5 MPa, FW and DW and roots increased with all concentrations (table 2).

Table 2
Average fresh and dry weights of foliage and roots, in the control and stressed *Medicago sativa* DK166 variety, after exposure to salt and drought conditions.

Weight in g Var. DK166 (ANOVA results)				
	Foliage		*Roots*	
NaCl (mM)	*FW*	*DW*	*FW*	*DW*
0	0.15 c	0.03 b	0.028 ab	0.028 ab
50	0.30 **ab**	0.06 **a**	0.042 a	0.042 a
100	0.38 **a**	0.07 **a**	0.018 ab	0.018 ab
200	0.25 **b**	0.06 **a**	0.018 ab	0.018 ab
PEG 6000 MPa				
0	0.23 b	0.02 b	0.02 b	0.01 b
-0.5	0.43 **a**	0.14 **a**	0.06 ab	0.03 **a**
-1	0.19 b	0.08 b	0.06 ab	0.04 **a**
-1.5	0.18 b	0.08 b	0.11 **a**	0.04 **a**

a,b,c Statistically significant differences are observed between the control and stress variants (within each stress treatment?) ($P \leq 0.05$ according to ANOVA, Tukey test) (FW: Fresh Weight, DW: Dry Weight).

Verdor under 100 and 200 mM NaCl increased its foliage FW significantly but decreased in roots; However, with 50 mM NaCl, but with concentrations of 100 and 200 mM NaCl, they died (table 3). DW in foliage with 100 mM NaCl increased; however at 200 mM it decreased significantly. With 100 and 200 mM of NaCl, the roots died (table 3). In drought stress conditions (PEG 6000), FW and DW from foliage decreased when they were watered with solutions of -0.5 and -1 MPa respectively. FW and DW from roots increased in the same concentrations however, at -1.5 MPa, they died (table 3).

Table 3
Average fresh and dry weights of foliage and roots, in the control and stressed *Medicago sativa* VERDOR variety, after exposure to salt and drought conditions.

Weight in g Var. VERDOR (ANOVA Results)				
	Foliage		*Roots*	
NaCl (mM)	*FW*	*DW*	*FW*	*DW*
0	0.13 c	0.003 c	0.05 a	0.006c
50	0.14 c	0.003 c	0.02 **b**	0.01a
100	0.23 **a**	0.02 **a**	death	death
200	0.17 c	0.006 c	death	death
PEG 6000 MPa				
0	0.30 a	0.10 a	0.02 b	0.01 b
-0.5	0.09 **b**	0.03 **b**	0.09 **a**	0.05 **a**
-1	0.11 **b**	0.04 **b**	0.08 **a**	0.04 **a**
-1.5	death	Death	death	Death

a,b,c Statistically significant differences are observed between the control and stress variants (within each stress treatment?) ($P \leq 0.05$ according to ANOVA, Tukey test) (FW: Fresh Weight, DW: Dry Weight).

The FW and DW of the Salina variety, against salt stress, showed no significant differences between control and other concentrations of NaCl for any fractions (table 4). FW in the foliage decreased in all concentration of PEG 6000 and in roots at -0.5 and -1 MPa of osmotic pressure (table 4). The DW in the foliage is similar in all treatments; however, in roots at -0.5 and -1 MPa *versus* control (table 4).

Table 4
Average fresh and dry weights of foliage and roots, in the control and stressed *Medicago sativa* SALINA variety, after exposure to salt and drought conditions

Weight in g Var. SALINA (ANOVA Results)				
	Foliage		*Roots*	
NaCl (mM)	*FW*	*DW*	*FW*	*DW*
0	0.22 a	0.04 a	0.045 a	0.02 a
50	0.40 a	0.05 a	0.085 a	0.04 a
100	0.34 a	0.04 a	0.095 a	0.04 a
200	0.38 a	0.05 a	0.055 a	0.03 a
PEG 6000 MPa				
0	0.36 a	0.03 a	0.04 c	0.01 c
-0.5	0.19 **b**	0.02 a	0.06 **b**	0.04 **b**
-1	0.15 **b**	0.02 a	0.09 **a**	0.07 **a**
-1.5	0.12 **b**	0.01 ab	0.04 c	0.01 c

a,b,c Statistically significant differences are observed between the control and stress variants (within each stress treatment?) ($P \leq 0.05$ according to ANOVA, Tukey test) (FW: Fresh Weight, DW: Dry Weight).

Growth

In Dkl66 under salt stress, the foliage showed no differences with regard to the control. The longitude of roots decreased to 45 % with 100 mM and 20 % with 200 mM of NaCl with regard to the control. The leaves and roots of this variety showed a significant increase in length under all osmotic pressures, doubling the control in some cases (table 5).

In Verdor under salt stress, leaves length increased significantly from the control at concentrations of 100 and 200 mM, and the radical portion showed a significant decrease of 0.50 mM of NaCl; however, higher concentrations caused necrosis and death. In drought stress, shoot and root fractions did not differ significantly, showing that growth stopped and in 1,5Mpa died (table 5).

Salina longitude foliage did not change under any treatment; the roots had a significant increase in all saline treatments though. Under PEG 6000 treatments, foliage length decreased and roots increased significantly in all treatments (table 5).

Table 5

Average length of foliage and roots, in *Medicago sativa* varieties, after exposure to salt and drought treatment.

Average length of foliage and root (cm)						
	Var. DK166		*Var. Verdor*		*Var. Salina*	
NaCl (mM)	LF	LR	LF	LR	LF	LR
0	4.55 ab	4.55 a	2.16 b	4.13 a	5.80 a	3.60 b
50	4.41 b	3.80 ab	2.40 b	3.40 **b**	5.90 a	6.80 **a**
100	5.20 a	2.43 **c**	5.03 **a**	death	5.76 a	4.76 b
200	5.00 a	3.63 **b**	4.06 **a**	death	5.23 a	5.23 a
PEG 6000 (MPa)						
0	4,56 c	2.10 b	5.23 a	4.66 a	5.80 a	3.6 b
-0.5	9,46 **a**	5.33 **a**	3,68 a	4,26 a	2,66 **b**	4.7 **a**
-1	7,50 **b**	6,43 **a**	4,16 a	4,40 a	2,96 **b**	3,83 **a**
-1.5	7,50 **b**	6.00 **a**	death	death	2,50 **b**	5,03 **a**

a,b,c Statistically significant differences are observed between the control and stress variants (within each stress treatment?) (P≤ 0.05 according to ANOVA, Tukey test). The percentage of sowing after day 3 is energy germination (EG) and after day 7 is power germination (FPG).)

Discussion

The salinity stress expressed as electrical conductance showed that in moderately salinity (4.5ds/m 0 50 mM NaCl) Dk166 had EG and FPG 50% above the control; in saline (9 ds/m or 100mM NaCl) had FPG higher than 50% but declined in extreme salty. EG and FPG in Verdor were less sensitive than Dk166 in moderately saline treatment (4.5 ds/m), although at higher salinity levels the behavior was worse than Dk166. In Saline alone decreased to strongly saline media (18 ds/m or 200 mM NaCl), showing more tolerance to salinity during germination (table 1). Saline stress had a lesser effect on the germination of seeds from *Vigna unguiculata* L. Walp than hydric stress simulated with PEG at the same osmotic potentials (Murillo-Amador *et al*., 2002) and in *Solanum melongena* seeds, also were more sensitive to extreme osmotic stress than to saline stress (Demir *et al*., 2003).

Drought is one of the most important natural phenomenons that affect plant growth. Alfalfa (*Medicago sativa* L.) is an essential fodder product with an extensive and deep roots system, which enables the plant to tolerate long term drought without any damage to its regrowing process (Safarmejad, 2008). Vast research showed that germination percentage in alfalfa was reduced under osmotic potential (Castroluna, 2009; Safarmejad, 2008; Younis *et al.*, 1963).

In our experiment in Dk166, EG and FPG diminished (minor 50%) under moderate water stress and under strongly stress. The differences as regards to the control were greater than 50%, indicating tolerance to moderate water stress, but not severe (Hsiao, 1973).The behavior of EG and FPG in the Verdor variety with slight to moderate water stress, indicated that it was less sensitive than Dk166. Salina was similar to the other varieties studied (table 1).

In *Digitaria eriantha*, both EG and FPG also decreased at high osmotic pressures, indicating a sensitivity to severe drought. These results are similar to those described for *T. crinita*, where the pressure osmotic of -0.5 MPa did not affect germination in any of the evaluated temperatures significantly (Di Giambatista *et al.*, 2003). However, the lower potentials significantly reduced the percentage of germination, in agreement with the observations made in wheat seeds (González and Argentel, 2005) and *Bulnesia retama* seeds (Rodríguez *et al.*, 2007).

The present study demostrated that salinity and drougth stress (simulated by PEG6000) decreased germination of *Medicago sativa* seeds, and these responses depended on the genotipe (variety) similar to that found in *Triticum durum* under similar estresses (table 1) (Khayatnezhad and Gholamin, 2011).

Germination and seedling emergence from laboratory results does not necessary represent germination and seedling emergence from field soils. The most important agronomic question is whether the observed differences in salt tolerance during early stages are representative of the salt tolerance of the cultivars during the whole growth cycle (Khayatnezhad and Gholamin, 2011). The Dk166 variety did not tolerate salt stress well. There was a tendency to increase fresh air mass (length and fresh weight) and maintain or reduce root mass, by toxic NaCl effects.This response indicated a stress avoidance strategy by modifying the production of roots in order to reduce the root surface, decreasing the permeability and preventing water loss in dry soil (Nobel and Huang, 1992).

The rise of DW in the canopy by the accumulation of solutes in cells increased the osmotic potential. This leads to a greater flow of water to the cells, becoming a tolerance strategy (Morgan, 1984). In a moderate and severe drought the FW and DW of the leaves did not vary; counteracting this situation the roots increased both parameters, as another form of stress avoidance (table 2). Dk166 continued its growth in leaves and roots with moderate water stress (table 5). This variety showed behavior differences in roots under a different stress.

The presence of NaCl, or Mannitol in the germination and emergence medium reduces water uptake of seedlings and inhibits the mobilization of the seed reserves to the growing embryonic axis (Khayatnezhad and Gholamin, 2011), thus affecting seedlings growth (Sayar *et al*. 2010). In the Verdor variety, although the FW, DW (table 3) and foliage length (table 5), is increased among moderately saline (4.5 ds/ m) and saline (9 ds/m) media, however the roots disappears in saline and extremely saline treatments (9ds/m and 18ds/m) indicating low tolerance to salt. Osmotic pressure of 0.5 MPa and -1 MPa promoted slight to moderate water stress (Hsiao, 1973), and in these media FW and DW declined and there was no canopy growth; the roots increased all these parameters as an avoidance system (Lewitt, 1980) consequently resulting in greater absorption of water. The shoot and root died with severe water stress (-1.5 MPa), indicating that Verdor has little tolerance to saline and semi-arid environments (table 3) and showed differential behavior in roots under drought and salt stress in Dk166 (table 5).

The Salina variety plants grew normally in all saline treatments, keeping the parameters FW, DW and length, both in air and root fraction, close to control, clearly demonstrating its salt tolerance (table 4) However the plants showed susceptibility to severe water stress, behaving similarly to Verdor in terms of root development as a means of compensation in moderating drought stress (table 5). This will probably show a stress avoidance mechanism, where the roots are elongated in search of greater moisture available at depth. The Salina variety shows a high tolerance to salinity, but sensitivity to severe drought.

Conclusions

The adverse effect of PEG-6000 and NaCl on germination and early seedling growth was due to the osmotic effect and to the specific ion. The response to the osmotic effect and salinity stress in each variety was different, showing damage to ion effect in any case. Seedling growth under normal conditions showed an increase in aerial biomass, and in severe drought and salinity death; except the Salina variety, which is considered as tolerant. In roots, there was a different strategy front for each stress depending of the level and genotype. In the genotypes more sensible front light salt stress stop their ground and under light drought increasing. According to our studies, we may define the commercial varieties of *Medicago sativa* as moderately tolerant to salt and drought: the Dk166 variety; as sensitive or intolerant to salinity and drought: the Verdor variety and as high tolerance to salinity and any sensitivity to drought: the Salinity variety.

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