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# Salt and Cadmium Stress Tolerance in Four Genotypes of *Medicago sativa* L.

## Tolerancia al estrés por sal y cadmio en cuatro genotipos de *Medicago sativa* L.

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## Abstract

**Objective**: To evaluate the tolerance of four genotypes of *Medicago sativa* L. to salinity and cadmium, through the study of germination, growth parameters, photosynthetic pigments, and osmocompatible response. **Materials and methods:** The experimental design was a completely randomized design with a factorial arrangement: type of genotype: CW 197, Trinidad 87, CW 660 and Salina PV and levels of saline (NaCl) and cadmium ($Cl_2Cd$) stress. Factorial ANOVA was performed to identify the influence of stress conditions on the measured variables, multiple comparisons analyse of means were performed when a significant effect was found. Twenty seeds were sown in triplicate irrigated with water (control) and solutions between 10 to 200 mM of NaCl and 100 to 500 $\mu$M of $Cl_2Cd$ for the germination study. Growth, proline, and photosynthetic pigments

## Resumen

**Objetivo:** Evaluar la tolerancia de cuatro genotipos de *Medicago sativa* L. a la salinidad y cadmio, mediante el estudio de la geminación, parámetros de crecimiento, pigmentos fotosintéticos y respuesta osmocompatible. **Materiales y métodos:** El diseño experimental fue completamente al azar con arreglo factorial: tipo de genotipo: CW 197, Trinidad 87, CW 660 y Salina PV y niveles de estrés salino (NaCl) y cadmio ($Cl_2Cd$) estrés. Se realizó ANOVA factorial para identificar la influencia de las condiciones de estrés en las variables medidas, se realizaron análisis de comparaciones múltiples de medias cuando se encontró un efecto significativo. Se sembraron 20 semillas por triplicado regadas con agua (control) y soluciones entre 10 a 200 mM de NaCl y 100 a 500 $\mu$M de $Cl_2Cd$ para el estudio de germinación. En plántulas de cuatro semanas crecidas en terri-

were measured in 4-week-old seedlings grown in pots irrigated with nutrient solution (control) and increasing concentrations of NaCl (50-200 mM) and $Cl_2Cd$ (25-100 $\mu$M). **Results:** Under salinity, the CW179 and CW660 genotypes were tolerant to salinity at germination, and the Salina PV and Trinidad 87genotypes were more tolerant during growth. In response to Cd, the genotypes showed tolerance during growth. Proline was osmoprotective against salinity, but not against Cd stress. Photosynthetic pigments decreased with salt stress; however, Cd did not affect them. **Conclusion:** The CW 660 genotype was the most tolerant to salinity and Cd during germination. It is concluded that the Trinidad 87 and Salina PV genotypes are tolerant to salt and the CW 197 and CW 660 genotypes are sensitive during growth. Under Cd stress, all genotypes showed tolerance during growth. Proline and photosynthetic pigments were indicators of tolerance to salt stress.

*Keywords*

Germination, growth, pigments, proline, salinity.

nas regadas con solución nutritiva (control) y concentraciones crecientes de NaCl (50-200 mM) y $Cl_2Cd$ (25$\mu$M-100 $\mu$M) se midieron crecimiento, prolina y pigmentos fotosintéticos. **Resultados:** Bajo salinidad, los genotipos CW179 y CW660 fueron tolerantes a la salinidad en la germinación, y los genotipos Salina PV y Trinidad 87 fueron más tolerantes durante el crecimiento. En respuesta a Cd, los genotipos mostraron tolerancia durante el crecimiento. La prolina fue osmoprotectora frente a la salinidad, pero no contra el estrés por Cd. Los pigmentos fotosintéticos disminuyeron con el estrés salino; sin embargo, Cd no los afectó. **Conclusión:** El genotipo CW 660 fue el más tolerante a la salinidad y al Cd durante la germinación. Se concluye que los genotipos Trinidad 87 y Salina PV son tolerantes a la sal y los genotipos CW 197 y CW 660 son sensibles, durante el crecimiento. Bajo estrés por Cd, todos los genotipos mostraron tolerancia durante el crecimiento. La prolina y los pigmentos fotosintéticos fueron indicadores de tolerancia al estrés salino.

*Palabras clave*

Crecimiento, germinación, pigmentos, prolina, salinidad.

## Introduction

Environmental pollution is one of the most critical problems affecting urban life in the 21st Century. The decrease of air quality, water resources, and land available for agricultural activities has increased exponentially (Chen *et al.*, 2013).

Climate change and the rapid development of irrigated agriculture have led to salinization of soils has suddenly increased worldwide, standing a threat to environmental sustainability and agriculture itself (Bui *et al.*, 2013; Singh *et al.*, 2015). In addition to the above, salinization is a problem of global concern, due to the reduction of the productivity of large arable areas, a decrease of land value (causing its abandonment) that it provokes (Guida-Jhonson *et al.*, 2017).

Beyond the well-known effects of salt accumulation, the derived heavy metal contamination is a severe global environmental issue. As a non-essential nutrient element, Cd is one of the common heavy-metal pollutants in the environment, being it sourced from industrial and/or anthropogenic activities, finding among the most severe the application of Cd-rich phosphate fertilizers, and the leather industry (Groppa *et al.*, 2012; Hayat *et al.*,

2019). Even in small amounts, exposure to Cd has adverse effects on plant growth and development (Sandalio *et al*., 2012). Furthermore, cadmium is one of the heavy metals with the most possibility to be accumulated by plants, causing severe imbalances in the nutrition, photosynthesis, and antioxidant metabolism (Perez Chaca *et al*., 2014a) and in growth, anatomy, and hormone levels (Perez Chaca *et al*., 2014b). Regarding plant adaptation to salt stressing conditions, there are available in literature many recent studies that have shown that halophytes are more adapted of abiotic stresses, including heavy metals, than those sensitive to salt (Amari *et al*., 2014; Taamalli *et al*., 2014). Among the most reported, forage plants that live in saline soils contaminated with heavy metals, are frequently used with the dual purpose of phytoremediation and production of biomass. Other examples are legumes, which can fix atmospheric nitrogen, reduce the need for nitrogen fertilization, and are especially important in the context of sustainable agriculture; features that make them more attractive a sustainable source of high-quality proteins.

On the other hand, *Medicago* species plants such as *M. polymorpha* and *M. orbicularis* were found in highly Hg-contaminated soils, indicating that there is a similarity among members of this genus in their ability to remediate contaminated soils (Nonnoi *et al*., 2012).

The nutritional quality, forage production, growth habit, perennials, plasticity, and capacity for symbiotic fixation of atmospheric nitrogen of alfalfa (*M. sativa*), make it a fundamental resource for many agricultural production schemes (Basigalupi *et al*., 2007).

This study aimed was to determine the effect of high salt and heavy metals added as NaCl and Cd, on germination and early growth of four varieties of the new alfalfa biotech products called CW 197, Trinidad 87, CW 660, and Salina PV, to determine the tolerance to these pollutants and select genotypes for growing in contaminated soils with bioremediation possibilities.

## Materials and Methods

Seeds were obtained from Cal / West Seed or Palo Verde CW 197 (Cal / West Seed), Trinidad 87 (Palo Verde), CW 660 (Cal / West Seed), Salina PV (Palo Verde). They were sterilized with 70% commercial sodium hypochlorite for 1 min, rinsed with sterile distilled water six times and left in sterile water for one hour for hydration.

### *Germination under salinity and cadmium stress*

The experimental design was a completely randomized design with factorial arrangement*: M. sativa* (CW 197, Trinidad 87, CW 660 and Salina PV)) and stress level (NaCl or $Cl_2Cd$). The stress factor was seven levels (0, 10, 25, 50, 100, 150 and 200 mM) of NaCl and six levels (0, 100, 200, 300, 400 and 500 $\mu$M) of $CdCl_{2.}$

In salinity tolerance, the germination experiment was conducted in Petri dishes with double filter paper watered with 5 mL of distilled water (control) or corresponding solution of NaCl, twice a week. In the cadmium tolerance assay, 1% agar Petri dishes different concentrations of $CdCl_2$ were used. In two experiments, were used twenty seeds for each Petri dishes with three repetitions each one and were placed in a stove at 25ºC in the dark (Castro-Luna *et al.*, 2014).

In both tests, seeds were considered germinated when radicles measured 2 mm size. Number of seeds germinated was recorded on the 3rd day, the germination energy percentage (GE) and on the 7th day for the germination power (GP). Germination percentage was calculated with the following formula:

$$\text{Germination percentage (\%)} = \frac{\text{N° of germinated seeds x 100}}{\text{N° of total seeds}}$$

### *Growth and biomass*

The experimental design was a completely randomized design with a factorial arrangement: *M. sativa* (CW 197, Trinidad 87, CW 660 and Salina PV)) and stress level (NaCl or $Cl_2Cd$). The stress factor with NaCl has four levels (0, 50, 100 and 200 mM) and the stress factor with $Cl_2Cd$ has five levels (0, 25, 50, 75 and 100 $\mu$M), with five independent replications per treatment, each replicate consisted of one pot.

The seeds were sown homogenously at a depth of 2 cm, with 1 g seeds per 500 mL pot with sterile vermiculite to ensure good colonization of the soil by plants. The treatment started one week after sowing. The control plants were watered with Hoagland nutrient solution (HNS). The treatments were: HNS + 50 mM NaCl; 100 mM and 200 mM (saline treatment) and HNS+ 25 $\mu$M; 50 $\mu$M: 75 $\mu$M; 100 $\mu$M $Cl_2Cd$ (Cd treatment).

The plants were watered three times a week with 100 mL of each solution. After five weeks of treatment, five samples of each treatment were collected to perform the growth studies; aerial part (AL) and root (RL) length (cm), fresh weight of aerial (FWA) and root (FWR) portion and aerial part (DWA) and root (DWR) dry weight (g). Proline and photosynthetic pigments were determined in all treatments for aerial portions.

### *Proline*

The proline concentration was expressed in $\mu$g proline/ml of sample. The analysis was performed with five biological replicates per treatment, determined according to the methodology of Bates *et al.* (1973) based on a L-proline standard curve (Synth). The samples were read on a 700 Plus spectrophotometer (FEMTO®), at length 520 nm waveform.

### *Photosynthetic pigments*

The determination of chlorophylls a, b totals, and carotenoids of fresh leaves was performed according to the methodology proposed by Porra (2002). The samples were read on the 700 Plus spectrophotometer (FEMTO®) at an absorbance of 663, 645 and 470 nm. The analysis was performed with five biological replicates per treatment. The calculation of the endogenous content of chlorophylls was carried out according to Porra (2002) and that of carotenoids according to Sims and Gamon (2002). The result was expressed in mg chlorophyll /l.

### *Statistical analysis*

A two-factorial ANOVA was carried out to analyze the effects of alfalfa genotypes, factors stress (NaCl and $Cl_2Cd$) and the interaction between them ($p \leq 0.05$). Tukey post-hoc tests were used to evaluate pairwise differences of means. Data manipulation and analysis were done by SPSS (Statistical Pack for Social Sciences) version 27.

## Results

### *Interaction salinity and genotypes*

There was a significant interaction between the four genotypes and the salinity stress factor (table 1).

Table 1

Interaction between two main factors: *M. sativa* (CW 197, Trinidad 87, CW 660 and Salina PV) and salinity stress (ANOVA)

| Factors | GE | GP | AL | AFW | ADW | RL | RFW | RDW | Chlr.a | Chlr.b | Carot. | Pro. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotype | *** | *** | *** | *** | *** | *** | *** | * | *** | *** | *** | *** |
| NaCl | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** |
| Gen x NaCl | *** | *** | *** | *** | *** | * | * | * | *** | *** | *** | *** |

GE: Germination Energy; GP: Germination Power; AL: Aerial Length; AFW: Aerial Fresh Weight; ADW: Aerial Dry Weight; RL: Root Length; RFW: Root Fresh Weight; RDW: Root Dry Weight; Chlr. a: Chlorophyll a; Chlr. b: Chlorophyll b; Carot.: Carotenoids; Pro.: Proline; Ns: Not Significant.
* Significance with $p \leq 0.05$; ** Significance with a $p \leq 0.01$; *** Significance with $p \leq 0.00$.

Table 2 shows that the Saline PV genotype was sensitive to salinity starting at 50mM NaCl. Trinidad is sensitive from 100mM NaCl; CW 197 is sensitive from 150 mM NaCl and CW 660 is sensitive from 200 mM NaCl, representing this genotype tolerance to salinity in germination.

Table 2

Germination Energy (GE) and Power (GP) in four genotypes of *M. sativa* under NaCl stress

| NaCl mM | Genotypes | GE | GP |
|---|---|---|---|
| 0 | CW 197 | 73.33 a | 80.00 a |
| | Trinidad 87 | 73.33 a | 80.00 a |
| | CW660 | 78.00 a | 83.00 a |
| | Salina PV | 77.00 a | 85.00 a |
| 10 | CW 197 | 75.00 a | 77.00 a |
| | Trinidad 87 | 71.66 a | 78.33 a |
| | CW660 | 67.00 a | 73.00 a |
| | Salina PV | 68.00 a | 77.00 a |
| 25 | CW 197 | 73.33 a | 80.00 a |
| | Trinidad 87 | 61.66 ab | 71.66 a |
| | CW660 | 73.00 a | 75.00 a |
| | Salina PV | 80.00 a | 83.00 a |
| 50 | CW 197 | 70.00 a | 78.33 a |
| | Trinidad 87 | 71.66 a | 83.33 a |
| | CW660 | 62.00 ab | 75.00 a |
| | Salina PV | 57.00 b | 60.00 b |
| 100 | CW 197 | 80.00 a | 85.00 a |
| | Trinidad 87 | 35.00 c | 65.00 b |
| | CW660 | 58.00 b | 70.00 a |
| | Salina PV | 45.00 c | 52.00 b |
| 150 | CW 197 | 11.66 de | 36.66 c |
| | Trinidad 87 | 13.33 de | 41.66 c |
| | CW660 | 57.00 b | 73.00 a |
| | Salina PV | 25.00 d | 35.00 c |
| 200 | CW 197 | 6.66 e | 6.66 e |
| | Trinidad 87 | 0.00 f | 16.66 de |
| | CW660 | 12.00 de | 20.00 d |
| | Salina PV | 0.00 f | 0.00 f |
| EEM | | 0.016 | 0.018 |
| P | | 0.00 | 0.00 |

The columns represent mean values (n=3). Different letters represent significant differences of the treatments (NaCl) in relation to the control determined by the analysis of variance (ANOVA) according to Tukey's test ($P \leq 0.05$).

Table 3 shows that AL and AFW decreased at 100 mM NaCl in CW 197 and CW660 genotypes, and photosynthetic pigments decreased in CW179 genotype too (table 4). Both genotypes showed a compensatory response to salt stress by increasing of proline (table 4). At 100mM NaCl, the Salina PV genotype remained unchanged although compensated with a significant increase in proline and Trinidad 87 showed absolute tolerance to this level of salinity. At 200mM NaCl all genotypes had decreased growth (table 3), photosynthetic pigments (table 4). The CW179 and CW 660 genotypes were the most affected (with 7 to 8 parameters decreased) and the Trinidad 87 and Salina PV genotypes the least affected (4 to 5 parameters decreased) (table 3 and 4). Proline had significantly increased in four genotypes, as a form of compensation to saline stress (table 4).

Table 3

Growth parameters in four genotypes of *M. sativa* under control and salinity stress

| ClNa mM | Genotype | AL (cm) | AFW (g) | ADW (g) | RL (cm) | RFW (g) | RDW (g) |
|---|---|---|---|---|---|---|---|
| 0 | CW 197 | 11.42a | 0.21a | 0.02a | 17.74a | 0.19a | 0.01a |
| | Trinidad 87 | 11.20a | 0.19a | 0.02a | 14.88a | 0.14ab | 0.01a |
| | CW 660 | 13.73a | 0.28a | 0.02a | 25.47a | 0.23a | 0.03ab |
| | Salina PV | 11.77a | 0.21a | 0.01a | 12.63ab | 0.18a | 0.01a |
| 50 | CW 197 | 9.64a | 0.17a | 0.02a | 14.54a | 0.19a | 0.01a |
| | Trinidad 87 | 10.32a | 0.14a | 0.01a | 13.51a | 0.15ab | 0.01a |
| | CW 660 | 11.78a | 0.27a | 0.04a | 24.73a | 0.21a | 0.03ab |
| | Salina PV | 9.45a | 0.18a | 0.02a | 13.86ab | 0.16a | 0.01a |
| 100 | CW 197 | 7.64b | 0.14b | 0.02a | 13.10ab | 0.15ab | 0.01a |
| | Trinidad 87 | 8.45a | 0.15a | 0.01a | 12.87ab | 0.15ab | 0.01a |
| | CW 660 | 10.25b | 0.15b | 0.05a | 25.20a | 0.22a | 0.02a |
| | Salina PV | 8.30a | 0.14a | 0.01a | 11.50ab | 0.13ab | 0.01a |
| 200 | CW 197 | 6.34c | 0.12c | 0.01b | 12.16b | 0.11b | 0.01a |
| | Trinidad 87 | 6.10c | 0.10c | 0.01a | 12.45b | 0.11ab | 0.01a |
| | CW 660 | 8.00b | 0.15b | 0.04b | 18.50b | 0.15b | 0.02a |
| | Salina PV | 6.73c | 0.16a | 0.01b | 11.33ab | 0.16a | 0.01a |
| EMM | | 0.37 | 0.01 | 0.002 | 0.85 | 0.006 | 0.001 |
| P | | 0.00 | 0.00 | 0.00 | 0.04 | 0.03 | 0.03 |

AL: Aerial Length; AFW: Aerial Fresh Weight; ADW: Aerial Dry Weight; RL: Root Length; RFW: Root Fresh Weight; RDW: Root Dry Weight.

The columns represent mean values (n=5). Different letters represent significant differences of the treatments (NaCl) in relation to the control determined by the analysis of variance (ANOVA) according to Tukey's test ($P \leq 0.05$).

Table 4

Photosynthetic pigments and proline in four genotypes of *M. sativa* under control and salinity stress

| Na Cl mM | Genotype | Chlor. a (mg/l) | Chlor. b (mg/l) | Carot. (mg/l) | Pro. ($\mu$g/ml) |
|---|---|---|---|---|---|
| 0 | CW197 | 7.80a | 3.16a | 4.35a | 545.50c |
| | Trinidad 87 | 7.40a | 3.00a | 3.92a | 269.00d |
| | CW 660 | 12.70a | 6.28a | 4.44a | 107.94d |
| | Salina PV | 6.40ab | 4.00a | 3.92a | 188.13d |
| 50 | CW197 | 8.22a | 3.31a | 4.37a | 503.63c |
| | Trinidad 87 | 10.40a | 4.53a | 4.57a | 335.62cd |
| | CW 660 | 12.09a | 5.59a | 4.55a | 556.16c |
| | Salina PV | 5.90ab | 3.53a | 4.57a | 235.90d |
| 100 | CW197 | 5.92b | 2.25ab | 3.61b | 1 336.16ab |
| | Trinidad 87 | 6.63ab | 3.04a | 3.81a | 696.20c |
| | CW 660 | 11.48a | 5.24a | 4.56a | 1 262.62ab |
| | Salina PV | 6.63ab | 2.54ab | 4.57a | 1 327.82ab |
| 200 | CW197 | 3.75c | 1.40c | 2.59b | 1 075.28ab |
| | Trinidad 87 | 3.58c | 1.53c | 2.53a | 2 683.48a |
| | CW 660 | 4.00c | 1.60c | 2.73b | 2 624.78a |
| | Salina PV | 3.58c | 2.03c | 3.23a | 3 551.15a |
| EEM | | 0.34 | 0.18 | 0.11 | 11.3 |
| P | | 0.00 | 0.00 | 0.00 | 0.00 |

Chlr. a: Chlorophyll a; Chlr. b: Chlorophyll b; Carot.: Carotenoids. Pro: Prolina.

The columns represent mean values (n=5). Different letters represent significant differences of the treatments (NaCl) in relation to the control determined by the analysis of variance (ANOVA) according to Tukey's test ($P \leq 0.05$).

### *Interaction cadmium and genotypes*

Table 5 shows that in germination, aerial morphological parameters, chlorophyll a and proline, there was a significant interaction between the four genotype levels and the applied stress factor with cadmium. Chlorophyll b, carotenoids and fresh and dry weight of roots, there was no significant interaction with genotypes and cadmium stress, the main factors did not have significant effects either.

Table 6 shows the germination of the four genotypes at increasing concentrations of $CdCl_2$. It is observed that all the genotypes with the exception of CW660, showed a significant decrease in EG and PG with respect to the control, from 300 $\mu$M of Cd. CW660 showed a decrease in germination parameters at 500 $\mu$M $Cl_2Cd$, for its tolerance to cadmium.

Table 5
Interaction between two main factors: *M. sativa* (CW 197, Trinidad 87, CW 660 and Salina PV) and cadmium stress (ANOVA)

| Factors | GE | PG | AL | AFW | ADW | RL | RFW | RDW | Chlr. a | Chlr. b | Carot. | Pro. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotypes | *** | *** | *** | *** | *** | * | Ns | Ns | *** | Ns | Ns | * |
| $Cl_2Cd$ | *** | *** | *** | *** | *** | *** | Ns | Ns | *** | Ns | Ns | *** |
| Gen x $Cl_2Cd$ | *** | *** | *** | *** | ** | ** | Ns | Ns | * | Ns | Ns | *** |

GE: Germination Energy; GP: Germination Power; AL: Aerial Length; AFW: Aerial Fresh Weight; ADW: Aerial Dry Weight; RL: Root Length; RFW: Root Fresh Weight; RDW: Root Dry Weight; Chlr. a: Chlorophyll a; Chlr. b: Chlorophyll b; Carot.: Carotenoids; Pro.: Proline; Ns: Not Significant.
* Significance with $p \leq 0.05$; ** Significance with a $p \leq 0.01$; *** Significance with $p \leq 0.00$.

Table 6
Four genotypes of *M. sativa* under $Cl_2Cd$ stress

| CdCl $\mu$M | Genotypes | GE (%) | GP (%) |
|---|---|---|---|
| 0 | CW 197 | 81.66a | 88.33a |
| | Trinidad 87 | 70.00a | 78.33a |
| | CW660 | 78.00a | 88.00a |
| | Salina PV | 82.00a | 90.00a |
| 100 | CW 197 | 68.33a | 71.67a |
| | Trinidad 87 | 65.00a | 73.33a |
| | CW660 | 77.00a | 83.00a |
| | Salina PV | 80.00a | 88.00a |
| 200 | CW 197 | 63.33a | 66.67a |
| | Trinidad 87 | 73.33a | 81.66a |
| | CW660 | 72.00a | 77.00a |
| | Salina PV | 73.00a | 82.00a |
| 300 | CW 197 | 60.00b | 60.00b |
| | Trinidad 87 | 48.33c | 50.00c |
| | CW660 | 82.00a | 85.00a |
| | Salina PV | 60.00b | 75.00b |
| 400 | CW 197 | 30.00c | 31.67c |
| | Trinidad 87 | 50.00c | 50.00c |
| | CW660 | 78.00a | 80.00a |
| | Salina PV | 45.00c | 65.00b |
| 500 | CW 197 | 21.66d | 23.33d |
| | Trinidad 87 | 35.00d | 38.33d |
| | CW660 | 68.00b | 70.00b |
| | Salina PV | 42.00c | 47.00c |
| EEM | | 0.016 | 0.018 |
| P | | 0.00 | 0.00 |

GE: Germination Energy; GP: Power (GP). The columns represent mean values (n=3). Different letters represent significant differences of the treatments ($CdCl_2$) in relation to the control determined by the analysis of variance (ANOVA) according to Tukey's test ($P \leq 0.05$).

Table 7 shows that under Cd stress, aerial parameters (AL, AFW and ADW) increased at 75 and 100 $\mu$M in Salina PV. ADW decreased significantly in Trinidad 87 and CW 660 at 100 $\mu$M $Cl_2Cd$. The pigment contents in all genotypes did not change under cadmium stress (table 8). Proline contents in CW 197 genotype increased at 75 $\mu$M $Cl_2Cd$; CW660 and Trinidad 87 genotypes decreased respect to control and Salina PV genotype did not show any change (table 8).

Table 7

Growth parameters in four genotypes of *M. sativa* under control and cadmium stress

| $CdCl_2$ $\mu$M | Genotype | AL (cm) | AFW (g) | ADW (g) | RL (cm) | RFW (g) | RDW (g) |
|---|---|---|---|---|---|---|---|
| 0 | CW 197 | 12.12 c | 0.19d | 0.04b | 8.38a | 0.05ab | 0.010a |
| | Trinidad 87 | 18.66b | 0.39c | 0.08a | 10.98a | 0.14a | 0.020a |
| | CW 660 | 13.74c | 0.23d | 0.05b | 8.02a | 0.07ab | 0.020a |
| | Salina PV | 16.92b | 0.29d | 0.05b | 8.66a | 0.11a | 0.010a |
| 25 | CW 197 | 9.60c | 0.13d | 0.02c | 7.38ab | 0.05ab | 0.030a |
| | Trinidad 87 | 17.84b | 0.30c | 0.06b | 9.04a | 0.09a | 0.020a |
| | CW 660 | 12.36c | 0.20d | 0.04b | 7.94ab | 0.07ab | 0.010a |
| | Salina PV | 16.92b | 0.21d | 0.04b | 8.34a | 0.07ab | 0.010a |
| 50 | CW 197 | 13.38c | 0.21d | 0.04b | 10.74a | 0.06ab | 0.010a |
| | Trinidad 87 | 16.84b | 0.28d | 0.05b | 9.30a | 0.08a | 0.010a |
| | CW 660 | 13.62c | 0.23d | 0.04b | 8.34a | 0.06ab | 0.010a |
| | Salina PV | 19.30b | 0.31c | 0.07b | 9.98a | 0.09a | 0.010a |
| 75 | CW 197 | 11.80c | 0.18d | 0.03c | 9.76a | 0.07ab | 0.005ab |
| | Trinidad 87 | 20.32b | 0.38c | 0.06b | 10.58a | 0.11a | 0.020a |
| | CW 660 | 12.21c | 0.14d | 0.03c | 7.26ab | 0.04ab | 0.006ab |
| | Salina PV | 27.24a | 0.57b | 0.11a | 12.26a | 0.09a | 0.010a |
| 100 | CW 197 | 13.90c | 0.18d | 0.04b | 8.88a | 0.07ab | 0.030a |
| | Trinidad 87 | 16.62b | 0.31c | 0.05b | 9.38a | 0.08a | 0.004ab |
| | CW 660 | 11.18c | 0.11e | 0.02c | 7.52ab | 0.03ab | 0.004ab |
| | Salina PV | 28.52a | 0.90a | 0.16a | 12.70a | 0.12a | 0.004ab |
| EEM | | 1.03 | 0.03 | 0.006 | 0.64 | 0.01 | 0.0024 |
| P | | 0.00 | 0.00 | 0.00 | 0.01 | 0.73 | 0.61 |

AL: Aerial Length; AFW: Aerial Fresh Weight; ADW: Aerial Dry Weight; RL: Root Length; RFW: Root Fresh Weight; RDW: Root Dry Weight.

The columns represent mean values (n=5). * Represent significant differences of the treatments ($CdCl_2$) in relation with the control determined by the analysis of variance (ANOVA) according to Tukey's test ($P \leq 0.05$).

Table 8

Photosynthetic pigments and proline in four genotypes of *M. sativa* under control and cadmium stress

| $CdCl_2$ $\mu$M | Genotype | Chlor. a (mg/l) | Chlor. b (mg/l) | Carot. (mg/l) | Pro. ($\mu$g/ml) |
|---|---|---|---|---|---|
| 0 | CW 197 | 7.21a | 5.37a | 3.50a | 187.29c |
| | Trinidad 87 | 8.05a | 5.62a | 3.36a | 345.62bc |
| | CW 660 | 9.56a | 6.52a | 4.37a | 771.51a |
| | Salina PV | 8.87a | 5.83a | 3.70a | 166.1c |
| 25 | CW 197 | 7.42a | 5.15a | 3.30a | 240.52c |
| | Trinidad 87 | 8.80a | 6.32a | 3.65a | 246.35c |
| | CW 660 | 7.25a | 4.92a | 3.20a | 709.36a |
| | Salina PV | 8.98a | 5.66a | 3.54a | 202.92c |
| 50 | CW 197 | 6.45a | 4.60a | 3.00a | 230.22c |
| | Trinidad 87 | 8.41a | 5.66a | 3.51a | 237.94c |
| | CW 660 | 6.51a | 4.31a | 2.94a | 246.26c |
| | Salina PV | 8.90a | 5.73a | 3.51a | 362.95bc |
| 75 | CW 197 | 5.98ab | 4.09a | 2.95a | 416.38ab |
| | Trinidad 87 | 8.18a | 5.40a | 3.40a | 100.24c |
| | CW 660 | 7.59a | 4.80a | 3.24a | 59.25d |
| | Salina PV | 7.97a | 5.04a | 3.27a | 187.95c |
| 100 | CW 197 | 6.26a | 4.29a | 3.11a | 89.04d |
| | Trinidad 87 | 4.50ab | 3.62a | 1.79a | 47.36d |
| | CW 660 | 8.43a | 5.31a | 3.46a | 2.89e |
| | Salina PV | 8.97a | 6.15a | 3.76a | 126.85c |
| EEM | | 0.88 | 0.32 | 0.16 | 12.36 |
| P | | 0.02 | 0.27 | 0.11 | 0.00 |

Chlr. a: Chlorophyll a; Chlr. b: Chlorophyll b; Carot.: Carotenoids. Pro: Prolina.
The columns represent mean values (n=5). Different letters represent significant differences of the treatments (NaCl) in relation to the control determined by the analysis of variance (ANOVA) according to Tukey's test ($P \leq 0.05$).

## Discussion

Differences in stress tolerance between different genotypes can be difficult to assess in the field where plants are exposed to both biological and climatic factors and variable conditions (Tal, 1993). Laboratory testing may be preferred to carry out a quick comparison of alfalfa varieties with respect to their tolerance to different stressors. In the first stage, the study allows the comparison of alfalfa varieties based on the characteristics of tolerance to salt and cadmium through a germination test, the study of growth parameters, photosynthetic pigments and proline.

It has been demonstrated in alfalfa that salt tolerance exhibited at all growth stages are highly correlated, indicating that the selected genotypes for salt tolerance at one developmental stage will generally not decrease tolerance at other stages (Campanelli *et al.*, 2012).

Germination has been reported as a potential indicator of salinity tolerance and phytotoxicity of metals (Bai *et al.*, 2018). The response to salinity and heavy metal stress varies among species and varieties (Ramos *et al.*, 2020; Rajasekar *et al.*, 2017).

Trinidad 87, CW 660 and Salina PV genotypes moderately decreased their GE from 100 mM NaCl; these results coincide with those reported by Maas (1990), who classified alfalfa as a moderately sensitive crop in electrical conductivities between 2.0 and 7.9 dS $m^{-1}$ (25 mM and 100 mM NaCl). The GP of the genotypes Trinidad 87, CW 660, and CW197, decreased between 150 mM and 200 mM NaCl. The delay time to start germination is in line with previous studies by Zhang *et al.* (2018), in which increased salinity delayed germination by 4 days, to 240 mM NaCl for *Medicago sativa*. These results coincide with those obtained in alfalfa genotypes DK 166 and Verdor, where it was verified that from 100 mM of NaCl, they showed sensitivity to salt (Castro-Luna *et al.*, 2014). Several researchers have shown that in front of an increase of osmotic potential (Safarmejad, 2008) and higher salt concentration, the percentage of germination in alfalfa decreases (Taffouo *et al.* 2009).

Results from the Cd tolerance trial indicate that all alfalfa varieties except CW 660 have similar germination rates at 3 and 7 days from planting, showing early germination when exposed to Cd. The nitric oxide overproduced in response to heavy metals can be involved in accelerating germination through the stimulation of enzymatic activities involved in the management of oxidative stress (Lefèvre *et al.*, 2009). GE and GP decreased in Trinidad 87 and Salina PV genotypes at 300 $\mu$M $Cl_2Cd$. GE and GP decreased in CW 197 genotype at 200 $\mu$M of $Cl_2Cd$. GE and GP decreased in CW 660 genotype at 500 $\mu$M. The tolerance of the genotypes to Cd was similar to that demonstrated by Lefèvre *et al.* (2009) in *Dorycnium pentaphyllum*, where high concentrations of Cd and Zn, 1000 $\mu$M, inhibited germination. Such inhibition may be the consequence of Cd interaction with calmodulin, immobilization of reserves through a decrease in a-amylase activities, or interference with the hormonal state of the seeds in germination. It is observed a decline in the elongation of the radicle, which agrees with the reports of (Kastori *et al.*, 2019), which refer that in vegetables the evidence of cadmium toxicity is a reduction in the growth of the aerial part and elongation of the roots, photosynthesis inhibition and chlorosis symptoms.

Abiotic stress due to salinity in low concentration suppresses plant growth and productivity, while in high concentration it can cause plant death. The influence can occur in two phases; the first phase is produced by the osmotic effect are due to the high salt concentration in the root zones, while the toxic effects due to the high salt accumulation in the leaf tissues (Munns *et al.*, 2006) govern the second phase. The studied genotypes have the capacity to grow in conditions of stress by salinity and cadmium, and, as it is shown in this work, these species can survive to high concentrations of NaCl and Cd, although this situation affects the growth of leaves as well as roots. The studies were conducted in seedlings because it is a crucial and particularly vulnerable stage in the life cycle of crops. Salt stress affects many aspects of plant growth, such as biomass production, yield, photosynthesis, and leaf metabolites (Munns and Gilliham, 2015; Negrão *et al.*, 2017).

The air morphological parameters of alfalfa seedlings showed differential response due to salinity, with higher values observed in the controls and lower values at 100 mM in the genotypes CW 660 and CW 197. These results coincide with those of Castro-Luna *et al.* (2014) where *M. sativa* plants respond to saline stress by decreasing growth rate, height, size, and the number of leaves. The results showed that increased tolerance to salt and cadmium resulted in higher growth. Munns *et al.* (2006) suggest that salinity tolerance is determined by height, which is attributed to the fact that salinity reduces water access and decreases growth, causing cellular damage through leaf transpiration, thus inhibiting growth. The RL behaved differently than the aerial part, as the RL only decreased at 200 mM NaCl in Trinidad 87 and CW 660, while RFW and RDW were not affected by the high salinity concentrations. Hussain *et al.* (2017) reported that root length, the number of roots, and length of rice seedlings decreased significantly in response to salt stress, and higher salt concentrations caused higher mortality in all rice cultivars at the early seedling stage.

The inhibitory effect of Cd on growth has been reported for different plant species (Nahar *et al.*, 2016; Deng *et al.*, 2017; Huybrechts *et al.*, 2019; Liu *et al.*, 2015; Huihui *et al.*, 2020). On the contrary, Salina PV genotype was not negatively affected by cadmium, where the leaf area was increased in its aerial length, unlike several authors who refer that in vegetables the evidence of cadmium toxicity is reduction in the growth of aerial part and elongation of roots, photosynthesis inhibition and symptoms of chlorosis (Maksimović *et al.*, 2007).

A decrease in chlorophyll content under salt stress is a well-described phenomenon and, in several studies, its concentration has been used as a precise indicator of cellular metabolic state. Previous studies in wheat, tomato, and cotton plants (Singh *et al.*, 2019; Xie *et al.*, 2015; Sikder *et al.*, 2020) agree with the findings of this work since all genotypes showed a decrease in the concentration of photosynthetic pigments as the salt concentration increased. The higher sensitivity of genotypes CW 197 and CW 660 was CW 660 genotype depicted in the decrease of all pigments including carotenoids, agree with the increase in the concentration of proline as osmocompatible against salt stress. The Trinidad 87 and Salina PV genotypes, only decreased chlorophyll a and b at 200 mM NaCl, whilst carotenoids remained unaltered. As a general trend, chlorophyll A expressed more than chlorophyll B in plants, and their levels turned similar when salinity increases (Mane *et al.*, 2010), and that behavior was the observed in the model described here; *i.e.*, at 200 mM NaCl there the determined values of chlorophyll A and chlorophyll B were closer than those observed at lower salt concentration.

Osmotic stress caused by soil salinization leads to a 'physiological drought' in plants, which decreases the capacity of roots to incorporate water, decreases the internal water supply and decelerates or inhibits metabolism. The low water content, causes the closure of the stomas, decreases the intake of $CO_2$ and $H_2O$, and therefore it decreases photosynthesis and synthesis of pigments (Sikder *et al.*, 2020).

Cadmium can affect the growth of plantae by changing chlorophyll synthesis, transpiration respiration process, and stoma opening, thus reducing photosynthesis (Gill *et*

*al.*, 2012). However, the responses of photosynthesis to Cd can be different and sometimes opposite according to the species (Siliang *et al.*, 2015). This result is similar to those found in *Robinia pseudoacacia*, in which the content of chlorophyll A in the leaves was maintained at a constant level when the seedlings were exposed to 250 mg/L Cd for 10 days, and a marked increase was also observed under Cd increase (Dezhban *et al.*, 2015).

Osmoprotectants or compatible solutes, assist plant survival under extreme stress through osmotic adjustment by stabilizing specific proteins and membranes and preventing dehydration within the cell organelles (Sikder *et al.*, 2020). In this study, all genotypes exposed to salinity increased the osmoprotectant molecule, proline, concentration in relation to increased stress by NaCl, which shows that osmotic regulation mechanisms are activated by adapting to stress and preventing dehydration and is consistent with that described by Chaparzadeh and Mehrnejad (2013) and Campanelli *et al.* (2013). However, the concentration of proline in plants exposed to cadmium stress decreases radically from 75 $\mu$M and 100 $\mu$M $Cl_2Cd$. This experimental observation could be attributed to the presence of cadmium, increasing the action of phytochelatines (PC), which sequester this heavy metal through formation of PC-heavy metal complexes that accumulate in vacuoles (Pál *et al.*, 2018) and drops Cd concentration in cytosol.

## Conclusions

In the study of the interaction between genotypes and abiotic factors, it was observed that the response to salinity and cadmium stress is influenced by the alfalfa variety, which promotes different tolerance mechanisms.

The germination was an indicator of tolerance to salinity and Cd. The CW 660 genotype was the most tolerant to salinity and Cd during the germination process.

Under salt stress, Trinidad 87 and Salina PV genotypes, showed tolerance; CW 197 and CW 660 showed sensitivity, in the growth parameters.

All genotypes showed tolerance to heavy metal stress during growth. Proline was an indicator of tolerance to salt stress, but it is not for Cd stress.

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