



Halotolerant *Sinorhizobium meliloti* Strain Confers Salinity Tolerance to *Medicago sativa* L.

Cepa de *Sinorhizobium meliloti* halotolerante confiere tolerancia a la salinidad a *Medicago sativa* L

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Resumen

Objetivo. Evaluar de manera integral el efecto de la salinidad en plantas de la variedad CW 660 de *Medicago sativa* L., sometidas a dos tratamientos: fertilización con nitrógeno e inoculación con una cepa halotolerante de *Sinorhizobium meliloti*. **Materiales y métodos.** Las plantas de *M. sativa* L. se dividieron en dos grupos: fertilizadas con nitrógeno, pero no inoculadas con *S. meliloti* (PF) e inoculadas con *S. meliloti* pero sin fertilización (PI). El estrés salino se indujo con solución de Hoagland y NaCl (50, 100 y 200 mM) para PF, misma solución con nitrógeno limitado para PI. Las variables repuestas evaluadas fueron longitud

Abstract

Objective. To comprehensively evaluate the effect of salinity on CW 660 *Medicago sativa* L. plants subjected to two treatments: nitrogen fertilization and inoculation with a halotolerant strain of *Sinorhizobium meliloti*. **Materials and methods.** *M. sativa* L. plants were divided into two groups: fertilized with nitrogen but not inoculated with *S. meliloti* (FP) and inoculated with *S. meliloti* but not fertilized (IP). Salt stress was induced with Hoagland's solution and NaCl (50, 100, and 200 mM) for FP, the same solution with limited nitrogen for IP. Response variables length (L), fresh weight (FW), and dry weight (DW) of roots

(L), peso fresco (PF) y peso seco (PS) de raíces y partes aéreas, pigmentos fotosintéticos (clorofilas a, b y carotenoides) y concentración de prolina tras cuatro semanas de tratamiento. **Resultados.** Mediante modelos aditivos generalizados (GAM, por sus siglas en inglés), se evaluó el efecto de salinidad e inoculación sobre las variables repuestas. Las PI mostraron mejoras significativas en la longitud aérea y radical, y en las clorofilas a y b bajo estrés salino, en comparación con PF. La concentración de prolina bajó en PI. La nodulación disminuyó debido al estrés salino, pero la inoculación promovió nódulos activos. **Conclusión.** La inoculación con *S. meliloti* halotolerante mejoró la resistencia al estrés salino, impulsando el crecimiento de las plantas.

Palabras clave

Suelos salinos, inoculación bacteriana, agricultura sostenible.

and aerial parts, photosynthetic pigments (chlorophylls a and b and carotenoids), and proline concentration were measured after four weeks. **Results.** Generalized additive models (GAM) were used to evaluate the effect of salinity and inoculation on the response variables. The inoculated plants showed significant improvements in aerial and radical length, and chlorophylls a and b under salinity stress compared to the fertilized plants FP. Proline concentration was decreased in IP. Nodulation decreased due to salt stress, but inoculation promoted active nodules. **Conclusion.** Inoculation with halotolerant *S. meliloti* improved salt stress resistance and growth in plant.

Keywords

Saline soils, bacterial inoculation, sustainable agriculture.

Introduction

Soil salinity poses a significant threat to agricultural sustainability, food production, and food security, particularly in arid and semi-arid regions (Shah *et al.*, 2022). The scale of the problem is considerable, with over one billion hectares of land affected by soil salinity and its spread continuing (Hopmans *et al.*, 2021). The crisis is intensifying at over two million hectares per year (Singh, 2018), and the consequences are evident in different regions.

Globally, the impact of soil salinity is remarkable. In Argentina, 34% of the irrigated area is affected, while the figures are 18% in South Africa and 33% in Egypt (Adejumobi *et al.*, 2016). On a larger scale, more than one-fifth of the world's total irrigated land is affected by salinity. Without intervention, the expansion of salt-affected land could exceed 50% within the next three decades (Wang *et al.*, 2020).

The impact of salinity on agricultural productivity is evident in drylands, where salts accumulate due to increased evaporation, leading to osmotic stress and reduced water availability for plants (Ramos *et al.*, 2020). This challenge is compounded by the complex interplay between salinity, nitrogen fertilization, and symbiotic interactions.

Fertilization management under saline conditions is complex. While efficient nitrogen nutrition can increase crop resilience to salinity stress by mitigating toxic effects, excessive nitrogen fertilization leads to environmental problems and negatively impacts air and water quality, biodiversity, and human health (Zhao *et al.*, 2013). Finding the right balance is critical.

Medicago sativa L., commonly known as alfalfa, stands out as an important forage legume due to its high protein content and nitrogen fixation capacity (Rokebul-Anower *et al.*, 2017). Its symbiotic association with *Sinorhizobium meliloti* is crucial, as this interaction leads to atmospheric nitrogen fixation, benefiting both the plant and the bacteria.

However, alfalfa is considered moderately salt tolerant and can tolerate 20 mM NaCl equivalent (Bertrand *et al.*, 2015). Rhizobia associated with alfalfa roots play a role in enhancing salt tolerance through mechanisms such as maintaining ion balance and producing osmo-protective compounds (Bertrand *et al.*, 2020).

This study focuses on how the interplay of salinity, nitrogen fertilization, and *S. meliloti* inoculation affects *M. sativa* L. plants. This research aims to provide important insights for improving salt stress tolerance in different agricultural management contexts.

Materials and methods

Experimental design

Eight treatments were compared under a randomized complete block design with five replicates. Treatments came from the combination of two factors: (1) inoculated and non-inoculated (fertilized) plants with *S. meliloti* (2) four growing conditions non-stressed (control), and any of three stresses: 50mM; 100 mM and 200 mM NaCl. The experimental unit was a pot.

Germination and inoculation of seeds

M. sativa L. seeds of CW 660 variety were washed with water for 5 min, 70% alcohol for 1 min, 10% sodium hypochlorite for 10 min, and three rinses with sterile water between each step. They were then sown in Petri dishes with double-moistened filter paper and kept for 48 hours in the dark at a temperature of 25 °C. After germination, the seeds were divided into two groups. One group of seeds was inoculated with *S. meliloti*, while the other group of seeds was not inoculated (fertilized) with *S. meliloti*.

To inoculate the seeds, a halotolerant strain of *S. meliloti* was previously selected, which was isolated from the saline soil of the Villa Mercedes Experimental Station (SL) of INTA (Pacheco *et al.*, 2019), with a (Minimum Inhibitory Concentration, MIC) of 600 mM NaCl (the minimum concentration of a substance that fully inhibits microbial growth) (Nonnoi *et al.*, 2012). The rhizobia grew in Tryptone-Yeast Extract TY culture medium (Vincent, 1970) for 24 h at 28 °C with constant agitation until it reached its exponential growth phase. This was verified with a spectrophotometer at a wavelength of 600 nm (0.8 λ). Before sowing, seeds were inoculated by immersion for 1 h in bacterial inoculum. Seeds were then transferred to 100 mL pots filled with sterile vermiculite. Plants were re-inoculated with 1 ml of a 108 cfu/mL (Colony Forming Units per milliliter) suspension of the corresponding bacterial strain, whereas 1 ml of water was added to non-inoculated plants.

Cultivation of plants and saline treatments

Cultivation was carried out using vermiculite sterilized in an oven at 80 °C for 48 h, distributed in 100 ml pots in which the previously germinated and inoculated seeds were placed. Plants were grown in a culture chamber at 25 °C with a photoperiod of 16 h light and 8 h dark for 5 weeks. The saline treatment was initiated one week after sowing: (1) fertilized plants without inoculum (FP) were irrigated with Hoagland's solution + 50, 100, and 200 mM NaCl, and (2) inoculated plants (IP) were irrigated with the same nitrogen-limiting solution + 50, 100, and 200 mM NaCl, control plants were only irrigated with Hoagland's solution.

Nodule production

After 5 weeks, roots were extracted from plants, washed, and nodules were counted, and activity was assessed by cross sections and coloration (Chmelíková and Hejcman, 2012). Active nodules were defined as those with pink coloration inside, and inactive nodules were defined as those with white coloration.

Biomass, plant growth, and determination of photosynthetic pigments

At the end of the treatments, length (L, cm), fresh weight (FW, g.), and dry weight (DW, g.) of roots and aerial parts were measured in quintuplicate. To calculate the DW, the samples were placed in an oven at a temperature of 30 °C for 7 days.

The determination of chlorophylls a, b, and carotenoids was carried out according to Porra (2002). One hundred mg of fresh aerial material (leaves) were collected, crushed in a mortar with 10 ml of 80% (v/v) acetone, and filtered. The extract was kept at 4 °C until the spectrophotometer reading. For the quantification of chlorophylls, the absorbance was measured at a wavelength of 646.6 nm (chlorophyll a) and 663.6 nm (chlorophyll b) and 470 nm for carotenoids, using 80% (v/v) acetone as a blank, expressing the results in µg/ml.

Proline determination

The Bates method (1973) was used for proline determination. We crushed 0.5 g of fresh aerial material (leaves) with a mortar and pestle with 10 ml of 3% aqueous sulfosalicylic acid solution. The homogenate was filtered and mixed with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid. The mixture was boiled in a water bath at 100 °C for one hour at a constant temperature, and the reaction was stopped by immersing the tube in cold water. For proline quantification, 0.5 ml of the samples were taken, and the absorbance of each sample was determined at a wavelength of 520 nm.

Statistical analysis

To examine the effects of stress and fertilization-inoculation on alfalfa plants, we used generalized additive models (GAMs), a robust statistical modeling technique. GAMs use smoothed functions of predictor variables, including factors such as salinity stress and inoculation, to identify their relationships with response variables (Hastie, 1986). These

smooth functions, often represented as splines, skillfully model and capture nonlinearities inherent in the data. The effect of salinity stress on nodule production was assessed using ANOVA. Data preprocessing and analysis were performed using *RStudio* version 4.1.

Results

Ten GAMs were created, each model using the two predictor variables or factors (salinity stress and inoculation) to predict different response variables and select the models with the lowest AIC and best R^2 .

Parameters morphology

Generalized additive models (GAM) with gamma distribution (logarithmic modality) were used to evaluate the effect of salinity and inoculation on aerial length (AL) and root length (RL). Regarding the effect of salinity stress, the associated coefficient μ was estimated to be -0.003 for AL and -0.001 for RL. This suggests that with each increase in salinity, there is an expected reduction in both aerial length (AL) and root length (RL). However, when plants are inoculated, the values of AL and RL show an approximate increase of 0.364 and 0.151, respectively. Significant effects of both salinity and inoculation were observed for both response variables.

To accurately capture the underlying response distribution of ADF, ADW, RFW, and RDW, we used GAM models fitted with an exponential distribution. The coefficient μ associated with salinity stress was -0.002, -0.002, -0.002, and -0.0005 for the response variables ADF, ADW, RFW, and RDW, respectively. Conversely, the coefficient μ associated with inoculation for these response variables increased by approximately 0.336, 0.439, 0.224, and 0.095, respectively. However, similar to the salinity results, these results did not reach statistical significance (table 1).

The quality of the models can be assessed by considering the adjusted R^2 values for the observed (response) and modeled (adjusted) values and the AIC value (table 2).

Table 1

P-values for morphological variables GAMs as predictors for salinity and inoculation

Factors	Parameters					
	AL	RL	AFW	RFW	ADW	RDW
Salinity	2.0*10⁻¹¹	1.9*10⁻⁷	0.25	0.28	0.34	0.79
Inoculation	5.1*10⁻⁰⁸	5.2*10⁻⁴	0.29	0.17	0.48	0.76

AL: aerial length, RL: root length, AFW: aerial fresh weight, RFW: root fresh weight, ADW: aerial dry weight, RDW: root dry weight. *P*-values ≤ 0.1 are in bold.

Table 2
AIC and R² values for six different GAMs used for morphological parameters

	Parameters					
	AL	RL	AFW	RFW	ADW	RDW
AIC	182.5	213.5	-11.6	-21.5	-154.7	-206.3
R ²	0.88	0.74	0.69	0.77	0.67	0.48

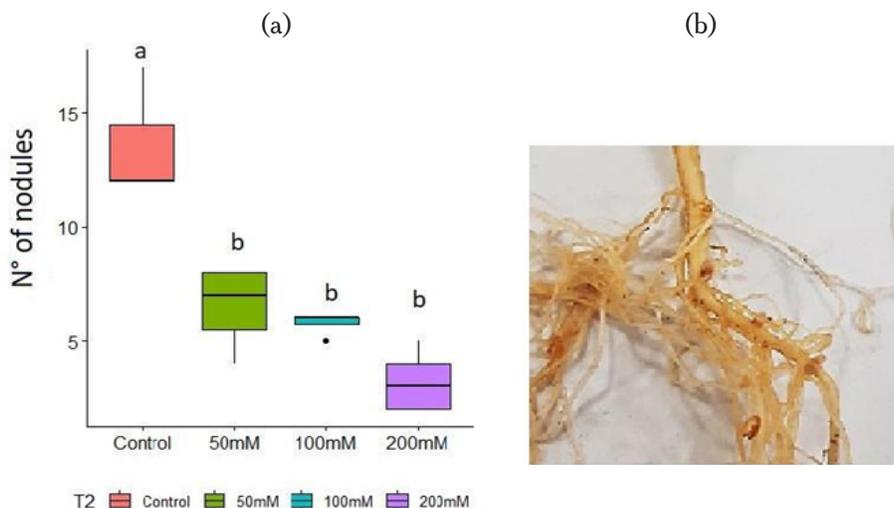
AL: aerial length, RL: root length, AFW: aerial fresh weight, RFW: root fresh weight, ADW: aerial dry weight, RDW: root dry weight. The lowest AIC value is in bold, and the highest R2 values are in bold.

Nodulation

Figure 1a shows a significant decrease in the number of active nodules from 50 mM onwards concerning the control. Figure 1b shows the roots of *M. sativa* L. plants with active nodules, with pink coloration, the plants without inoculation did not present nodules.

Figure 1

(a) Number of *M. sativa* L. var CW 660 active nodules inoculated with *S. meliloti* at different NaCl concentrations. b) Root of *M. sativa* L. var. CW 660 with active *S. meliloti* nodules



Different letters represent significant differences in the treatments (NaCl) about the control determined by the analysis of variance (ANOVA) according to Tukey's test ($p \leq 0, 05$).

Determination of photosynthetic pigments and proline content in SF and SI

GAMs fitted to a normal distribution (NO) were used to assess chlorophyll a, b, carotenoids, and proline concentrations to ensure adequate capture of the underlying distribution of responses.

For chlorophylls “a” and “b”, the coefficient μ associated with salinity stress was -0.0001 and -0.0004, respectively. This suggests that a decrease in these parameters can be expected with each increase in salinity, but these results were not significant (Table 3). The coefficient μ associated with the inoculation for chlorophylls “a” and “b” increased by approximately 0.613 and 0.783, respectively. These results were significant.

The μ coefficients associated with salinity stress -0.002 and inoculation -0.652 for carotenoids showed negative values. This means that as salinity increases, the values are likely to decrease, and inoculation is likely to have a negative effect on carotenoids. The results were statistically significant for both salinity and inoculation (table 3).

Table 3

P-values for pigments. GAMs as predictor variables for salinity and inoculation

Factors	Pigments		
	Chlorophylls a	Chlorophylls b	Carotenoids
Salinity	0.77	0.01	0.01
Inoculación	1.6*10⁻⁵	2.3*10⁻⁵	0.0001

P values ≤ 0.1 are in bold.

The adjusted R^2 values for the observed (response) and modeled (adjusted) values and the AIC value were used to assess the quality of the models (table 4).

Table 4

AIC and R^2 values for four different GAMs used for the pigments

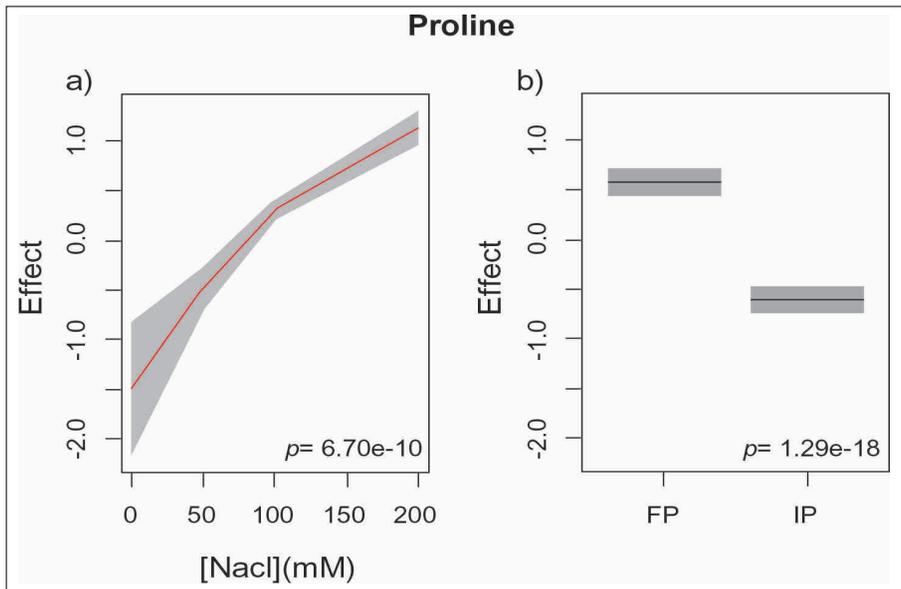
	Pigments		
	Chlorophylls a	Chlorophylls b	Carotenoids
AIC	120.6	109.4	63.3
R^2	0.83	0.79	0.78

The lowest AIC value is indicated in bold, and the highest R^2 values are indicated in bold.

In the case of proline, the μ coefficient associated with salinity stress was 0.010. This indicates that with each increase in salinity, an increase in proline can be expected (figure 2a). However, the μ coefficient associated with inoculation decreases by approximately -1.187 (figure 2b). In both cases, these results were significant (figure 2a y 2b).

Figure 2

- a) Smooth function showing the effect of salinity on proline concentration. The marks on the x-axis are different treatment salinities. b) Effect of proline in fertilized and inoculated plants



The quality of the model can be assessed by considering the confidence interval (gray shaded area) and significance (p -values) of each smoothed term for the predictor variable used (Figure 2a y 2b). The adjusted R^2 values for the observed (response) and modeled (adjusted) 0.976. The AIC value corresponds to 329.2136.

Discussion

In this study, we used generalized additive models (GAMs) to investigate the effects of salinity stress and inoculation with the halotolerant strain of *Sinorhizobium meliloti* on the *Medicago sativa* L variety CW 660. During the early stages of development, our observations revealed a significant interaction between these factors and their influences on various physiological and morphological parameters of plants.

The calculated coefficient (μ) associated with salinity stress showed that with each incremental increase in salinity, a decrease in both aerial length (AL) and root length (RL) is expected. Interestingly, our results showed a distinct pattern when plants were inoculated. This highlights the potential mitigating effect of inoculation against the negative effects of salinity on plant growth.

The derived coefficients (μ) associated with salinity stress indicated that these response variable (ADF, ADW, RFW, RDW) all experienced a reduction with increasing salinity. This result is in line with expectations, as saline conditions are known to inhibit plant biomass production and development.

In general, IP showed better performance in both aerial and root morphological parameters compared to FP. This improvement in growth and salinity stress in IP with salt-tolerant bacteria may involve different mechanisms such as antioxidant enzymes, phosphate solubilization, siderophores, and secretion of different phytohormones (Alizadeh and Parsaeimehr 2011, Chakraborty *et al.*, 2011, Nabti *et al.*, 2015).

However, high salinity can negatively affect nodulation ability by inhibiting the initial steps of rhizobium-legume symbiosis establishment (Zahran, 1999). When analyzing the number of active nodules, it is observed that it decreases significantly with increasing salinity concentrations; these results are similar to those obtained for *Gliricidia sepium* (Clavero and Razz, 2002).

Pigment content provides information on the effect of abiotic stress caused by NaCl on the photosynthetic apparatus, since photosynthetic tissues are very sensitive to environmental conditions (Esteban *et al.*, 2015), making its use as a biological indicator of stress useful.

When analyzing the coefficient (μ) associated with salinity stress, it was observed that the concentration of chlorophyll a and b with each increase in salinity decreased significantly. These results are consistent with those reported for sunflowers, where salinity stress causes pigment degradation and reduces the activities of enzymes related to chlorophyll biosynthesis, consequently affecting chlorophyll fluorescence and net photosynthesis (Santos, 2004).

On the other hand, when analyzing the coefficient (μ) associated with inoculation, it was observed that IP significantly alleviated salt stress by increasing chlorophyll a and b levels compared to FP plants. These results are in agreement with those of Irshad *et al.*, (2021), who showed that active nodulation increased chlorophyll a and b by 37.18% and 44.51%, respectively, in *Medicago truncatula* inoculated with *Rhizobium meliloti* and exposed to salt stress.

The μ coefficients related to salinity stress and inoculation on carotenoids showed negative values, indicating higher concentrations of FP than in IP. This is because salinity stress induces abscisic acid (ABA) biosynthesis from carotenoids through the mevalonate pathway, which is responsible for regulating plant development in response to water tolerance (Lim *et al.*, 2012). Therefore, the accumulation of carotenoids in FP compared to IP could be the result of stimulating the mevalonate pathway to induce ABA.

Proline acts as an osmoprotectant, can also act as a protein stabilizer, acts as a scavenger of hydroxyl radicals, stabilizes cell membranes by interacting with phospholipids, and serves as a source of carbon and nitrogen (Kishor *et al.*, 2005). It is believed that plants accumulate high levels of proline when exposed to salt stress to maintain cell turgor and chlorophyll levels, allowing for efficient protection of photosynthetic activity (Silva-Ortega *et al.*, 2008). When analyzing the coefficients (μ) for proline, it was observed that these concentrations increased with increasing salinity; this increase was lower in IP, indicating a higher tolerance to salinity. These results are similar to those reported for *Triticum durum* inoculated with rhizobacteria (Silini *et al.*, 2016, Cherif-Silini *et al.*, 2019).

Conclusion

While the growth of *M. sativa* L. is indeed affected by salinity, the results of this study offer a promising avenue for improving both the growth and yield of alfalfa crops in saline soils. In addition, these results shed light on the physiological importance of halotolerant bacteria in saline soil ecosystems. This research not only contributes to our basic understanding of plant responses to salinity stress, but also has practical implications for sustainable agriculture in regions facing salinity problems.

By demonstrating the potential of plant-bacterial symbiosis in the face of nitrogen fertilization, this study lays the groundwork for future research and agricultural advances aimed at improving plant resilience under adverse growing conditions.

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