

## Article

# Genetic Variation in Responses to Salt Stress in Tunisian Populations of *Medicago ciliaris*

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**Abstract:** Soil salinity is one of the most serious environmental factors affecting crop productivity around the world. We used a morpho-physiological approach to investigate the salt responses of four Tunisian natural populations of *Medicago ciliaris*. Forty-six lines of *M. ciliaris* were grown under a control treatment and 100 mM NaCl. We measured 11 quantitative traits of shoot and root growth during harvest. An analysis of variance showed that the variations in salt response can be explained by the effects of the population, line, treatment, and interactions between the population and treatment and the line and treatment. Most of the measured traits showed significant differences between the studied populations under the control treatment and salt stress. High to moderate broad-sense heritabilities ( $H^2$ ) were noted for most of the parameters under the control treatment and salt stress, implying that salt tolerance is highly heritable. Most correlations between the measured traits under the two treatments were positive, of which the strongest correlations were recorded between characteristics related to development. The 46 studied lines formed three groups in both conditions, while their genetic structure was dependent on the treatment factor. The results from this study can provide a basis for identifying and breeding salt-tolerant lines of *M. ciliaris*.

**Keywords:** *Medicago ciliaris*; natural variation; morpho-physiological traits; populations; lines; NaCl



**Citation:** Aloui, M.; Mahjoub, A.; Cheikh, N.B.; Ludidi, N.; Abdelly, C.; Badri, M. Genetic Variation in Responses to Salt Stress in Tunisian Populations of *Medicago ciliaris*. *Agronomy* **2022**, *12*, 1781. <https://doi.org/10.3390/agronomy12081781>

Academic Editor: Aisheng Xiong

Received: 1 June 2022

Accepted: 15 July 2022

Published: 28 July 2022

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## 1. Introduction

Soil salinity is a major problem for agriculture throughout the world, and salinity affects 7% of the world's land area, which amounts to 930 million hectares [1,2].

In the Mediterranean region, the climate is mainly characterized by low rainfall, high temperatures in summer, and low temperatures in winter [3]. This, together with the scarcity and low quality of the water resources, poses challenges to vegetation [4]. In Tunisia, the area affected by salinity is estimated to be 9.13% of the total surface of the country and approximately 25% of the cultivated areas [5]. The saline soils are mainly located in semi-arid and arid regions. Soil salinity is one of the most significant abiotic stresses for crop plants, including legumes [3]. These plants are very important both ecologically and agriculturally because legume roots are able to interact symbiotically with soil microorganisms to form nodules that fix atmospheric nitrogen. Hence, legumes are interesting candidates for improving soil fertility and incorporating salty soils into agriculture. According to their ability to grow in high-salt medium, plants have been classified as glycophytes or halophytes, of which glycophytes cannot grow well under

high salinity, whereas halophytes grow well under high salinity [6]. Most crop species are glycophytes and cannot tolerate salt stress.

The selection of salt tolerant varieties is a promising way to create plants with high yields under this constraint [7]. The genetic engineering of key regulatory genes appears to be one of the most promising strategies to minimize the deleterious effects associated with various stresses, including salt stress. The impact of salinity on plant growth and development is correlated with different morphological and physiological characteristics, such as biomass [8]. Morpho-agronomic characteristics reflect the combined genetic and environmental impacts on plants and parameters, such as survival under unfavorable conditions, plant height, leaf area, injury to salt stress, relative growth rate, and relative growth reduction, which are considered the selection criteria for salt tolerance [9]. Salt tolerant plants can reduce the detrimental effects of high salinities through anatomical, morphological, and physiological adaptations [10].

Among the *Medicago* annual species, *Medicago ciliaris* is a diploid species ( $2n = 16$ ) and a predominantly autogamous plant found in the Mediterranean region [11]. In Tunisia, *M. ciliaris* grows spontaneously in Northern areas, restricted to humid, sub-humid, superior, and inferior semi-arid bioclimatic areas. Cherifi et al. [12] reported that in addition to its good forage quality, *M. ciliaris* has the capacity to improve the nitrogenation of salted lands. It is a glycophyte plant that can grow in moderately saline soils in association with halophytes, producing up to 40% of the vegetative cover in rainy years [13]. Ben Salah et al. [14] reported that *M. ciliaris* is a good candidate for the improvement of marginal or degraded lands with low fertility, and/or high salinity lands, such as 'Sebkha'. In saline conditions, species are threatened and emergency measures must be taken to preserve these plants. To overcome this problem, the selection of varieties tolerant to salt remains the best approach for the rehabilitation of regions affected by this constraint. The effectiveness of such an approach depends on the existence of the genetic diversity of salt tolerance and its use for the screening and selection of plants with the best agronomic performance. For many species, significant intraspecific diversity in salt tolerance has been noted between cultivars and spontaneous species [15–17]. The most sensitive species show reduced growth, often considerable at low salt concentrations. However, tolerant species react to the harmful effect of salt by accumulating toxic ions and improving the mobilization of active organic compounds, such as glycinebetaine and proline.

Although responses and physiological mechanisms to salinity have been extensively studied in different plant species [18–21], research exploring the diversity in the salt tolerance of natural populations is still limited for many plant species. Only a few studies have explored the natural variation of responses to abiotic stresses in some species. Among these studies, natural variations for responses to drought stress [22,23] and salt [16] have been analyzed in Tunisian natural populations of *M. truncatula*. This study forms a part of a large legume improvement program focusing on the use of *M. ciliaris* to expand genetic variability, develop alternate plant types and physiological processes, and increase salt in *M. sativa*. The current work aims to (i) explore the variability of morpho-physiological responses in this collection of lines of *M. ciliaris* under a control treatment and 100 mM NaCl, and (ii) identify the important parameters affected by salinity and determine whether parental genotypes could be used in genetic studies for salinity tolerance programs.

## 2. Materials and Methods

### 2.1. Plant Material and Growing Conditions

A total of 46 lines of *M. ciliaris* from four Tunisian populations randomly collected from different eco-geographic sites were used: 12 lines of the population from Soliman (TNC8), 12 lines from Enfidha (TNC1), 11 lines from Rhayet (TNC10), and 11 lines from Mateur (TNC11) (Supplementary Table S1). The studied lines were created by three generations of spontaneous selfing in the greenhouse. The selection of the geographical locations of the sites from which the populations were collected and the protocol for the sampling of the pods of *M. ciliaris* were performed as described by Badri et al. [11]. The seeds of

the lines were produced by Prof. Mounawer Badri's research team in the Laboratory of Extremophile Plants in the Centre of Biotechnology of Borj Cedria. The seeds were scarified using sandpaper and transferred to 5 L pots (20 cm in diameter and 16 cm deep) filled with a mixture of sand and compost of sphagnum (2:1). Each plant was grown in a separate pot under greenhouse conditions at the Centre of Biotechnology of Borj Cedria (CBBC), Tunisia. No pesticides were applied, and weeds were manually eliminated. During the twenty-one first days, the plants were watered every two days with a nutritive solution of Fahræus medium [24]. Salt stress was applied at the sixth leaf stage (after 21 days of seedling growth). The plants were cultivated under a control treatment (0 mM NaCl) and 100 mM NaCl. Sodium chloride was added gradually at three concentrations of 50 mM, 75 mM, and 100 mM; the final concentration (100 mM) was reached after one week. Four replicates per line and per treatment were carried out, providing a total of 368 plants. The experimental design was organized in a split-plot design with four blocks.

### 2.2. Measured Traits

The harvest of the plants was performed after 81 days of cultivation in May 2017. The plants did not flower in the same week so we removed the flowers from the plants until the late plants bloomed. The plants were separated into roots and shoots (stems and leaves), and a set of morph-agronomic parameters measurements was established. For each individual plant, we measured 11 quantitative traits, including the flowering date (FLOR, days), length of stems (LS, cm), length of roots (LR, cm), number of leaves (NL), aerial fresh weight (AFW, g), aerial dry weight (ADW, g), root dry weight (RDW, g), root fresh weight (RFW, g), root dry weight on aerial dry weight (ratio), chlorophyll content (Chl,  $\text{mg}\cdot\text{g}^{-1}$  FW), and relative water content (RWC, %). The relative water content (RWC) was measured before harvest using one leaf per plant and weighed three times in the fresh, turgid, and dry states. The RWC was estimated as follows:  $\text{RWC} (\%) = 100 [(LFW - LDW) / (LTW - LDW)]$ , where LFW is the leaf fresh weight, LDW is the leaf dry weight, and LTW is the leaf turgid weight.

The flowering time (FLOR) corresponds to the number of days from the planting of the plants until the first flower color appearance. For the dry weight biomass analysis, the plant organs were dried at 65 °C for 48 h. The concentrations of leaf chlorophylls were determined for the fresh mass (FM) in 80% (v/v) acetone using the method of Lichtenthaler [25].

The sensitivity index of trait expression to salt stress (SSI) was estimated as follows [26]:  $\text{SSI} = 100 (S - C) / C$ , where S and C are the performances under salt stress and the control treatment, respectively.

### 2.3. Statistical Analyses

Three-way (population, line, and treatment) analysis of variance was performed using Proc GLM in SPSS version 20 (2007 Rel 1600 SPSS Inc., Chicago, IL, USA). The phenotypic mean values of each population were compared for all quantitative traits using the Duncan multiple range test at 5%. The variance components were estimated using the procedure VARCOMP in the SPSS software. The within-line variance can be assumed as the effect of the environment, while the variation among the lines is assumed to be solely genetic. The broad-sense heritability ( $H^2$ ) of the measured parameters was estimated as the ratio of genetic variance to the sum of genetic and environmental variances [19].

$H^2 = \sigma^2g / (\sigma^2g + (\sigma^2e/k))$ , where  $\sigma^2g$  is the genotypic variance,  $\sigma^2e$  is the environmental variance, and k is the number of replicates.

Phenotypic correlations between all trait combinations for each treatment were estimated by computing the Pearson correlation coefficient (r) using the SPSS Correlate procedure.

To represent the relationships between lines within populations in the control treatment and salt condition, cluster analysis was performed to generate phenograms based on the squared Euclidean distance by the Ward method using SPSS software. A discriminant analysis (DA) was also performed on the control and salt stress values for the groups of *M. ciliaris* lines.

### 3. Results

#### 3.1. Morpho-Physiological Responses of Studied Lines

The results from the ANOVA show that the trait variation is explained by the effects of the population, the line nested within the population, the treatment, and the interactions between the population and treatment and the line and treatment (Table 1). The maximum effect was observed for the treatment factor. Thus, *M. ciliaris* lines differ in their response to salt treatment. Four out of 11 traits, eight out of 11 traits, and nine out of 11 traits are explained by the effects of the population, the lines nested within the populations, and the treatment, respectively. Furthermore, the variabilities of only four (LR, RFW, RDW, and ratio) of the eleven measured parameters are explained by the interaction of the populations  $\times$  treatment, and four (FLOR, LR, RFW, and RDW) parameters are explained by the interaction of the line  $\times$  treatment. This result indicates that the line performance under the control treatment and salt stress did not have similar trends for these measured traits.

**Table 1.** Contributions of population, line nested within population (line(pop)), treatment (treat), and population  $\times$  treatment (pop  $\times$  treat) and treatment  $\times$  line nested within population (treat  $\times$  line(pop)) interactions to the total variance of the measured traits for the studied populations of *M. ciliaris* under the control treatment and salt stress.

	Population		Line(pop)		Treatment		Pop $\times$ Treat		Treat $\times$ Line(pop)	
	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>
FLOR	2.96	0.036	3.06	0.000	1.04	0.310	0.44	0.727	1.63	0.031
LS	6.89	0.000	2.30	0.000	293.16	0.000	1.36	0.259	1.25	0.190
LR	0.16	0.922	3.50	0.000	49.25	0.000	3.33	0.023	3.16	0.000
NL	2.88	0.040	1.09	0.352	133.71	0.000	0.84	0.474	0.57	0.970
AFW	0.80	0.497	1.53	0.045	114.56	0.000	0.81	0.490	0.75	0.837
ADW	0.80	0.497	1.53	0.045	114.56	0.000	0.81	0.490	0.75	0.837
RFW	0.93	0.432	4.81	0.000	44.58	0.000	6.17	0.001	3.27	0.000
RDW	3.92	0.011	5.24	0.000	185.50	0.000	9.35	0.000	3.17	0.000
ratio	2.14	0.100	2.04	0.002	3.11	0.081	4.90	0.003	1.13	0.312
Chl	0.86	0.465	1.13	0.305	4.21	0.043	0.43	0.733	1.07	0.390
RWC	2.31	0.081	1.39	0.094	13.75	0.000	0.96	0.417	1.48	0.066

F: Coefficient of Snedecor–Fisher, significant ( $p \leq 0.05$ ). Flowering date (FLOR) (days), length of stems (LS) (cm), length of roots (LR) (cm), number of leaves (NL), aerial fresh weight (AFW) (g), aerial dry weight (ADW) (g), root dry weight (RDW) (g), root fresh weight (RFW) (g), root dry weight on aerial dry weight (ratio), chlorophyll content (Chl) ( $\text{mg}\cdot\text{g}^{-1}$  FW), relative water content (RWC).

Most measured traits showed a significant difference between the studied populations of *M. ciliaris* under both treatments (Table 2).

In the control treatment, seven out of the eleven traits showed significant differences among the studied populations. The Rhayet populations exhibited the lowest value of the length of stems (LS = 60.43 cm) and the highest ratio of RDW/ADW (0.67).

Under salt stress, only three out of the eleven measured characteristics showed significant differences among the populations. The largest values of LS (39.98 cm) and NL (64.74) were registered for the Enfidha populations, while the lowest levels (LS = 33.33 cm; NL = 51.00) were for those of Rhayet.

Overall, the Soliman populations exhibited the lowest levels of reduction for AFW (−43%) and ADW (−43%), while Rhayet had the largest values (AFW = −52%; ADW = −52%).

**Table 2.** Comparison of means of measured traits for studied populations of *M. ciliaris* under control treatment and salt stress.

Control Treatment											
	FLOR	LS	LR	NL	AFW	ADW	RFW	RDW	Ratio	Chl	RWC
TNC1	67.04 ± 0.73 ab	70.44 ± 1.80 a	28.77 ± 0.65 abcd	114.55 ± 4.94 a	16.18 ± 0.77 a	2.86 ± 0.14 a	5.10 ± 0.27 bcd	1.28 ± 0.07 ab	0.49 ± 0.03 ab	2.74 ± 0.37 a	64.66 ± 2.38 ab
TNC8	68.33 ± 0.70 a	61.31 ± 2.42 ab	31.73 ± 0.77 ab	111.18 ± 4.75 a	13.97 ± 0.76 a	2.46 ± 0.13 a	4.09 ± 0.20 d	1.02 ± 0.05 bcd	0.47 ± 0.03 b	2.84 ± 0.34 a	63.85 ± 2.11 abc
TNC10	66.00 ± 0.90 b	60.43 ± 2.17 b	32.08 ± 0.68 a	98.40 ± 4.42 b	14.53 ± 1.02 a	2.56 ± 0.18 a	6.14 ± 0.32 abc	1.54 ± 0.08 a	0.67 ± 0.05 a	3.03 ± 0.45 a	70.50 ± 1.87 a
TNC11	65.54 ± 0.72 ab	67.14 ± 2.17 a	30.36 ± 0.64 abc	112.69 ± 4.42 a	15.51 ± 0.68 a	2.74 ± 0.12 a	4.95 ± 0.25 cd	1.24 ± 0.06 abc	0.47 ± 0.02 b	3.16 ± 0.46 a	66.93 ± 2.66 abc
F	1.33	6.37	4.03	1.90	1.66	1.66	15.91	15.91	7.93	0.69	0.83
p	0.272	0.001	0.011	0.138	0.184	0.184	0.000	0.000	0.000	0.562	0.482
Salt stress											
	FLOR	LS	LR	NL	AFW	ADW	RFW	RDW	ratio	Chl	RWC
TNC1	65.74 ± 0.56 ab	39.98 ± 1.25 c	25.68 ± 1.73 de	64.74 ± 3.13 c	7.33 ± 0.33 b	1.29 ± 0.06 b	6.49 ± 0.35 abc	0.72 ± 0.04 e	0.58 ± 0.04 ab	2.21 ± 0.27 a	52.97 ± 1.48 d
TNC8	68.01 ± 0.62 a	35.39 ± 1.50 cd	24.53 ± 1.04 cde	58.60 ± 2.50 d	7.96 ± 0.33 b	1.40 ± 0.06 b	6.10 ± 0.47 a	0.68 ± 0.05 de	0.55 ± 0.05 ab	1.98 ± 0.20 a	61.76 ± 1.87 cd
TNC10	64.44 ± 0.86 ab	33.33 ± 1.90 d	25.67 ± 1.90 e	51.00 ± 2.33 f	6.98 ± 0.41 b	1.23 ± 0.07 b	5.95 ± 0.51 ab	0.66 ± 0.06 e	0.54 ± 0.05 ab	2.16 ± 0.25 a	62.50 ± 2.37 abcd
TNC11	64.05 ± 1.11 ab	38.40 ± 1.71 c	24.39 ± 1.09 bcde	57.73 ± 1.93 e	6.88 ± 0.36 b	1.21 ± 0.06 b	6.28 ± 0.62 abc	0.70 ± 0.07 e	0.51 ± 0.05 ab	2.17 ± 0.20 a	61.06 ± 1.72 abcd
F	2.32	5.75	0.93	7.63	0.35	0.35	0.78	0.78	0.61	1.45	3.06
p	0.094	0.003	0.437	0.001	0.792	0.792	0.514	0.514	0.612	0.246	0.042
Salt sensitivity index (SI)											
	FLOR	LS	LR	NL	AFW	ADW	RFW	RDW	ratio	Chl	RWC
TNC1	-1.9	-43	-11	-43	-55	-55	27.3	-44	18.4	-19.3	-18
TNC8	-0.5	-42	-23	-47	-43	-43	49.1	-33	17	-30.3	-3.3
TNC10	-2.4	-45	-20	-48	-52	-52	-3.1	-57	-19	-28.7	-11
TNC11	-2.3	-43	-20	-49	-56	-56	26.9	-44	8.51	-31.3	-8.8

Flowering date (FLOR) (days), length of stems (LS) (cm), length of roots (LR) (cm), number of leaves (NL), aerial fresh weight (AFW) (g), aerial dry weight (ADW) (g), root dry weight (RDW) (g), root fresh weight (RFW) (g), root dry weight on aerial dry weight (ratio), chlorophyll content (Chl) ( $\text{mg}\cdot\text{g}^{-1}$  FW), relative water content (RWC). The means of each trait followed by the same or common letters are not significantly different among the studied populations under both treatments according to Duncan's multiple range test at 5%. Enfidha (TNC1), Soliman (TNC8), Rhayet (TNC10), and Mateur (TNC11).

The heritability ( $H^2$ ) values of the measured traits ranged from 0.00 to 0.93 and from 0.39 to 0.89 under the control treatment and salt stress, respectively (Table 3). In the control treatment, high heritabilities ( $H^2 > 0.4$ ) were recorded for the flowering time (FLOR), length of roots (LR), aerial fresh weight (AFW), aerial dry weight (ADW), root fresh weight (RFW), root dry weight (RDW), and the ratio of RDW/ADW. Moderate values ( $0.2 < H^2 \leq 0.4$ ) were found for the length of stems (LS) and relative water content (RWC). Lower levels of heritability ( $H^2 \leq 0.2$ ) were registered for the number of leaves (NL) and chlorophyll content (Chl). Furthermore, under salt stress, high levels of heritability ( $H^2$ ) were observed for all measured traits except Chl, which exhibited a moderate level of heritability ( $H^2 = 0.39$ ). Overall, our results show higher levels of heritability for most of the analyzed traits under salt stress than those under the control treatment.

**Table 3.** Genetic variance (Vg), environmental variance (Ve), and heritabilities ( $H^2$ ) for measured traits in *M. ciliaris* lines under control and salt condition.

	Control Treatment			Salt Stress		
	Vg	Ve	$H^2$	Vg	Ve	$H^2$
FLOR	6.66	20.80	0.56	21.02	10.79	0.89
LS	31.07	196.39	0.39	48.45	66.34	0.74
LR	9.04	10.71	0.77	32.77	55.16	0.70
NL	0.00	990.20	0.00	91.49	191.4	0.66
AFW	6.50	24.21	0.52	2.294	2.568	0.78
ADW	0.20	0.75	0.52	0.071	0.08	0.78
RFW	2.79	0.86	0.93	3.253	4.925	0.73
RDW	0.17	0.05	0.93	0.04	0.061	0.72
ratio	0.02	0.05	0.57	0.022	0.047	0.65
Chl	0.00	5.95	0.00	0.251	1.569	0.39
RWC	30.53	210.94	0.37	66.37	108.7	0.71

Flowering date (FLOR) (days), length of stems (LS) (cm), length of roots (LR) (cm), number of leaves (NL), aerial fresh weight (AFW) (g), aerial dry weight (ADW) (g), root fresh weight (RFW) (g), root dry weight (RDW) (g), root dry weight on aerial dry weight (Ratio), chlorophyll content (Chl) ( $\text{mg}\cdot\text{g}^{-1}$  FW), relative water content (RWC).

In the control treatment, out of the 55 possible correlations among the measured traits, 23 were significant and 18 of them were positive (Table 4). Positive significant correlations were observed between the flowering time (FLOR) and length of stems (LS), and between the LS and NL ( $r = 0.36$ ;  $p \leq 0.05$ ), LS and AFW ( $r = 0.43$ ;  $p \leq 0.05$ ), LS and ADW ( $r = 0.43$ ;  $p \leq 0.05$ ), LS and RFW ( $r = 0.15$ ;  $p \leq 0.05$ ), and LS and RDW ( $r = 0.15$ ;  $p \leq 0.05$ ). In addition, higher positive correlations were also observed between the number of leaves (NL) and AFW ( $r = 0.56$ ;  $p \leq 0.05$ ), NL and ADW ( $r = 0.56$ ;  $p \leq 0.05$ ), ADW and RFW ( $r = 0.40$ ;  $p \leq 0.05$ ), and ADW and RDW ( $r = 0.40$ ;  $p \leq 0.05$ ). Negative correlations were registered between the FLOR and RWC ( $r = -0.17$ ;  $p \leq 0.05$ ), the ratio and LS ( $r = -0.27$ ;  $p \leq 0.05$ ), the ratio and NL ( $r = -0.38$ ;  $p \leq 0.05$ ), the ratio and AFW ( $r = -0.44$ ;  $p \leq 0.05$ ), and the ratio and ADW ( $r = -0.44$ ;  $p \leq 0.05$ ).

Under salt stress, out of the 55 correlations among the measured characteristics, 25 were significant and 19 of them were positive (Table 4). Positive correlations were observed between the LS and LR ( $r = 0.20$ ;  $p \leq 0.05$ ), LS and NL ( $r = 0.56$ ;  $p \leq 0.05$ ), LS and AFW ( $r = 0.41$ ,  $p \leq 0.05$ ), LS and ADW ( $r = 0.41$ ;  $p \leq 0.05$ ), LS and RFW ( $r = 0.30$ ;  $p \leq 0.05$ ), LS and RDW ( $r = 0.30$ ;  $p \leq 0.05$ ), NL and LR ( $r = 0.30$ ;  $p \leq 0.05$ ), NL and AFW ( $r = 0.37$ ;  $p \leq 0.05$ ), NL and ADW ( $r = 0.37$ ;  $p \leq 0.05$ ), NL and RFW ( $r = 0.21$ ;  $p \leq 0.05$ ), NL and RDW ( $r = 0.21$ ;  $p \leq 0.05$ ), AFW and RFW ( $r = 0.30$ ;  $p \leq 0.05$ ), AFW and ADW ( $r = 1.00$ ;  $p \leq 0.05$ ) and ADW and RDW ( $r = 0.30$ ;  $p \leq 0.05$ ). Negative correlations were recorded between the ratio and AFW ( $r = -0.33$ ;  $p \leq 0.05$ ), the ratio and ADW ( $r = -0.33$ ;  $p \leq 0.05$ ), RWC and AFW ( $r = -0.20$ ;  $p \leq 0.05$ ), RWC and ADW ( $r = -0.20$ ;  $p \leq 0.05$ ), RWC and RFW ( $r = -0.23$ ;  $p \leq 0.05$ ), and RWC and RDW ( $r = -0.23$ ;  $p \leq 0.05$ ).



**Table 4.** Correlations between measured traits for lines of *M. ciliaris* under control treatment (below diagonal) and salt stress (above diagonal).

	FLOR	LS	LR	NL	AFW	ADW	RFW	RDW	Ratio	Chl	RWC
FLOR	1.00	−0.04	0.05	0.01	−0.04	−0.04	−0.09	−0.09	−0.07	0.16	0.04
LS	0.16 *	1.00	0.20 *	0.56 *	0.41 *	0.41 *	0.30 *	0.30 *	−0.10	−0.04	−0.07
LR	0.06	0.00	1.00	0.30 *	0.05	0.05	−0.04	−0.04	−0.12	0.17	0.02
NL	0.01	0.36 *	0.00	1.00	0.37 *	0.37 *	0.21 *	0.21 *	−0.05	0.05	−0.05
AFW	−0.11	0.43 *	−0.02	0.56 *	1.00	1.00 *	0.30 *	0.30 *	−0.33 *	0.01	−0.20 *
ADW	−0.11	0.43 *	−0.02	0.56 *	1.00 *	1.00	0.30 *	0.30 *	−0.33 *	0.01	−0.20 *
RFW	−0.11	0.15 *	−0.12	0.14	0.40 *	0.40 *	1.00	1.00 *	0.76 *	−0.14	−0.23 *
RDW	−0.11	0.15 *	−0.12	0.14	0.40 *	0.40 *	1.00 *	1.00	0.76 *	−0.14	−0.23 *
ratio	−0.09	−0.27 *	−0.15	−0.38 *	−0.44 *	−0.44 *	0.52 *	0.52 *	1.00	−0.16	−0.10
Chl	−0.02	−0.03	0.02	−0.03	−0.08	−0.08	0.04	0.04	0.13	1.00	0.03
RWC	−0.17 *	−0.07	0.03	−0.05	0.06	0.06	0.15 *	0.15 *	0.07	0.04	1.00

\* Correlation is significant at the 0.05 level (2-tailed). Flowering date (FLOR)(days), length of stems (LS) (cm), length of roots (LR) (cm), number of leaves (NL), aerial fresh weight (AFW) (g), aerial dry weight (ADW) (g), root fresh weight (RFW) (g), root dry weight (RDW) (g), root dry weight on aerial dry weight (ratio), chlorophyll content (Chl) ( $\text{mg}\cdot\text{g}^{-1}$  FW), relative water content (RWC).

Comparison between the two matrices of the correlations in the control treatment and salt stress showed that: (i) several correlations are specific to the control treatment, such as the correlations between the FLOR and LS ( $r = 0.16$ ;  $p \leq 0.05$ ), FLOR and RWC ( $r = -0.17$ ;  $p \leq 0.05$ ), the ratio and LS ( $r = -0.27$ ;  $p \leq 0.05$ ), and the ratio and NL ( $r = -0.38$ ;  $p \leq 0.05$ ), and (ii) correlations specific to salt stress were noticed, such as a positive correlation between the LS and LR ( $r = 0.20$ ;  $p \leq 0.05$ ), NL and RFW ( $r = 0.21$ ;  $p \leq 0.05$ ), NL and RDW ( $r = 0.21$ ;  $p \leq 0.05$ ), RWC and AFW ( $r = -0.20$ ;  $p \leq 0.05$ ), and RWC and ADW ( $r = -0.20$ ;  $p \leq 0.05$ ).

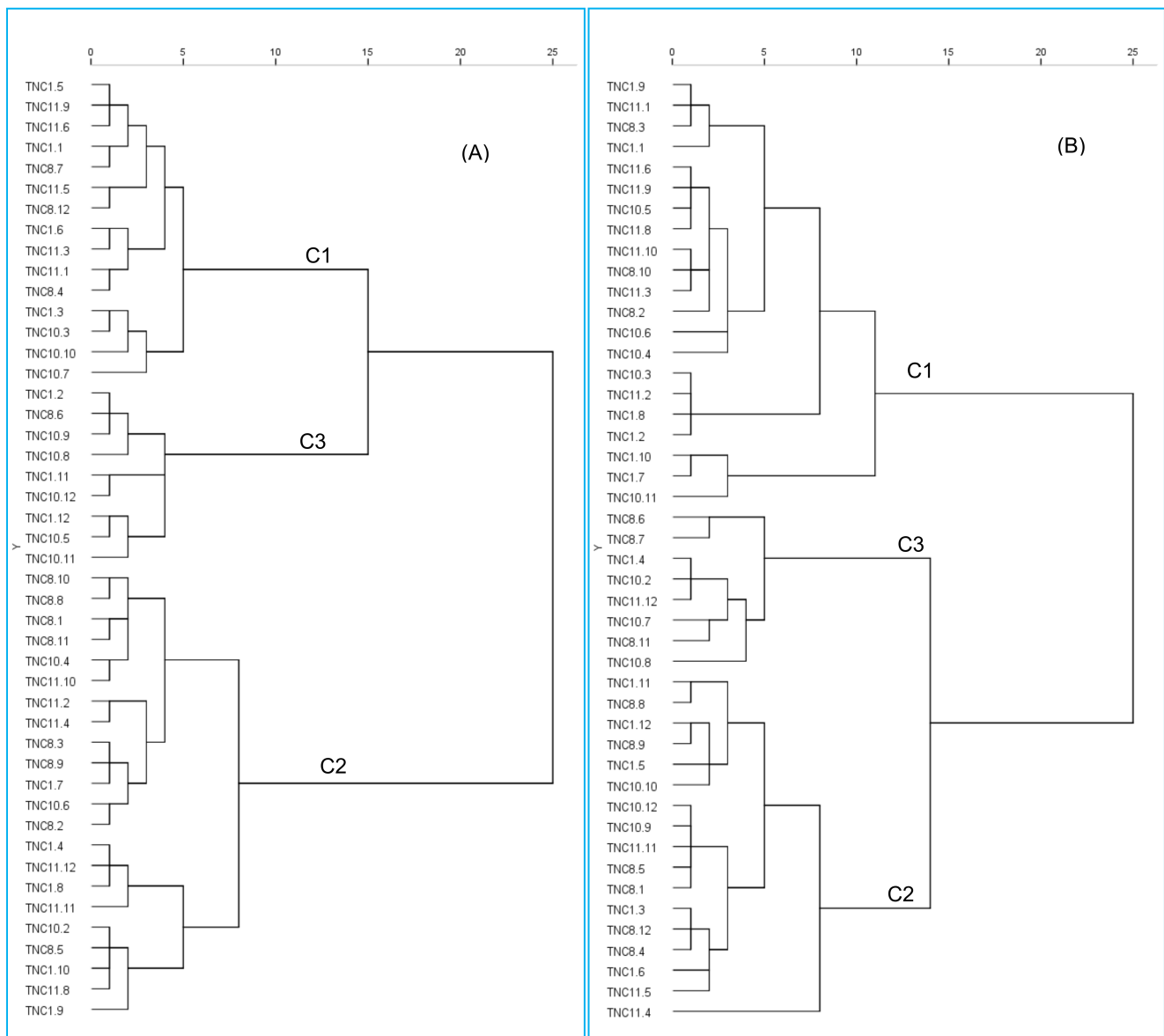
### 3.2. Clustering and Discriminant Analyses

In the control treatment, the studied lines of *M. ciliaris* were clustered into three groups (Figure 1). The first group consisted of 15 lines, with five from Mateur, four from Enfidha, three from Soliman, and three from Rhayet. A second group constituted 22 lines, with eight from Soliman, six from Mateur, five from Enfidha, and three from Rhayet. A third group was composed of nine lines, with five lines from Rhayet, three from Enfidha, and one from Soliman.

Under salt stress, the studied lines formed three groups. The first group was composed of 21 lines, with seven lines from Mateur, six from Enfidha, five from Rhayet, and three from Soliman. The second group constituted 17 lines, with six lines from Soliman, five from Enfidha, three from Rhayet, and three from Mateur. The third group consisted of eight lines, with three lines from Soliman, three from Rhayet, one from Enfidha, and one from Mateur.

Overall, our results show that the genetic structures of the studied lines were dependent on the treatment factor.

The discriminant power of the analyzed parameters for this classification was estimated using the Wilks lambda index (Table 5). In the control treatment, our results show that FLOR, NL, AFW, ADW, RFW, RDW, and the ratio of RDW/ADW were the main determinant traits for the classification of the studied lines into three clusters. The first group of lines flowered earlier and exhibited higher values of NL (125.79), AFW (18.59 g), and ADW (3.28 g) than those of the remaining groups.



**Figure 1.** Clustering of *M. ciliaris* lines based on square Euclidean distances using Ward's method on three groups under control treatment (A) and salt stress (B). Enfidha (TNC1), Soliman (TNC8), Rhayet (TNC10), and Mateur (TNC11).

Under salt stress, the traits of NL, AFW, ADW, RFW, RDW, the ratio of RDW/ADW, and Chl were the main determinants for the classification of the studied lines into three groups. The third group of lines exhibited the highest values for AFW (10.29 g) and ADW (1.82 g), while those of the second group showed the highest levels for NL (66.36), RFW (7.73 g), RDW (0.86 g), and the ratio of RDW/ADW (0.73).

Overall, the lines of the first group exhibited the highest values for AFW (18.59 g) and ADW (3.28 g) under the control treatment, while those of the third group had the highest levels for these two parameters (AFW = 10.29 g; ADW = 1.82 g) under salt stress. The comparison of the composition of these two groups showed that (i) certain lines (TNC1.1, TNC1.3, TNC1.5, TNC1.6, TNC8.12, TNC8.4, TNC10.3, TNC10.10, TNC11.1, TNC11.3, TNC11.5, TNC11.6, and TNC11.9) were specific to the first group, (ii) other lines (TNC1.4, TNC8.6, TNC8.11, TNC10.2, TNC10.8, and TNC11.12) were specific to the second group, and (iii) two lines (TNC8.7 and TNC10.7) were common to groups 1 and 3.



**Table 5.** Means of measured traits for classes of *M. ciliaris* lines under control treatment and salt stress.

	Control Treatment						Salt Stress					
	1	2	3	$\lambda$	F	$p$	1	2	3	$\lambda$	F	$p$
FLOR	64.22 b	67.42 a	69.59 a	0.67	10.68	0.000	65.41 a	65.67 a	66.03 a	1.00	0.05	0.955
LS	66.87 a	62.93 a	66.3 a	0.96	1.00	0.375	33.93 a	39.53 a	39.49 a	0.88	2.87	0.068
LR	29.79 a	31.70 a	30.02 a	0.93	1.57	0.220	23.23 a	27.39 a	24.43 a	0.92	1.85	0.170
NL	125.79 a	104.44 b	96.22 b	0.49	22.58	0.000	53.46 b	66.36 a	55.95 b	0.76	6.80	0.003
AFW	18.59 a	13.19 b	13.68 b	0.50	21.30	0.000	6.55 b	7.20 b	10.29 a	0.42	29.22	0.000
ADW	3.28 a	2.33 b	2.41 b	0.50	21.27	0.000	1.16 b	1.27 b	1.82 a	0.42	29.27	0.000
RFW	6.15 a	3.59 b	6.78 a	0.32	45.67	0.000	4.31 c	7.73 a	6.50 b	0.47	23.99	0.000
RDW	1.54 b	0.90 a	1.70 b	0.32	45.71	0.000	0.48 c	0.86 a	0.72 b	0.48	23.76	0.000
ratio	0.54 b	0.41 c	0.77 a	0.36	39.03	0.000	0.45 b	0.73 a	0.43 b	0.51	21.07	0.000
Chl	2.74 a	2.81 a	3.39 a	0.96	0.83	0.441	2.56 a	1.90 ab	1.68 b	0.85	3.71	0.033
RWC	67.39 a	64.57 a	69.15 a	0.96	0.94	0.399	60.36 a	59.11 a	57.93 a	0.99	0.19	0.824

$\lambda$ : Lambda of Wilks; F: coefficient of Snedecor–Fisher,  $p$ : probability value. The means of each trait followed by the same or common letters are not significantly different among the groups of the studied lines under the control treatment and salt stress according to Duncan’s multiple range test at 5%. Flowering date (FLOR) (days), length of stems (LS) (cm), length of roots (LR) (cm), number of leaves (NL), aerial fresh weight (AFW) (g), aerial dry weight (ADW) (g), root fresh weight (RFW) (g), root dry weight (RDW) (g), root dry weight on aerial dry weight (ratio), chlorophyll content (Chl) ( $\text{mg}\cdot\text{g}^{-1}$  FW), relative water content (RWC).

#### 4. Discussion

Solving the problem of salinity has global importance. The short-term relief of salt stress can be achieved by water management. However, the long-term solution to this problem relies on the improvement of salt tolerance for cultivated crop species [27]. The impact of salinity on plant growth and development is correlated with different morphological and physiological attributes, such as biomass [8]. In this study, variations in morpho-physiological traits were mainly explained by the treatment factor for four Tunisian populations of *M. ciliaris*, indicating that the measured characteristics were significantly affected by salt stress.

Our results show that salinity reduced plant growth. The magnitude of the reduction varied among the traits (the most affected were LS, NL, AFW, and ADW), and the severity of stress symptoms was different among populations (the population from Soliman (TNC8) was the most tolerant) and lines. A similar trend was also obtained in the natural populations of *M. truncatula* [16] and *Hordeum marinum* under different levels of salt stress [17].

The significant genotype  $\times$  treatment interaction observed for four traits (FLOR, LR, RFW, and RDW) suggests that these studied lines may have developed an adaptive response to salt stress that can be exploited to further determine the genetic basis of this physiological adaptation. Accordingly, Friesen et al. [28] showed that four populations of *M. truncatula* from Northern Tunisia have an adaptive response to salt stress. In addition, Tunisian natural populations of *M. truncatula* showed a large variation in salt tolerance [16].

Under salt stress, higher to moderate broad-sense heritability ( $H^2$ ) levels were found for the measured traits, indicating that a substantial part of phenotypic variation was due to genetic factors. Ten out of eleven traits showed higher heritability under salt stress. This indicates that these characteristics are good determinants that can be used in the selection of salt-tolerant lines for breeding programs of *M. ciliaris*. Accordingly, most morphological traits exhibited higher  $H^2$  for Tunisian populations of *Hordeum marinum* under salt stress [17].

In the current study, some specific correlations were found among the analyzed characteristics under control treatment or salt stress. Similarly, Arraouadi et al. [26] showed that significant correlations among the measured traits were noted for Tunisian and reference lines of *M. truncatula* under salt stress. In the same sense, Badri et al. [22] reported that the relationships among the analyzed traits for populations of *M. truncatula* under drought stress were dependent on the treatment effect.

Cluster analysis based on morpho-physiological traits can facilitate the choice of candidate lines in future breeding programs. Farmers interested in high biomass production under control conditions can choose lines belonging to the first group, while lines from the third group can be used when the objective is to have a good yield under salt stress. Furthermore, the two lines TNC8.7 and TNC10.7 can be used if a higher production of biomass under both conditions is desired. However, the phenotype of these lines should also be tested in the field in order to validate the results obtained in the laboratory. A pair of contrasting lines, one of which would come from the third group and the other from the first or second group, can be used to analyze the genetic determinants of salt tolerance in *M. ciliaris*. Moreover, the genetic basis for salt tolerance could also be analyzed using the genome-wide association study (GWAS) approach and including the entire collection of the studied lines.

## 5. Conclusions

Our results show that the variations in the traits in the Tunisian natural populations of *M. ciliaris* are mainly explained by the treatment factor. Most measured traits can be used as good determinants for the selection of *M. ciliaris* lines. The majority of correlations among the measured traits under the control and salinity treatments were positive, of which the strongest correlations were registered among characteristics related to development. The two lines TNC8.7 and TNC10.7 were the most vigorous under both treatments. Phenotypic characterization of the lines should be conducted in the field to validate the phenotypic behavior observed under greenhouse conditions. Overall, the large variation found in *M. ciliaris* under salinity conditions could be useful for the identification of genetic determinants of tolerance to this constraint.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/agronomy12081781/s1>, Table S1: List of lines from the four populations of *M. ciliaris* used in the study.

**Author Contributions:** Conceptualization, M.A., A.M., C.A. and M.B.; data curation, M.A., N.B.C. and M.B.; writing—original draft preparation, M.A., A.M. and M.B.; writing—review and editing, M.A., A.M., N.L. and M.B.; supervision, C.A. and M.B.; project administration, M.B.; funding acquisition, N.L. and M.B. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was supported by the Tunisian–Algerian collaborative project (2013–2015), the Tunisian Ministry of Higher Education and Scientific Research (CBBC02 LR15), and the National Research Foundation of South Africa in the framework of the Tunisian–South African Joint Research Collaboration Program (2019–2023).

**Data Availability Statement:** The data are contained within the article and Supplementary Material.

**Acknowledgments:** We thank Marshall Keyster (UWC, South Africa) for his helpful comments on the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

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