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Full Length Research Paper

Variability salt stress response analysis of Tunisian natural populations of *Medicago truncatula* (Fabaceae) using salt response index (SRI) ratio

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We evaluated the responses to salt stress of 106 *Medicago truncatula* lines from 11 Tunisian natural populations collected from areas that varied in soil composition, salinity and water availability. Five references lines were also included in this study. Plants were cultivated in two treatments (0 and 50 mM of NaCl) during a period of 60 days. At harvest, we measured 14 quantitative traits of the aerial and root growth to identify genotypic variability in salt response. Analysis of variance showed that, the response to salt lines within populations was dependent on the effects of treatment, population, line within population and their interaction with maximum value recorded for treatment (93.78%). This study also analyzed heritability of the salt response index (SRI), defined as the ratio between the observed values with and without salt treatment. SRI of most measured traits had high broad-sense heritability (H^2). Most of established correlations among SRI values of measured traits were positive. SRI means revealed that, Soliman and Bulla Regia are the most salt-tolerant populations. Based on Ward's estimated distance, all lines were classified into 4 clusters showing similarity and dissimilarity in response to salt stress. The eco-geographical factors that influence more the variation of SRI of measured traits among populations are assimilated P_2O_5, % organic matter and carbon and mean annual rainfall. Findings from this study will provide the basis for identifying and breeding of salt-tolerant lines in *M. truncatula*.

Key words: Environmental factors, lines, *Medicago truncatula*, NaCl stress, populations, quantitative traits.

INTRODUCTION

The legume family (Leguminosae or Fabaceae) is the second most important food and forage source after grasses. Nearly 33% of all human nutritional requirements for nitrogen comes from legumes and in many developing countries, legumes serve as the single most important source of proteins (Roe and Kupfer, 2004). In the Mediterranean basin, native legumes play a central role in the enhancement of natural spaces since they can be fed to animals while simultaneously protecting the soil, stabilizing dunes and representing a source of medicinal products (Howieson et al., 2000). Most cultivated legumes have big genomes and they are allogamous, which makes their genetic analysis extremely complex. Thus, a model legume is needed to facilitate genetics, genomics and breeding for legume crops. Among the *Medicago* annual species, *Medicago truncatula* is widely used as a model plant for legume genetics and genomics (Barker et

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Abbreviations: SRI, Salt response index; **LO,** length of orthotropic axis; **LP,** length of plagiotropic axes; **LS,** length of stems; **NIN,** number of internodes; **NL,** number of leaves; **LA,** leaf area; **RWC,** root water content; **GCV,** genotypic coefficient of variation; **PCV,** phenotypic coefficient of variation; **RDW,** root dry weight; **AWC,** aerial water content.

al., 1990; Young and Udvardi, 2009). The close phylogenetic relationship of *M. truncatula* with crop legumes increases its value as a resource for understanding tolerance to abiotic stress. *M. truncatula* is an autogamous self-fertile plant with a small (500 to 550 Mb) diploid (2n=16) genome, a short life cycle and prolific seed production (Blondon et al., 1994). It is a forage legume commonly grown in Australia with more than 10 million hectares.

Among the abiotic stresses, salinity even caused by irrigation, is one of the major agricultural constraints affecting 20% of the world's irrigated cropland (Kim et al., 2008) and will soon become even more severe (Allakhverdiev, 2000). It is expected that >50% of all arable land will have salinity problems by the year 2050 (Vinocur and Altman, 2005). Solving the problem of salinity has a global importance. 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 the cultivated crops species (Dalton et al., 2001). 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 the salt stress. The impact of salinity on plant growth and development was correlated with different morphological and physiological attributes as biomass (Waheed et al., 2006) number of leaves (Mohammed et al., 1998), leaf area (Marcelis and van Hooijdonk, 1999), plant water relations (Soria and Cuartero, 1997), chlorophyll and carotenoids contents (Lycoskoufis et al., 2005). Morpho-agronomic characters reflect the combined genetic and environmental impacts on plants and parameters like survival under unfavorable conditions, plant height, leaf area, injury to salt stress, relative growth rate and relative growth reduction are considered as selection criteria for salt tolerance (Ashraf and Harris, 2004). Salt tolerant plants can reduce the detrimental effects of high salinities by producing a series of anatomical, morphological and physiological adaptations (Poljakoff-Mayber, 1988; Ashraf and Foolad, 2007); such as an extensive root system (Hameed and Ashraf, 2008) and reduction in growth in terms of leaf area (Monteverdi et al., 2008). Climate is a potent selective force in natural populations, yet the importance of adaptation in the response of plant species to past climate change has been questioned. As many species are unlikely to migrate fast enough to track the rapidly changing climate of the future, adaptation must play an increasingly important role in their response (Alistair and Penuela, 2005).

Salt tolerance is controlled by multiple genes and regulated by different types of proteins (Bohnert and Jensen, 1996; Ashraf, 2009). To improve the reliability and selection efficiency for salt tolerance, it is necessary to identify the salt-induced characteristic changes in multiple traits among different genotypes. Local populations comprise important genetic resources for *M. truncatula* breeding schemes and can be maintained as inbred lines. Inbred lines from different germplasm pools can be used as parents in ''wide crosses'' for the development of high yielding synthetic varieties (Link, 1990). Salt response index (SRI) is the ratio between the observed values with and without salt treatment; it is a measurement of change for plant traits caused by salt stress (Chen et al., 2007). The SRI value was considered as the indicator for salt tolerance.

In Tunisia, most salty soils are mainly located in the semi-arid and arid regions. In these regions, crops productivity was substantially impeded by salinity. This study aims to (1) Evaluate the variability of responses to salt stress within and between 11 Tunisian populations of *M. truncatula*, and (2) assess associations between these responses with site-of-origin environmental factors.

MATERIALS AND METHODS

Plant material and growing conditions

One hundred and six lines of *M. truncatula,* from 11 Tunisian populations, collected in different eco-geographical sites were used (Figure 1; Table 1) (Arraouadi et al., 2009; Lazrek et al., 2009). Selfing is known to reduce diversity within population and self-fertilization may also increase between-population differentiation due to reduced pollen dispersal. Thus, to minimize the chance of sampling the same individual more than once, the minimum distance between sampling individuals was 4 m. Lines were created by at least two generations of spontaneous selfing in the greenhouse. Five reference lines were also used in this study, including one line from Australian collection (Jemalong A17 (JA17)), one Moroccan line (A20) (Ané et al., 2002), 2 Algerian lines (DZA45.5 and DZA315.16) and one French line (F83005.5) from the Var region. Seeds were surface-sterilized and scarified with concentrated H_2SO_4 for 7 min and rinsed 10 times with sterile distilled water (Badri et al., 2007). The soaked seeds were sown in Petri dishes on agar 0.9% medium before being vernalized at 4°C for 96 h. Once the emerging root attained a length of 4 mm, seedlings were transferred to 33 cl pots (8 cm diameter and 10.5 cm deep) filled with sterilized sand which was previously washed using HCl 0.05%. Each plant was grown in an individual pot in greenhouse under controlled conditions: A temperature of 25°C, 80% of relative humidity and a photoperiod of 16/8 h (120.74 Lux during 5 h). The experimental design was completely randomized. During 60 days, plants were irrigated twice per week. For control treatment, we used a nutritive solution as described by Vadez et al. (1996) while the source of iron was modified by adding Fe-ethylenediaminetetraacetic acid (EDTA).

In the salt stress treatment, we added 50 mM of NaCl to the nutritive solution. The choice of 50 mM of NaCl to induce salt stress was based on a preliminary work using a range of salt concentrations (15, 45 and 75 mM of NaCl) and using 5 references lines (Arraouadi et al., unpublished results). 50 mM of NaCl causes 50% inhibition (I50) for most of measured traits for studied lines. Salt stress was applied at seedling stage directly after germination. For both treatments, the whole retention capacity was maintained by weighting pots and adding the nutritive solution to compensate the decrease in volume. To avoid NaCl accumulation problem in substrate, the pots were irrigated with distilled water twice per week. Five replicates for each line per treatment were used, giving a total of 1110 plants. For each individual plant we measured, at harvest, 14 quantitative traits related to shoot and root growth (Table 2). For weight biomass, plant organs were dried at 70°C for 48 h.

Table 1. Bioclimatic characteristic of the collection sites of Tunisian populations of *M. truncatula.*

Collection site	Code	E.C (mmho/cm)	Ann rain (mm)	Altitude (m)
Enfidha	TN1	0.6	350	2
Jelma	TN2	1.4	250	300
Amra	TN3	0.6	150	400
Deguache	TN4	15.6	50	25
Thala	TN6	2.7	350	800
El Kef	TN7	0.8	450	500
Soliman	TN8	1.4	600	2
Bulla Regia	TN9	0.8	600	200
Gabès	TN11	2.3	225	58
Djerba	TN12	0.6	150	18
Tataouine	TN14	0.5	138	137

E.C: Electro-conductivity; Ann rain: mean annual rainfall.

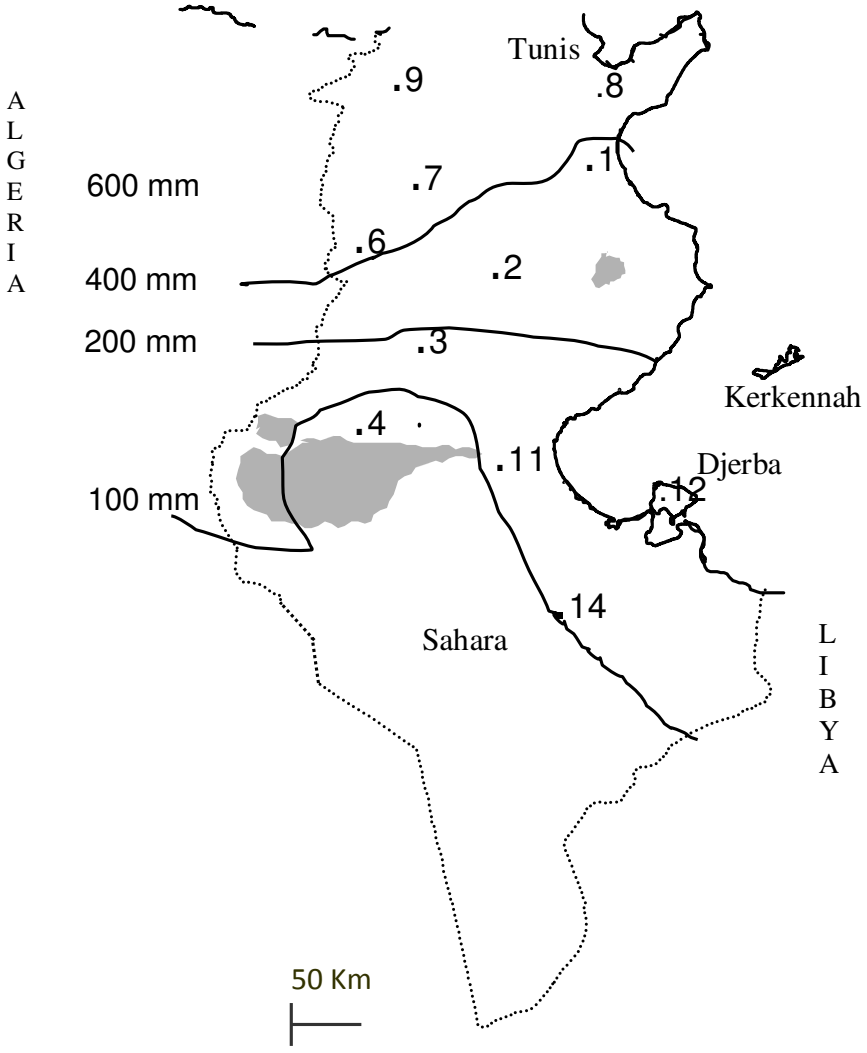

Figure 1. Geographic distribution of the 11 populations of *M. truncatula* collected in Tunisia. 1: Enfidha (TN1); 2: Jelma (TN2); 3: Amra (TN3); 4: Deguache (TN4); 6: Thala (TN6); 7: El Kef (TN7); 8: Soliman (TN8); 9: Bulla Regia (TN9); 11: Gabès (TN11); 12: Djerba (TN12); 14: Tataouine (TN14).

Table 2. List of measured quantitative traits and their abbreviations.

Trait	Abbreviation
Length of orthotropic axis (cm)	LO
Length of plagiotropic axes (cm)	LP
Total length of stems (cm)	LT = LO + LP
Length of roots (cm)	LR
Number of internodes	NIN
Number of leaves	NL
Leaf area (cm^2)	LA
Aerial fresh weight (g)	AFW
Aerial dry weight (g)	ADW
Aerial water content (%)	AWC = ((MFA-MSA)/MFA) x 100
Root fresh weight (g)	RFW
Root dry weight (g)	RDW
Root water content (%)	RWC = ((MFR-MSR)/MFR) x 100
Root dry weight and aerial dry weight ratio	RDW/ADW

Estimation of genetic parameters for SRI values

Presumed lines were created by 2 and 3 generations of spontaneous selfing in isolation in the greenhouse, even if we know that maternal effects could persist after 2 or 3 generations of selfing. Each population consisted of ten lines except for Jelma, Amra, Deguache and Djerba populations with only 9 lines. Offspring in each presumed line were considered genetically identical. Consequently, the within-line variance can be considered as environmental, while the among-lines variance component is assumed to be solely genetic (Bonnin et al., 1997). Analysis of variance of populations, lines within population, treatment, block and their interaction effects was conducted, using the Gene Stat software version 11.1.0.1504 (www. vsni.co.uk). The calculation procedure of genetic parameters is based on single-factors completely stochastic variance model and using the following formulas:

Genotypic coefficient of variation (GCV %) = genetic variance (δ^2_g) / total mean value ($\overline{X}$)

Phenotypic coefficient of variation (PCV %) = $\sqrt{\delta^2_p}/(\overline{X})$

Phenotypic variance (δ^2_p) = genetic variance (δ^2_g) + environmental variance (δ^2_e)

Broad-sense heritability (H^2) = δ^2_g/δ^2_p

For our design, using samples of lines from a selfing species, the genetic variance within populations, δ^2_g, is estimated by among line nested within population component of variance. However, this genetic variance includes both additive and non-additive components thus, leading to an overestimation of H^2 values.

Clustering analysis and correlations between SRI of measured traits

Correlations between SRI of measured traits for *M. truncatula* lines were estimated by computing the Pearson correlation coefficient (r). An agglomerative hierarchical clustering (AHC) was performed. To represent the relationships between responses to NaCl stress of studied lines, cluster analysis was performed to generate dendrograms. These dendrograms are based on the Ward's distance of dissimilarity. In short, this method attempts to minimize the sum of squares of any two (hypothetical) clusters that can be formed at each step (Ward, 1963). Clustering of each line was based on the sum of square variance of Ward's distance with the XLSTAT 1.02 software (www.xlstat.com).

Associations of SRI values with environmental factors

Eleven eco-geographical factors of sampling sites of natural populations of *M. truncatula* were examined: pH, saturation (ml/100 g), electro-conductivity (mmho/cm), percentage active calcareous, percentage organic matter, percentage carbon, assimilated P_2O_5 and K_2O, mean annual rainfall (mm) and altitude (m). As suggested by Badri et al. (2007; 2008a,b), to determine the influence of environmental factors on quantitative genetic variation among analyzed populations, correlations between SRI of measured traits and eco-geographical factors were computed using Pearson correlation coefficients (*r*). Significant level was set to 0.05 and adjusted for multiple comparisons by Bonferroni correction.

Statistical analyses

SRI value represents the relative change for each trait caused by salt treatment. It was calculated using the following formula (Chen et al., 2007):

SRI= (Value from salt treatment/ Value from control)*100%

RESULTS

Variance analysis showed that responses in salt were explained by the effects of treatment, population, lines within population and their interactions with maximum value recorded for treatment factor (Table 3).

Table 3. Proportions (%) and significance levels of population, treatment, line within population and population x treatment x line interaction effects on measured traits for studied populations of *M. truncatula.*

Trait	Effect	Population	Treatment	Pop x treat	Pop x line	Pop x treat x line
LO	F	214.88***	1502.71***	10.18 ***	16.41 ***	4.49 ***
	%	12.29	85.93	0.58	0.94	0.26
LP	F	29.12 ***	594.23 ***	30.74 ***	3.12 ***	2.35 ***
	%	4.42	90.09	4.66	0.47	0.36
LR	F	12.06 ***	6.17 **	8.73 ***	2.12 ***	1.28 *
	%	39.72	20.32	28.75	6.98	4.22
LS	F	35.33 ***	1089.18 ***	29.16 ***	4.92 ***	2.77 ***
	%	3.04	93.78	2.51	0.42	0.24
AFW	F	51.38 ***	1074.38 ***	33.47 ***	3.36 ***	2.58 ***
	%	4.41	92.21	2.87	0.29	0.22
RFW	F	2.82 **	9.01 **	1.47 ns	1.04 ns	1.02 ns
	%	18.36	58.66	9.57	6.77	6.64
ADW	F	56.62 ***	761.36 ***	28.36 ***	3.30 ***	2.22 ***
	%	6.65	89.38	3.33	0.39	0.26
RDW	F	25.70 ***	188.24 ***	5.64 ***	2.74 ***	1.73 ***
	%	11.47	84.02	2.52	1.22	0.77
RDW/ADW	F	5.00 ***	22.09 ***	3.09 ***	1.51 **	1.19 ns
	%	15.21	67.18	9.4	4.59	3.62
NIN	F	19.07 ***	525.79 ***	24.84 ***	3.38 ***	1.96 ***
	%	3.32	91.44	4.32	0.59	0.34
NL	F	38.68 ***	720.06 ***	27.62 ***	3.93 ***	2.39 ***
	%	4.88	90.84	3.48	0.5	0.3
LA	F	68.48 ***	397.77 ***	15.04 ***	5.45 ***	1.97 ***
	%	14.01	81.39	3.08	1.12	0.4
AWC	F	21.95 ***	32.04 ***	3.99 ***	2.72 ***	1.96 ***
	%	35.03	51.13	6.37	4.34	3.13
RWC	F	2.86 **	7.43 **	2.79 **	0.98 ns	0.91 ns
	%	19.1	49.63	18.64	6.55	6.08

Significance levels; *P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, ns no significant, F: coefficient of Snedecor-Fisher. Length of orthotropic axis (LO) (cm), length of plagiotropic axes (LP) (cm), length of roots (LR) (cm), length of total stems (LS) (cm), aerial fresh weight (AFW) (g), root fresh weight (RFW) (g), aerial dry weight (ADW) (g), root dry weight (RDW) (g), the RDW/ADW ratio, number of internodes (NIN), number of leaves (NL), leaf area (LA) (cm^2), aerial water content (AWC) (%) and root water content (RWC) (%).

Means comparison of SRI values

Lines from Soliman and Bulla Regia populations seemed to be the most tolerant showing the highest SRI values for shoot part; besides, a stimulation of growth in salt was observed for some lines. Moreover, Soliman and Bulla Regia populations exhibited the highest vigor for most measured traits (Table 4). For the rest of the studied populations, salt stress affects the length of orthotropic axis (LO), length of plagiotropic stems (LP), length of stems (LS), number of internodes (NIN) and the number of leaves (NL) with SRI values ranging from 5.98 to 78.9%. Indeed, SRI % values increase and varied between 60.39 and 85% for leaf area (LA). For AWC, SRI % values were more important, ranging from 96.41 to 99.93%.

As far as roots are concerned, SRI values of root length (LR) for all lines ranged from 92.81 to 114.79%. SRI of water content (RWC) values varied between 97.49 and 105.62%. Roots salt effect seems to be less important than shoot part. Indeed, length root stimulation was observed for Soliman and Bulla Regia populations. References lines of *M. truncatula* showed an intermediate behavior under salt stress regarding Tunisian lines. TN8.28 line from Soliman exhibited the largest SRI value, with the majority of measured traits varying between 79.1 and 288%. This line seems to be the most tolerant. Furthermore, TN3.20 from Amra population was the most susceptible line exhibiting the SRI values ranging from 5.7 to 137.2%.

Heredity parameters of SRI values

Table 4 summarizes the estimation of all the heredity

Table 4. SRI averages for individual trait of the 11 populations of *M. truncatula* and references lines.

Population	LO	LP	LS	NIN	AFW	ADW	NL	LA	LR	RFW	RDW	RDW/ADW	AWC	RWC
TN1	64.60^{abcd}	5.98^{b}	35.26^{fg}	43.2^{g}	37.89^{ef}	39.23^{efg}	53.48^{def}	84.15^{abc}	124.28^{a}	80.18^{bcd}	73.46^{bcd}	196.35^{bc}	99.40^{ab}	100.64^{abc}
TN2	42.61^{ef}	14.74^{b}	31.88^{fg}	54.39^{defg}	38.15^{ef}	44.84^{defg}	61.78^{bcdef}	84.73^{abc}	97.44^{c}	66.06^{de}	71.63^{bcd}	155.63^{bcde}	96.41^{b}	99.19^{bc}
TN3	43.56^{def}	15.09^{b}	28.21^{fg}	44.47^{g}	26.95^{f}	27.96^{g}	47.43^{f}	60.39^{d}	98.44^{bc}	48.88^{e}	50.82^{d}	181.80^{bcd}	99.57^{ab}	99.89^{abc}
TN4	53.65^{cde}	20.41^{b}	31.93^{fg}	53.73^{efg}	30.86^{f}	32.41^{fg}	49.74^{ef}	69.73^{cd}	88.90^{cd}	46.30^{e}	57.23^{cd}	166.23^{bcde}	99.87^{ab}	97.49^{bc}
TN6	31.74^{fg}	25.50^{b}	26.11^{g}	47.91^{fg}	37.23^{ef}	41.10^{defg}	58.14^{cdef}	74.65^{cd}	101.06^{bc}	61.22^{de}	55.27^{d}	130.20^{cde}	98.39^{ab}	101.22^{abc}
TN7	61.^{25abcde}	35.29^{b}	45.16^{ef}	65.06^{bcdefg}	50.83^{bcde}	53.56^{cdef}	67.98^{bcde}	85.74^{abc}	101.49^{bc}	90.13^{abcd}	78.60^{bcd}	148.97^{bcde}	98.61^{ab}	101.72^{abc}
TN8	67.36^{abc}	421.24^{a}	83.36^{ab}	109.79^{a}	93.79^{a}	96.62^{a}	109.73^{a}	95.78^{ab}	85.92^{cd}	111.89^{a}	102.48^{bc}	111.17^{de}	99.49^{ab}	101.22^{abc}
TN9	81.77^{a}	215.85^{ab}	84.52^{a}	111.14^{a}	91.58^{a}	89.72^{ab}	110.03^{a}	98.18^{a}	85.83^{cd}	102.76^{ab}	78.21^{bcd}	88.99^{e}	100.27^{a}	103.43^{ab}
TN11	55.60^{cde}	15.21^{b}	45.15^{ef}	60.20^{cdefg}	43.46^{cdef}	48.18^{cdefg}	57.27^{cdef}	75.05^{cd}	114.79^{ab}	67.02^{cde}	49.60^{d}	125.34^{cde}	99.93^{ab}	105.62^{a}
TN12	47.14^{cdef}	61.31^{b}	53.92^{de}	78.90^{bc}	56.18^{bcd}	61.58^{cd}	68.59^{bcde}	79.95^{bc}	103.70^{bc}	87.60^{abcd}	81.05^{bcd}	129.92^{cde}	98.66^{ab}	102.95^{abc}
TN14	66.06^{abcd}	68.18^{b}	66.49^{bcd}	77.81^{bc}	60.93^{bc}	69.31^{bc}	71.08^{bcde}	70.44^{cd}	92.81^{cd}	74.44^{bcde}	74.52^{bcd}	107.01^{de}	97.67^{ab}	100.38^{abc}
JA17	56.31^{bcde}	72.08^{b}	62.26^{cde}	85.32^{b}	67.06^{b}	70.19^{bc}	76.76^{bc}	74.35^{cd}	90.45^{cd}	89.43^{abcd}	101.96^{bc}	145.23^{bcde}	98.33^{ab}	98.76^{bc}
DZA45.5	63.46^{abcd}	38.24^{b}	58.81^{cde}	67.09^{bcdef}	51.97^{bcde}	52.15^{cdef}	60.48^{bcdef}	84.90^{abc}	74.30^{d}	97.23^{ab}	77.11^{bcd}	149.58^{bcde}	99.63^{ab}	102.97^{abc}
DZA315.16	31.63^{fg}	33.33^{b}	32.41^{fg}	51.15^{fg}	41.61^{def}	39.04^{efg}	57.92^{cdef}	73.99^{cd}	85.07^{cd}	63.71^{de}	84.79^{bcd}	213.14^{b}	101.30^{a}	96.75^{c}
F83005.5	19.58^{g}	150.00^{b}	36.65^{fg}	75.64^{bcde}	62.60^{b}	61.45^{cde}	72.50	62.19^{d}	90.70^{cd}	95.33^{abc}	242.33^{a}	374.69^{a}	100.30^{a}	88.70^{d}
A20	78.45^{ab}	28.92^{b}	71.12^{abc}	77.94^{bcd}	57.15^{bcd}	56.74^{cde}	78.45^{b}	72.90^{cd}	88.41^{cd}	83.45^{abcd}	111.56^{b}	217.84^{b}	99.28^{ab}	96.94^{c}

SRI means followed by the same letters are not significantly different between studied populations of *M. truncatula* at *P=0.05* based on Duncan's multiple-range test.1, Enfidha (TN1); 2, Jelma (TN2); 3, Amra (TN3); 4, Deguache (TN4); 6, Thala (TN6); 7, El Kef (TN7); 8, Soliman (TN8); 9, Bulla Regia (TN9); 11, Gabès (TN11); 12, Djerba (TN12); 14, Tataouine (TN14); length of orthotropic axis (LO) (cm); length of plagiotropic axes (LP) (cm); length of roots (LR) (cm); length of total stems (LS) (cm); aerial fresh weight (AFW) (g); root fresh weight (RFW) (g); aerial dry weight (ADW) (g); root dry weight (RDW) (g); the RDW/ADW ratio; number of internodes (NIN); number of leaves (NL); leaf area (LA) (cm^2); aerial water content (AWC) (%); root water content (RWC) (%).

parameters for SRI of investigated traits. The genotypic coefficient of variation (GCV) of SRI was the highest for length of plagiotropic axes (LP). The lowest ones were observed for SRI of root (RWC), shoot water content (AWC), leaf area (LA) and root length (LR). For other traits, GVC% was ranged from 9.03 to 16.07%. Phenotypic coefficient of variation (PCV) of SRI of measured traits ranged from 7.03 to 15.07%. SRI of most measured traits had high broad heritability, indicating that these traits have high genetic stability and are not much affected by environmental factors. Furthermore, the lowest broad-sense heritability for SRI values was observed for RDW, root/aerial dry weight ratio and RWC (Table 5).

Correlation among SRI of measured traits

Among the 91 possible correlations between measured traits, 55 were significant and 43, positive (Table 6). Negative correlation was observed for length of roots (LR) and root/aerial dry weight ratio (RDW/ADW). Root water content (RWC) is negatively correlated with root dry weight (RDW) and root/aerial dry weight ratio (RDW/ADW), while no significant association was noticed for these traits with aerial water content (AWC) (Table 6).

Clustering analysis based on the SRI values

All studied lines were classified into 4 clusters on the basis of Ward's distance range and each cluster was further divided into 2 sub-clusters (Table 7; Figure 2). The first cluster is formed by 37 lines from different localities, only one line TN8.3, TN9.24

Table 5. Estimation of heredity parameters for SRI values of measured traits for *M. truncatula* in 50 mM NaCl.

Trait	Ve	Vg	H^2	GCV (%)	PCV (%)
LO	75.53	503.90	0.87	9.03	8.01
LP	1688.75	18780.49	0.92	231.93	9.39
LS	7.56	597.41	0.99	12.23	7.03
NIN	53.49	750.91	0.93	10.94	8.57
AFW	4.47	651.09	0.99	12.43	7.26
ADW	16.59	766.84	0.98	13.81	7.53
NL	23.89	660.50	0.97	9.58	8.45
LA	22.11	213.25	0.91	2.67	9.38
LR	0.44	239.91	1.00	2.43	9.94
RFW	109.70	719.83	0.87	9.36	9.41
RDW	406.63	669.62	0.62	9.18	10.83
RDW/ADW	1353.13	2296.28	0.63	16.07	15.07
AWC	1.32	5.97	0.82	0.06	11.00
RWC	7.50	16.84	0.69	0.17	12.08

Length of orthotropic axis (LO) (cm); length of plagiotropic axes (LP) (cm); length of roots (LR) (cm); length of total stems (LS) (cm); aerial fresh weight (AFW) (g); root fresh weight (RFW) (g); aerial dry weight (ADW) (g); root dry weight (RDW) (g); the RDW/ADW ratio; number of internodes (NIN); number of leaves (NL); leaf area (LA) (cm^2); aerial water content (AWC) (%); root water content (RWC) (%).

Table 6. Correlation between SRI values of measured traits for *M. truncatula* 60 days seedlings in 50 mM NaCl.

Trait	LO	LP	LS	NIN	AFW	ADW	NL	LA	LR	RFW	RDW	RDW/ADW	AWC	RWC
LO	**1**													
LP	0.232	**1**												
LS	**0.698**	**0.559**	**1**											
NIN	**0.425**	**0.493**	**0.875**	**1**										
AFW	**0.532**	**0.581**	**0.934**	**0.920**	**1**									
ADW	**0.536**	**0.503**	**0.904**	**0.883**	**0.956**	**1**								
NL	**0.427**	**0.572**	**0.852**	**0.920**	**0.956**	**0.904**	**1**							
LA	**0.399**	0.311	**0.516**	**0.487**	**0.581**	**0.565**	**0.586**	**1**						
LR	-0.086	-0.243	**-0.363**	**-0.415**	**-0.403**	**-0.424**	**-0.393**	-0.087	**1**					
RFW	**0.429**	**0.378**	**0.680**	**0.694**	**0.737**	**0.730**	**0.733**	**0.624**	-0.124	**1**				
RDW	0.198	0.262	**0.455**	**0.543**	**0.545**	**0.523**	**0.552**	**0.399**	-0.166	**0.719**	**1**			
RDW/ADW	-0.306	-0.235	**-0.476**	**-0.397**	**-0.455**	**-0.501**	**-0.399**	-0.216	**0.326**	-0.044	**0.358**	**1**		
AWC	-0.105	0.121	-0.037	0.005	0.016	-0.228	0.044	-0.080	0.149	-0.111	-0.063	0.221	**1**	
RWC	0.059	0.044	0.065	-0.008	0.050	0.054	0.024	0.121	0.071	0.126	**-0.353**	**-0.376**	0.004	**1**

Values in bold are significantly different from 0 with a significance level alpha = 0.001; length of orthotropic axis (LO) (cm); length of plagiotropic axes (LP) (cm); length of roots (LR) (cm); length of total stems (LS) (cm); aerial fresh weight (AFW) (g); root fresh weight (RFW) (g); aerial dry weight (ADW) (g); root dry weight (RDW) (g); the RDW/ADW ratio, number of internodes (NIN); number of leaves (NL); leaf area (LA) (cm2); aerial water content (AWC) (%); root water content (RWC) (%).

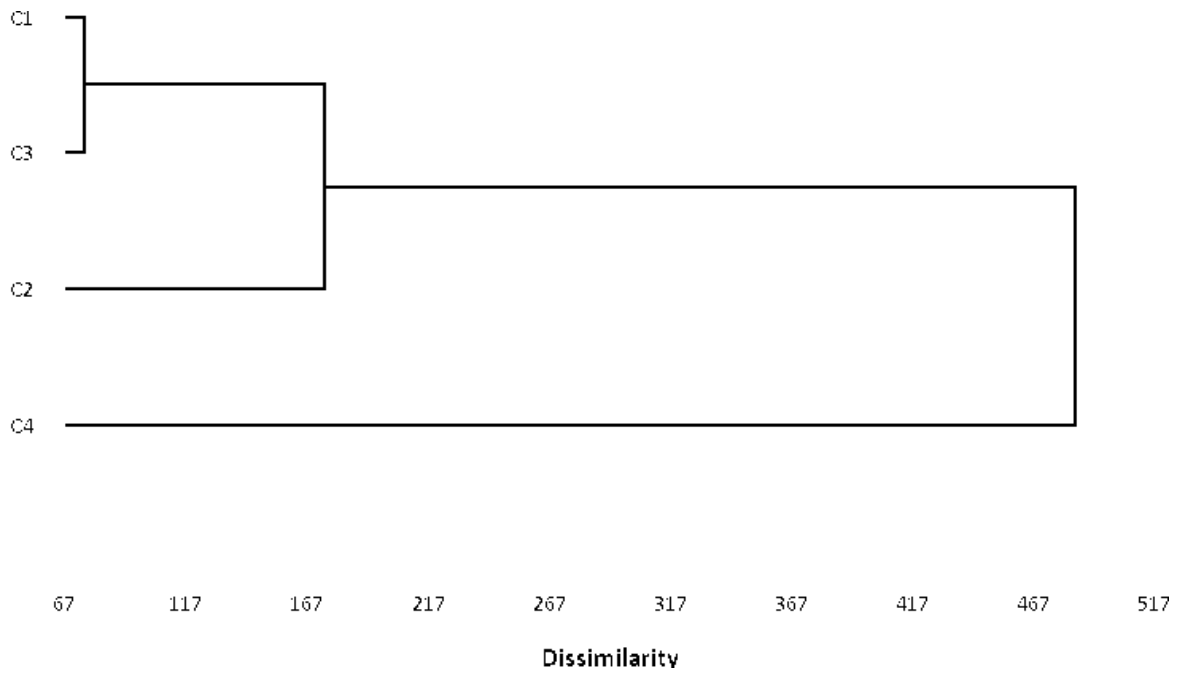

Figure 2. Dendrogram of the 113 lines of *M. truncatula* clustered using the sum of variance squares of Ward's distances. Lines were grouped into 4 clusters with XLSTAT software.

and TN12.4 from respectively Soliman, Bulla Regia and Djerba were grouped in this cluster. Lines formed this first cluster were more attached with the third one at a Ward's distance of 73.801 (Table 7) which is composed of 24 lines, when the majority was coming from Djerba and Tataouine. The second one is formed by 30 lines from Enfidha, Jelma, Amra, Deguache, Thala, Gabès and Algerian reference line (DZA315.16). As observed with SRI values, Soliman and Bulla Regia lines take specificity and they are grouped on the fourth group with only one line TN11.2 and TN14.10 from respectively, Gabès and Tataouine. Clustering of lines gives information on their similarity and dissimilarity of responses in salt stress (Table 7) which facilitate the choice of lines to be involved in future breeding programs.

Associations of SRI values with environmental factors

For a set of 140 possible correlations between SRI values of measured traits and environmental factors, 47 are significant ($P < 0.05$) and 45 of them, positive (Table 8). The environmental factors that influence more the SRI values of measured traits among populations of *M. truncatula* is assimilated P_2O_5 (20.83% of significant correlations), followed by % organic matter, % carbon and means annual rainfall (16.66%), saturation (14.58%), assimilated K_2O_5 (10.41%) and electro-conductivity and altitude (2.08%).

DISCUSSION

The relative change for each investigated trait caused by salt stress is presented by SRI estimation. There were differences between genotypes, but treatment effect was the most important reference explaining this variability. Two kinds of explanations can be proposed for correlation between SRI of measured traits. Most established correlations between above-ground traits were significant and positive; indicating that, response to salt stress of lines follows the same general pattern. However, traits related to plant root growth are negatively correlated. Further-more, these correlations may also be associated with the developmental stages of the lines when the samples were collected. For some lines, plants performed better in 50 mM NaCl than in non-saline conditions.

This significant variation in the capacity of salt tolerance explained by SRI between studied lines, coming from different geographical sites, suggests that a rich genetic resource for salt tolerance exists in different genotypes and it is feasible to select salt tolerant lines in *M. truncatula*. Furthermore, breeding scheme should be based on heritability of traits. High levels of genotypic variance and heritability were found for SRI of LO, LP, LS, NIN, AFW, ADW, NL, RFW, RDW and RDW/ADW. It will be relatively easier to select for these traits and pass them onto offspring. In contrast, leaf area, roots length and aerial water content had SRI values of high heritability but low genotypic variance; it will be very difficult to

Table 7. *M. truncatula* lines composition of each cluster.

Class	1	2	3	4
Objects	37	30	24	20
Sum of weights	37	30	24	20
Within-class variance	5827.417	4355.540	8823.397	144714.168
Minimum distance to centroid	29.377	26.042	12.389	48.694
Average distance to centroid	69.617	60.650	77.687	256.849
Maximum distance to centroid	142.316	104.834	285.502	1335.887
Lines composed each group	TN1.3	TN1.11	TN1.17	TN8.4
	TN1.16	TN1.1	TN2.1	TN8.5
	TN1.13	TN1.5	TN2.15	TN8.15
	TN1.19	TN1.15	TN4.22	TN8.21
	TN1.18	TN2.14	TN6.15	TN8.22
	TN2.4	TN2.12	TN7.2	TN8.23
	TN2.18	TN2.21	TN7.4	TN8.24
	TN2.13	TN3.16	TN12.10	TN8.25
	TN2.17	TN3.18	TN12.11	TN8.28
	TN3.3	TN3.19	TN12.9	TN9.17
	TN3.23	TN3.20	TN12.8	TN9.21
	TN3.17	TN3.22	TN12.7	TN9.3
	TN4.20	TN3.21	TN12.6	TN9.5
	TN4.21	TN4.13	TN12.2	TN9.4
	TN6.5	TN4.14	TN12.12	TN9.12
	TN6.16	TN4.16	TN14.12	TN9.15
	TN6.23	TN4.17	TN14.9	TN9.19
	TN6.2	TN4.18	TN14.8	TN9.20
	TN7.5	TN4.12	TN14.7	TN11.2
	TN7.11	TN6.1	TN14.6	TN14.10
	TN7.17	TN6.14	TN14.2	
	TN7.18	TN6.18	JA17	
	TN7.19	TN6.22	F83005.5	
	TN7.20	TN6.3	A20	
	TN7.22	TN11.10		
	TN7.23	TN11.9		
	TN8.3	TN11.8		
	TN9.24	TN11.5		
	TN11.11	TN11.7		
	TN11.4	DZA315.16		
	TN11.1			
	TN11.12			
	TN12.4			
	TN14.11			
	TN14.3			
	TN14.1			
	DZA45.5			

select a line with desirable traits. However, once the desirable traits are obtained, they can be easily stabilized.

Cluster analysis based on salt stress response was performed by Ward's method. This method uses an analysis of variance approach to evaluate the distances between clusters. In general, this method is regarded as very efficient; however, it tends to create clusters of small size. A net discrimination of Soliman (TN8) and Bulla Regia (TN9) populations was shown for their salt stress

Table 8. Estimated correlations between SRI values of measured traits for *M. truncatula* populations and eco-geographical factors.

Eco-geographical factor	pH	Sat	E.C	Calc	O.M (%)	Carbon	Assi P2O5	Assi K2O5	Ann rain	Altitude
LO	0.12	0.15	-0.06	0.00	0.12	0.13	0.37*	0.25	0.30	-0.31
LP	0.12	0.34*	-0.10	0.21	0.43*	0.43*	0.42*	0.26	0.41*	-0.17
LS	0.19	0.31	-0.23	0.04	0.39*	0.40*	0.58*	0.39*	0.48*	-0.35*
NIN	0.21	0.39*	-0.17	0.06	0.48*	0.49*	0.63*	0.41*	0.50*	-0.28
AFW	0.19	0.46*	-0.26	0.18	0.53*	0.54*	0.64*	0.39*	0.60*	-0.25
ADW	0.20	0.43*	-0.26	0.12	0.47*	0.48*	0.54*	0.31	0.53*	-0.24
NL	0.16	0.52*	-0.23	0.24	0.59*	0.60*	0.67*	0.43*	0.63*	-0.18
LA	0.21	0.51*	-0.17	0.20	0.41*	0.41*	0.47*	0.18	0.57*	-0.15
LR	0.01	-0.30	-0.19	0.03	-0.32	-0.33	-0.32	-0.28	-0.11	-0.05
RFW	0.31	0.37*	-0.34*	0.20	0.35*	0.36*	0.44*	0.19	0.55*	-0.19
RDW	0.31	0.26	-0.17	0.08	0.20	0.20	0.22	0.04	0.32	-0.22
RDW/ADW	0.12	-0.24	0.12	0.02	-0.31	-0.32	-0.35*	-0.21	-0.24	0.00
AWC	-0.07	-0.07	0.10	0.11	0.09	0.09	0.19	0.17	0.09	-0.13
RWC	0.01	-0.03	-0.24	0.04	0.08	0.08	0.15	0.09	0.17	-0.02

*Significant correlation after using Bonferroni correction at α = (0.05/140 = 0.000357). pH, saturation (ml/100g) (Sat); electro-conductivity (mmho/cm) (E.C); % active calcareous (Calc), % organic matter (O.M), % carbon (Carbon), assimilated P_2O_5 (Assi P_2O_5), (Assi K_2O_5), K_2O_5 assimilated (Assi K_2O_5), mean annual rainfall (mm) (Ann rain); altitude (m). Length of orthotropic axis (LO) (cm); length of plagiotropic axes (LP) (cm); length of roots (LR) (cm); length of total stems (LS) (cm); aerial fresh weight (AFW) (g); root fresh weight (RFW) (g); aerial dry weight (ADW) (g); root dry weight (RDW) (g); the RDW/ADW ratio, number of internodes (NIN); number of leaves (NL); leaf area (LA) (cm^2); aerial water content (AWC) (%); root water content (RWC) (%).

tolerance in this study. Lines from these populations were clustered separately in the fourth group, indicating that they display a homogenous phenotypic variance between them and different from the others. This design illustrated in Table 7 of studied lines could be a useful database for breeding programs.

In breeding process, it would be possible to improve aerial measured traits. However, many traits were positively correlated and together; these traits may act in concert to result in salt tolerance. Based on the hereditary characteristics of the investigated traits, we suggest that breeding for salt tolerant lines should be easier to speedup stability of the important traits. Our finding is in contrast with Chen et al. (2007), who studied salt stress response in Asparagus bean. This difference in results may be because we did not take into account photosynthetic and physiological traits associated with salt stress.

Salt tolerance is regulated by multigenes and proteins (Bohnert and Jensen, 1996). Breeding for salt tolerance needs reliable indicators or selection markers (Shannon, 1985; Noble and Rogers, 1992). The correlation analysis performed in this study indicates that, different salt tolerance traits are inter-correlated; it is thus, more appropriate to evaluate a breeding strategy comprehendsively based on multiple traits. The selection of traits such as LP is easy to observe and measure; the results should have very important values in practical breeding for salt tolerance. The fresh weight also had high genotypic variance and broad heredity in all lines. Together with the results from Greenway and Munns (1980), Murillo-Amador et al. (2001, 2006) and Chen et al. (2007), plant fresh weight and production of biomass have shown to be significantly correlated with salt tolerance characteristics and thus, should have more consideration in breeding process.

Correlations between SRI values of measured traits and environmental factors, in this study, suggest that these particular characters are adapting in response to the regional differences detected in eco-geographical factors. High influence on SRI values of quantitative traits was generally observed for soil mineral composition. Large effects were also observed for saturation and mean annual rainfall. The high effect of soil composition parameters on SRI values suggests that the response in salt stress traduces damage in plant growth according to ions propriety of soils. Indeed, salinity is a soil condition characterized by a high concentration of soluble salts (Munns and Tester, 2008). Because NaCl is the most soluble and widespread salt, it is not surprising that all plant have evolved mechanisms to regulate its accumulation and to select against it in favor of other nutrients commonly present in low concentration, such as K^+ and NO_3^-. Accordingly, significant micro-geographical genetic differentiation in response to climate (solar radiation, temperature and aridity stress) was found in populations of *Triticum dicoccoides* (wild emmer wheat) (Li et al., 2001) and *Hordeum spontaneum* (wild barley) (Huang et al., 2002). This micro-geographical adaptive differentiation with respect to climate was also reported for tree species such as *Pinus edulis* (pinon pine) (Mitton and Duran, 2004).

Overall, we found that the variation of salt stress responses among lines was essentially dependent on the

treatment effect. SRI of most measured traits had high broad-sense heritability indicating that these characters can be used as good descriptors in the genetic determinism of the tolerance to salt stress in *M. truncatula*. Most established correlations between SRI of measured traits were positive suggesting that lines behaviors in salt stress follow the same general pattern. On the other hand, the classification of lines into 4 clusters based on their responses in salt will facilitate the choice of candidate lines for any future breeding program. Established correlations between SRI of scored traits and environmental factors suggest that, lines response in salt was dependent in part on local adaptation of these lines in their original sites. Further study is needed to analyze the genetic basis of the tolerance to salt stress in *M. truncatula* using an association genetics approach. Association mapping is receiving increasing attention as a method complementary to traditional bi-parental mapping to associate the genotype to phenotype by exploiting the genetic variation present in germplasm collections. To date, association studies in plants have mainly been performed in species for which extensive sequence data is available. *M. truncatula* is the well example for these future studies because of its genome sequencing (http://www.medicago.org); in addition, the total genomes of 384 lines of this species from worldwide including the Tunisian lines involved in this study, will be sequenced (http://www.hapmap.org).

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