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Molecular morphogical and physiological study in Tunisian wild wheat relative *Aegilops geniculata* Roth and wheat (*Triticum durum* Desf.) under salt stress

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Abstract

In this investigation, an attempt was made to assess the genetic diversity among thirteen *Aegilops geniculata* Roth populations and three durum wheat varieties originated from different bioclimatic areas using 19 RAPD markers. For morpho-physiological traits we have selected three populations of *Aegilops geniculata* and one variety of durum wheat from each bioclimatic area. This study has shown a high degree of variation of these characters mainly related to geographical origin. It was observed also that the Sbeitla population was less affected by the imposed salt stress than all the others while Ain zana was the most affected one. The CO_2 assimilation rate, stomatal conductance, and intercellular CO_2 for the three *Ae. geniculata* populations and wheat variety significantly decreased with increasing salt. Among the factors inhibiting photosynthetic activity, those of a stomatal nature had a greater effect. The molecular study indicated an important inter-specific polymorphism between *Aegilops* and durum wheat. *Ae. geniculata* populations revealed a high level of polymorphism (71.27%) than wheat cultivars (39.76%). Two main groups were represented by cluster analyses. The first group is formed by *Ae. geniculata* populations and the second is constituted only by durum wheat cultivars.

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Introduction

salinity 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. Morpho-agronomic characters reflect the combined genetic and environmental impacts on plants like survival under unfavorable conditions. Salt tolerant plants can reduce the detrimental effects of high salinities by producing a series of anatomical, morphological and physiological adaptations (Ashraf and Foolad, 2007).

The wild species of *Triticeae* family, especially the genus *Aegilops* L. are valuable sources of genetic variation for wheat improvement since they possess the genetic background of all the cultivated wheat having still unidentified important characters such as resistance to different biotic and abiotic stress, (Farooq *et al.,* 1996; Zaharieva *et al.,* 2004). Moreover, it is one of the widespread species of the genus (Van Slageren, 1994) showing adaptations to a large range of environmental constraints. Three annual species of *Aegilops* were reported in Tunisia (Cuénod *et al.,* 1954): *Ae. geniculata* Roth*, Ae. triuncialis* L and *Ae. ventricosa* Taush. *Aegilops geniculata* Roth. (= *Ae. ovata* L.) is an annual, selfing allo-tetraploid species (2n = 4x = 28) with MU genome (Van Slageren, 1994). This species grows in Mediterranean regions characterized by a dry summer season with high temperature and high irradiance. As with other wild species, it can acclimate to these constraints by escape, avoidance and tolerance (Colmer *et al.,* 2006). *Aegilops geniculata* Roth. should be also considered an important source of variability for breeding genetic bases of cultivated crops (Nevo *et al.*,2002). Moreover, molecular markers have been especially used for studying the genetic diversity among a number of species of the tribe *Triticeae* (Charmers *et al.*, 2001).

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. For this reason, we initiated a research program to assess, conserve, update the genetic diversity of Tunisian species (*Aegilops geniculata* Roth. and *Triticum durum* Desf.) and evaluate morpho-physiological factors associated with sensitivity to salinity. The objective of the present study is, to evaluate the growth of the two species (Morphological and Yield-related traits) under experimental salinity concentrations from 0 to 200 mM NaCl, gas exchange of leaves, select tolerant population to saltiness, assess the genetic diversity of the two species and estimate their relationships and similarities in order to evaluate the potential of molecular markers in breeding programs as well as in germplasm conservation.

Material and methods

Plant material and collection areas

Thirteen *Aegilops geniculata* populations and three durum wheat varieties (*Triticum durum* Desf.) were used in this study. These populations were collected in various bioclimatic and ecological conditions of northern and central Tunisia (Table 1). The collecting sites extended from Kroumiry mountains and the coastal region to the Dorsal areas, including the Cap-Bon, Mogodses, Zaghouan and the steppic highlands, along a gradient of increasing aridity from North to South. Each sampling site was characterized by the main ecological factors of Mediterranean climate using the bioclimatic coefficient (Q_2). The average annual rainfall (P) and the average temperatures of the hottest month in summer (M) and in particular those of the coldest month in winter (m) correlated

with altitude were indicating continentality and bioclimatic variants.

Table 1. Characteristics climatic of the origin sites of *Ae. geniculata* Roth populations in Tunisia.

Code Pop	Site	Province	Alt. (m)	P (mm)	m	M	Q_2	Bioclimate
T	Tabarka	Littoral N/W	20	1032	7.2	31.5	148.1	Worm H
N	Nefza	Littoral N/W	10	960	7.5	32	131.2	Worm H
Z	Aine Zana	Kroumery	641	830	1.9	31.8	95.7	Fresh SH
M	Mekna	Kroumery	630	780	2.6	32.5	91.8	Fersh SH
B	Bizerte	N/E	300	653	7.2	30.9	94.3	Worm SH
G	Goussa	N/W	100	663	6.2	35.6	74.3	Mild SH
Abd	Djebel Abderrahmen	Dorsal	637	520	4.9	30.3	69.1	Mild SH
R	Djebel Ressas	N/E	795	453	5.4	33.1	55.3	Temperate Upper SA
Zg	Zaghouan	N/E	175	518	6.6	32.9	67.3	Worm Upper SA
O	Djebel Oust	Dorsal	400	435	5.9	35.6	49.7	Worm Upper SA
J	Souk jemaa	Dorsal	900	503	4.8	32.6	63.1	Temperate Upper SA
S	Djebel Serj	Dorsal	1357	400	3.3	33.7	44.8	Temperate Lower SA
Sb	Sbeitla	Center	670	328	4.5	34.4	37.7	Temperate Upper A
K	Karim	Béja						
Ch	Chili	Béja						
Mah	Mahmoudi	Béja						

Pop population, N/W north-west, N/E north east, Alt altitude, *P* annual rainfall, *M* and *m* are the of the average maximum temperature of the hottest month and the average of the minimum of the coldest month, respectively, Q_2 Emberger coefficient, H humid, SH subhumid, SA semiarid, A arid.

Genotyping

Total DNA was extracted from young leaves of *Ae. geniculata* populations and durum wheat varieties (*Triticum durum* Desf.) by the cetylmethylam-moniumm bromide (CTAB) method with minor modification Murray and Thompson (1980). After purification, the DNA concentration was spectrophotometrically estimated. DNA integrity was assessed by 0.8% agarose gel electrophoresis (Sambrook *et al.,* 1989). The extracted DNA was diluted to 20 ng/µl and used for PCR amplifications. Nineteen primers purchased from Operon Technologies inc. (Alameda, USA) were used for the amplification of random DNA sequences in this study (Table 2).

Table 2. Comparison of the averages of the 11 characters studied (Morphological, and yield-related traits) measured on three *Ae. geniculata* Roth populations "Ain zana", "Zaghouan", "Sbeitla" and one wheat variety 'Chili' (of Duncan Test) grown under salt stress (0, 50, 100, 150, and 200 mM) conditions at Ariana, Tunisia.

Characters	Abbrev.	Ae. Az	Ae. Zg	Ae. Sb	W
Morphological traits					
Plant height (cm)	PH	27.65^{c}	33.45^{b}	35.81^{b}	80.90^{a}
Leaves length (cm)	LL	5.48^{c}	6.88^{b}	7.11^{b}	23.75^{a}
Spikes length (cm)	SL	2.57^{c}	2.96^{b}	3.16^{b}	6.58^{a}
Awns number	AN	10.90^{b}	10.69^{b}	11.34^{b}	45.00^{a}
Yield-related traits					
Tillers number	NT	35.41^{b}	41.53^{ab}	45.15^{a}	3.04^{c}
Leaves number	NL	5.69^{c}	6.35^{b}	6.69^{b}	7.88^{a}
Spikes numbers	SN	26.60^{b}	29.99^{ab}	33.54^{a}	2.61^{c}
Spiklets number	NS	3.86^{b}	3.84^{b}	4.03^{b}	16.15^{a}
Plant weight (g)	PW	15.22^{b}	18.81^{a}	19.58^{a}	15.81^{b}
Grain yield per plant (g)	GYP	1.51^{c}	2.21^{b}	$2.06b^{c}$	2.93^{a}
100 grains weight (g)	100GW	0.92^{b}	0.94^{b}	1.15^{b}	2.87^{b}

For each column, values with the same letter indicate no-significant differences at 5%

Az: Ae. population "Ain zana"; Zg: Ae.population "Zaghouan"; Sb: Ae.population "Sbeitla"; W: Wheat variety 'Chili'.

PCR reactions were performed, in a 25 µl volume reaction mixture containing between 20-40 ng of total cellular DNA, 5 µl of 5x Taq DNA polymerase buffer, 0.5 µl dNTP (200 µM), 0.2 µTaq DNA polymerase (5U/µl) et 1 µL de $MgCl_2$ (2.5 mM), 25 µM of primer. The reaction mix was overlaid with a drop

of mineral water. PCRs were performed using a Biometra UNO II thermal-cycler and involved an initial denaturation step (94 °C, 5 min), 40 amplification cycles (each 94 °C, 30 s; 38 °C, 1 min and 72 °C, 1 min) and a final extension step (72 °C, 10min). The PCR products were separated on 2% agarose gels using 1x Tris-Acetate-EDTA buffer (TAE) and visualized under UV light after ethidium bromide staining (Sambrook *et al.,* 1989). Molecular sizes of the amplification products were estimated using a 1kb DNA ladder (from Promega).

Morpho-physiological traits

Physiological factors associated with sensitivity to salinity were evaluated of three *Ae. geniculata* populations (Ain Zana, Zaghouan and Sbeitla) and one variety of *Triticum durum* (Chili) selected from each bioclimatic area

Growing conditions

Experiments were carried in a greenhouse at National Institute of Agronomic Research of Tunisia (INRAT) in Ariana. The seedling was achieved in pots, of 28 cm of diameter and 25 cm of height. No pesticides were applied and weeds were manually eliminated. The pots were filled by the substratum of culture formed by a mixture 50 % loam (organic matter content 2 %, pH 7.8) and 50 % sand. Each pot contained 10 kg of soil a layer of 5 cm of gravel was placed in the bottom of every pot to guarantee a good drainage. After replenishment of the pots, 10 seeds per pot were sowed in a homogeneous way to a depth of 2–3 cm, and after emergence (7 days), the seedlings were thinned to six plants per pot. Salt treatment started when the seedling had approximately three to four leaves (4 weeks after seedling). Sodium chloride was added in four concentrations of 0, 50, 100, 150, and 200 mM. To avoid osmotic shock, saline treatment was progressively imposed; increasing the concentration, by 50 mM, every second irrigation until the final concentration (200 mM NaCl) was reached. Prior salt treatment, nutrient solution was supplied instead of water. The nutrient solution was prepared according to Maas *et al* (1986). Pots were irrigated at each stage of plant development (tillering, shooting, heading, flowering and grain filling (milk and dough stage) with nutrient solution. The experiment was set up as completely randomized design of five salt levels, four populations (three *Aegilops geniculata* populations and one durum wheat variety) and four replicates. Each pot (six seedlings) was considered as one replicate. Each treatment contained 24 seedlings per accession or variety and the total number of plant was evaluated to 480.

Gas exchange

Gas exchange parameters were measured on the flag of the tagged tillers using an open portable system ADC model LCA-4 infrared gas analyser (Analytical Development Co., Hoddesdon, UK), leaf temperature 32 ± 2 C°, relative humidity was from 40 to 50 %, and ambient CO_2 (Ca) concentration 365 ± 5 $cm^3\ m^{-3}$. CO_2 assimilation rate (P_N), stomatal resistance (r_s) and intercellular CO_2 (C_i) were automatically recorded by the machine (about 2 min) and three sub-measurements were made on four plants (one plant per replicate) in each treatment. The stomatal conductance of (g'_s) was calculated from the ratio 1/(1.6rs) (Béjaoui, 2006). The internal conductance of CO_2 ($g'_i=P_N/C_i$) affected the CO_2 between the intercellular spaces and carboxylation sites in chloroplast and Rubisco activity (El Aouni, 1980).

Data analysis

RAPD bands were scored for their presence (1) or absence (0) and then transformed into a binary matrix. Each marker band was assumed to represent a single locus. Population differentiation was analysed for polymorphism between populations by G_{st}. The amount of gene flow between populations (N_m) was estimated using the following formula (Boeger *et al.,* 1993): $N_m = 0.5(1 - G_{st})/G_{st}$. These calculations were performed by using POPGENE 32 program ver. 1.32 (Yeh *et al.*,1999). To estimate differences between populations, Nei's genetic distance (Nei, 1978) based on RAPD frequencies was calculated. Phylogenetic diagram was drowning using the Unweighted Pair Group Method with the Arithmetic Averaging (UPGMA) algorithm.

Statistics analysis

The data were analysed using appropriate procedures of the SAS software 6.12 (Khawarizmi Center, Tunis version 1998). Analysis of variance (ANOVA) was performed with the statistical programme Minitab (Minitab Inc.; College Park, PA), involving two levels of classification (salinity and population) with interactions. A Duncan's multiple range test was carried out to determine if significant ($P<0.05$) difference occurred between populations and treatments.

Results

Morpho-physiological study

The analysis of variance has been pursued then by a comparison of trait means, between populations (Table 2) and between treatments (Table 3) using Duncan's test at 5%. It revealed a highly significant species; population and treatment effects (Table 4). Population effect was significant in all traits (morphological and gas exchange treatments).

Table 3. Comparison of the averages of the 15 characters studied (Morphological, and yield-related traits) measured on control and salt stress (0, 50, 100, 150, and 200 mM) conditions (of Duncan Test) grown at Ariana, Tunisia.

Characters	0 mM	50 mM	100 mM	150 mM	200 mM
Morphological traits					
PH (cm)	65.24^{a}	52.66^{b}	41.54^{c}	34.76^{c}	27.47^{d}
LL (cm)	15.33^{a}	12.71^{b}	10.55^{bc}	8.49^{cd}	6.94^{d}
SL (cm)	4.84^{a}	4.22^{b}	3.51^{c}	3.09^{c}	2.29^{d}
AN	28.23^{a}	21.19^{b}	16.89^{bc}	13.20^{cd}	11.24^{d}
Yield-related traits					
TN	65.43^{a}	43.82^{b}	25.42^{c}	13.8^{d}	7.96^{d}
SN	54.84^{a}	34.51^{b}	17.01^{c}	7.09^{d}	4.72^{d}
LN	9.25^{a}	7.64^{b}	6.31^{c}	5.31^{d}	4.76^{e}
NSL	9.52^{a}	8.42^{a}	5.7^{b}	4.73^{b}	4.17^{b}
PW (g)	32.72^{a}	18.66^{b}	13.95^{c}	7.06^{d}	5.33^{e}
GYP (g)	5.23^{a}	2.40^{b}	1.16^{c}	0.35^{d}	0.07^{e}
100GW (g)	2.63^{a}	1.48^{b}	1.12^{c}	0.85^{cd}	0.62^{d}

For each column, values with the same letter indicate no-significant differences at 5%.

Table 4. analysis of variance for morphological parameters and gas exchange traits of three *Ae. geniculata* Roth. populations 'Ain Zana, Zaghouan and Sbeitla' and durum wheat variety 'Chili' grown under different salinity levels (0, 50, 100, 150, 200 mM) at Ariana, Tunisia.

Parameters	Abbr	Sources of variations		
		Populations (pop)	Treatment (Tr)	Pop and Tr
Morphological traits				
Plant height	PH	289.65**	40.07***	1775.2**
Leaves length	LL	704.93***	17.56***	2005.48***
Spikes lengths	SL	370.43***	210.7***	738.99***
Awns number	AN	370.96***	12.52***	1371.76***
Tillers number	NT	73.73***	120.94***	1493.77***
Leaves number	NL	27.34***	190.19***	167.4***
Spikes number	SN	54.83***	103.97***	1113.85***
Spiklets number	SLN	112.47***	7.19***	61.66***
Plant weight	PVT	4.75*	519.70***	409.14***
Grain yield per plant	GYP	8.18**	479.99***	559.93***
100 grains weight	100GW	119.17***	53.95***	2276.24***
Physiological traits				
Photosynthesis rate	PN	35.808***	2,398.26***	6.05***
Stomatal conductance	gs	0.0063***	0.327***	0.0012***
Intercellular CO_2	C	2,143.1***	89,191.1***	601.24***
Internal conductance	gi	0.017*	0.879***	0.011***
Stomatal limitation	Ls	0.0152***	0.67***	0.0047***

F-probabilities are indicated by symbols: ns = non-significant differences; * significant differences at $P<0.05$; ** significant differences at $P<0.01$; *** significant differences at $P<0.001$.

The expression of 12 traits showed significant difference ($P<0.05$) between *Aegilops* populations and wheat variety indicating a high level of genetic variability (Table 4). This included seven morphological traits (PH, LL, SL, SLN, PVT, GYP, 100GW) and five gas exchange parameters measured (P_N, g_s, C_i, g'_i, and L_s). The proportion of the inter-population variation explained by the geographical origin was high for all morphological traits. The parameters were significantly affected by the salt treatments ($P<0.05$). Population x treatment interactions were also indicated variable performance of populations in different salt levels for all traits.

Salt effects were highly significant for all traits (Table 3). Indeed, all traits were affected by the increasing levels of NaCl (0, 50, 100, 150, and 200 mM). During the vegetative phase, salt leaded a delay in the setting up of the leaves and decreased especially the tillering. On the other hand during the reproductive phase, salinity treatment accelerated spikes emergence and reduced sizes and spikes number (Table 3). For all levels of NaCl, the height of the plants for the three *Aegilops* populations and the variety of the durum wheat showed variation. Wheat plants present the most elevated height compared to the *Aegilops* plants (Table 2). For the *Aegilops*, Sbeitla and Zaghouan populations present the most elevated height and Ain zana population displayed the weakest value. The plants height decreased progressively with the acuteness of the saline stress. However, the difference between 100 mM and 150 mM is not significant (Table 3). The tillers number varied significantly between the *Aegilops* and wheat (Table 2). Indeed, the wheat variety presented the weakest tillers number. The salinity decreased significantly the tillers number that passed from 68.00 for 0 mM to 7.95 for 200 mM, with a reduction of 76%. The biomass varied significantly between the *Ae. geniculata* Roth populations. Sbeitla population was characterized by the most important dry weight (19.54 g). However, dry weight is not significantly different between Zaghouan population and wheat. The salt stress provoked a reduction of biomass reaching 84.41% at 200 mM (Table 3). The output in grains varied significantly according to the populations and the species (*Aegilops* and wheat). The saltiness exercised a depressive effect very marked on the output in grains that passes from 5.23 g/plant for 0 mM to 0.07 g/plant for 200 mM, with a reduction of 98.7% (Table 3).

Gas exchange parameters

Photosynthesis rate (P_N) of the flag leaf of all *Aegilops* population and wheat variety was reduced to a similar extent by salinity. P_N in the control was higher in the wheat variety than in *Aegilops* populations (Fig. 1A). However, the effect of salinity on P_N was similar in the tow species. An increasing NaCl treatment led to a decrease P_N. Under salt stress, Sbeitla population had the highest photosynthetic rate, followed by Zaghouan and wheat and Ain Zana had the least P_N. At 200 mM, P_N decreased with 96, 88, 86 and 92 % for Ain Zana, Zaghouan, Sbeitla and wheat variety (Chili), respectively, compared with the control.

Table 5. Limit stomatal (Ls) of three *Ae. geniculata* Roth. populations 'Ain Zana., Zaghouan and Sbeitla' and one durum Wheat variety 'Chili' grown under salt stress (0, 50, 100, 150, 200 mM) conditions.

Salinity levels of Ls (mM)	Ae. Ain Zana	Ae. Zaghouan	Ae. Sbeitla	Wheat
0	0.45	0.46	0.46	0.42
50	0.56	0.55	0.54	0.51
100	0.69	0.67	0.63	0.63
150	0.76	0.74	0.72	0.72
200	0.86	0.78	0.78	0.81

Stomatal conductance (g'_s) in flag leaf was similar among the three *Aegilops* populations in the control, but g'_s in this treatment was higher in the Wheat variety 'Chili'. g'_s decreased progressively with increasing salt concentration (Fig. 1b). Indeed, at 200 mM these decreases of g'_s were 89, 82, 77 and 85 % for Ain Zana, Zaghouan, Sbeitla and wheat variety 'Chili', respectively. However, the reductions of g'_s of *Aegilops* populations and wheat variety under salt stress were associated with the increase of C_i (Fig. 1c) and the reduction of g'_i (Fig. 1d). Indeed, at 200 mM, C_i was increased by 30, 24, 21 and 23 % and g'_i was decreased by 97, 92, 87 and 94 % for Ain Zana, Zaghouan, Sbeitla and wheat variety 'Chili',

respectively, compared with the control. stomatal limitation (L_s) increased with increasing salt treatment, with the greatest increment in Ain Zana, and the least in Sbeitla (Table 5). Strong relationships between P_N and g'$_s$ (Fig. 2a) and between P_N and g'$_i$ (Fig. 2b) for the plants studied were showed.

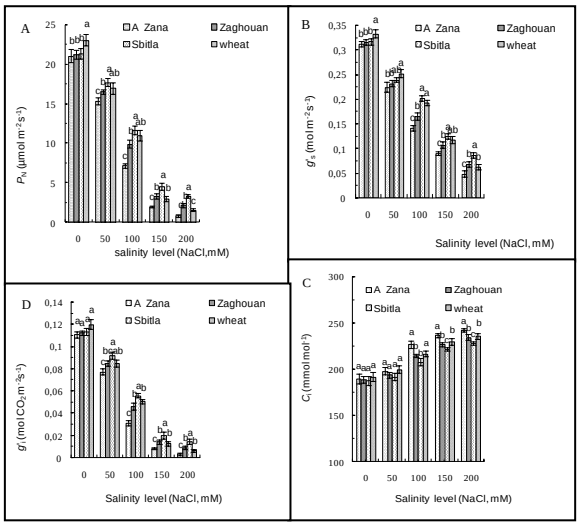

Fig. 1. A CO_2 assimilation rate (P_N), B stomatal conductance of CO_2 (g's), C intercellular of CO_2 (C_i), D internal conductance of CO_2 (g'i) of three *Ae. geniculata* Roth. populations 'Ain Zana, Zaghouan and Sbeitla' durum Wheat variety 'Chili' grown under different salinity levels (0, 50, 100, 150, 200 mM NaCl) the data are mean values of four replications with three measurements per replicate and vertical bars are LSD0.05.

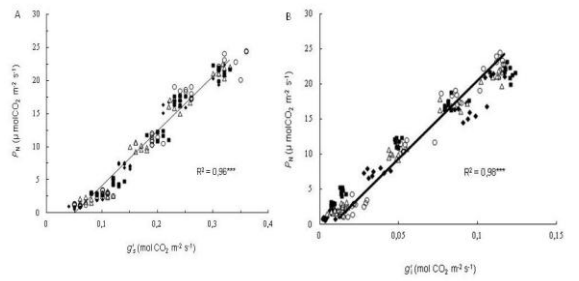

Fig. 2. Relationships A between assimilation rate (P_N) and stomatal conductance of CO_2 (g's), and B between assimilation rate (P_N) and internal conduction of CO_2 (g'$_i$) for three *Ae. geniculata* populations A. zana open triangle, Zaghouan filled diamond, Sbeitla filled square and wheat variety open circle grown at different salinity levels. Data points represent individual measurement.

Genetic diversity

The data for RAPD analysis were scored from photographs of the ethidium bromide stained agarose gels. Analysis of the amplification patterns of *Ae. geniculata* populations and *T. durum* showed a difference by position and number of generated bands. The nineteen primers chosen for analysis were assumed to be a random sample of the genome and generated a total of 212 bands ranging from 9 with (OPM14, OPD10, OPM12, OPJ06, OPD18, OPA12) to 14 with (OPG12, OPA06, OPE14), an average of 9.57 bans per primers that ranged in size from 0.5 to 3 Kb. The patterns of RAPD bans produced by the primer OPB05 is shown in fig. 3 as exemple. Of the 212 bands, 86% (182 in total) were polymorphic (Table 6). The level of polymorphism differed between *T. durum* and *Ae. geniculata* with different primers. 153 polymorphic fragments (71.27%) were shared in *Ae. geniculata,* and 7 in *T. durum* (39.76%) (Table 7). As a result, *Ae. geniculata* populations had the largest number of polymorphic fragments compared to *T. durum* varieties.

Table 6. Nucleotide sequence of primers with the number of amplified products and percentages of polymorphic fragments in the set of *Triticum durum* and *Ae. geniculata*.

Prim-ers	Sequences (5'-3')	Total amplified fragments	Polymorphic fragments	Polymorphism (%)
OPM14	AGGGTCGTTC	9	6	67
OPB13	TTCCCCCGCT	12	9	75
OPJ18	TGGTCGCAGA	11	7	64
OPG02	GGCATGAGC	13	13	100
OPF10	GGAAGCTTGG	12	11	92
OPD10	GGTCTACACC	9	6	67
OPG10	AGGGCCGTCT	11	10	91
OPG12	CAGCTCACGA	14	14	100
OPM12	GGGACGTTGG	9	9	100
OPA06	GGTCCCTGAC	14	14	100
OPJ16	CTGCTTAGGG	11	10	91
OPJ06	TCGTTCCGCA	9	9	100
OPM16	GTAACCAGCC	11	5	45
OPD20	ACCCGGTCAC	11	9	82
OPD18	GAGAGCCAAC	9	9	100
OPE14	TGCGGCTGAG	14	14	100
OPA12	TCGGCGATAG	9	9	100
OPB05	TGCGCCCTTC	13	10	77
OPJ04	CCGAACACGG	11	8	73
Total		212	182	86

Table 7. Number and percentages of polymorphic products for every primer to each species with respect to the total number of amplification fragments (212) are given.

Primers	Sequences (5'-3')	Total amplified fragments	Polymorphic fragments		Polymorphism (%)	
			Ae. geniculata	*T. durum*	*Ae. geniculata*	*T. durum*
OPM14	AGGGTCGTTC	9	3	2	33.33	22.22
OPB13	TTCCCCCGCT	12	4	4	33.33	33.33
OPJ18	TGGTCGCAGA	11	7	1	63.63	9.09
OPG02	GGCATGAGC	13	12	5	92.30	38.46
OPF10	GGAAGCTTGG	12	10	7	83.33	58.33
OPD10	GGTCTACACC	9	4	1	44.44	11.11
OPG10	AGGGCCGTCT	11	10	3	90.90	27.27
OPG12	CAGCTCACGA	14	14	6	100	42.85
OPM12	GGGACGTTGG	9	9	5	100	55.55
OPA06	GGTCCCTGAC	14	14	10	100	71.42
OPJ16	CTGCTTAGGG	11	9	1	81.81	9.09
OPJ06	TCGTTCCGCA	9	8	4	88.88	44.44
OPM16	GTAACCAGCC	11	3	1	27.27	9.09
OPD20	ACCCGGTCAC	11	6	3	54.54	27.27
OPD18	GAGAGCCAAC	9	7	4	77.77	44.44
OPE14	TGCGGCTGAG	14	9	11	64.28	78.57
OPA12	TCGGCGATAG	9	7	5	77.77	55.55
OPB05	TGCGCCCTTC	13	10	7	76.92	53.84
OPJ04	CCGAACACGG	11	7	7	63.63	63.63
Total		212	153	87	71,27	39,76

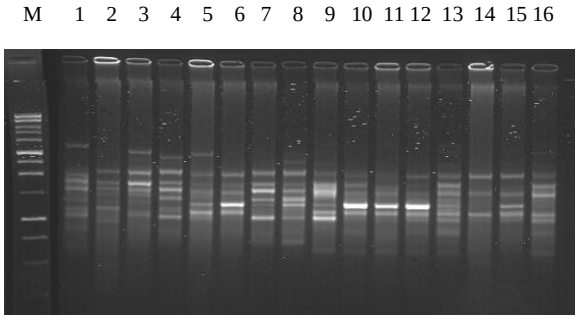

Fig. 3. Amplification profile of 13 *Aegilops* populations and 3 wheat varieties with OPB05 primer. 1= Djebel Ressas, 2= Djebel Serj, 3= Sbeitla, 4= Karim, 5= Souk jemaa, 6= Djebel Abderahmen, 7= Mahmoudi, 8= Chili, 9= Ain Zana, 10= Mekna, 11= Goussa, 12=Bizerte, 13= Djebel Oust, 14=Nefza, 15=Tabarka, 16=Zaghouan, M= Molecular weight markers: 1 Kb DNA Ladder.

The total genetic diversity (H_T) and the intra-population genetic diversity (H_S) are respectively 0.3195 and 0.1516. The M14 and M16 locus showed the lowest (H_S) values (0.0787, 0.0732) but J18 and M16 locus indicated the lowest (H_T) values (0.2166, 0.1651). As expected, total genetic diversity (H_T) was upper estimated compared with (H_S). Therefore, the genetic diversity values for each locus were very considerable and levels of inter-population genetic diversity were close. Across all RAPD markers the G_{st} value was 0.5255, indicating that about 52.50% of the total genetic variation could be explained by RAPDs differences while the remaining 47.5% might be attributable to differences among the populations. These results indicate that genetic differentiation was low in the two closely related species (*Ae. geniculata* populations and *T. durum*). The number of migrants per generation (N_m) is an estimated value from G_{st} to measure the gene flow, the higher its value, the less genetic differentiation among populations. According to the previous study and estimation of gene flow (Wright 1931), if $N_m > 1$, it can prevent the differentiation among populations caused by genetic drift. And, if $N_m < 1$, local populations tend to differentiate. The amount of gene flow (N_m) among (*Ae. geniculata* populations and *T. durum*) varied from 0.1802 in M16 to 1.4353 in F10, and was found to be 0.4515(<1) (Table 8), indicating negligible gene flow between the populations and showing that they tend to differentiate in over all locus.

Table 8. Differentiation between *Ae. geniculata* populations and *T. durum* based on RAPD markers.

Locus	Simple Size	H_t	H_s	G_{st}	N_m
M14	16	0,2312	0,0787	0,4255	0,3070
B13	16	0,2301	0,1143	0,3797	0,4267
J18	16	0,2166	0,1142	0,4526	0,4567
G02	16	0,4004	0,1804	0,5072	0,4063
F10	16	0,3682	0,1988	0,4414	1,4353
D10	16	0,2419	0,1191	0,3096	0,2208
G10	16	0,3407	0,1628	0,5078	0,3694
G12	16	0,3592	0,1877	0,4148	0,3527
M12	16	0,4221	0,2183	0,4659	0,5369
A06	16	0,3778	0,2212	0,3836	0,4790
J16	16	0,2797	0,1693	0,3395	0,4990
J06	16	0,4286	0,1785	0,5523	0,3838
M16	16	0,1651	0,0732	0,2810	0,1802
D20	16	0,3403	0,1244	0,5447	0,3579
D18	16	0,3885	0,1398	0,5448	0,2719
E14	16	0,3317	0,1582	0,4020	0,3082
A12	16	0,3752	0,1597	0,5531	0,2891
B05	16	0,3184	0,1381	0,5216	0,3730
J04	16	0,2444	0,1062	0,5317	0,2657
Mean	16	0.3195	0.1516	0.5255	0.4515
St.deviation (SD)	16	0.0241	0.0106		

H_S: genetic diversity within population (intra-population GD);

H_T: Total genetic diversity; G_{ST}: Coefficient of population differentiation

(inter-population GD); Nm: estimate of gene flow G_{st}; $N_m = 0.5(1 - G_{st})/G_{st}$.

Similarity indices were calculated within and among species (Table 9). The genetic diversity of *Ae. geniculata* populations was low, the estimated similarity coefficients ranged from 68.1% to 82.1%. In addition, durum wheat varieties exhibited a low level of intra-specific similarity that varied from 71.3% to 80.2 %. A high similarity at the level of the DNA was showed between two combinations Mekna and Souk jemaa populations with 82.1% and between Mahmoudi and Chili varieties with 80.2%. An inter-specific similarity was observed by a low of 52.2% for the two closely related species. Dendrogram based on Nei's (1978) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated segregation of two main clusters (fig. 4). The first cluster A is formed by two sub-clusters constituted by *Ae. geniculata* populations. The first sub-cluster is formed by five populations (Dj Ressas, Ain Zana, Bizerte, Dj Oust and Zaghouan), their similarities percentage varied between 0.70 and 0.73. The second sub-cluster is composed by eight populations (Dj Serj, Sbeitla, Souk jemaa, Mekna, Goussa, Dj Abderhamen, Nefza and Tabarka). The similarities percentage of this one ranged between 0.72 and 0.73. The second cluster B is constituted only with durum wheat varieties (Karim, Mahmoudi and Chili) with percentage similarity that varied between 0.52 and 0.71.

Table 9. Genetic similarity matrix among *Aegilops geniculata* Roth populations and durum wheat varieties based on RAPD markers.

	R	S	Sb	K	J	Abd	Mah	Ch	Z	M	G	B	O	N	T	Zg
R	1															
S	0.681	1														
Sb	0.681	0.798	1													
K	0.522	0.522	0.522	1												
J	0.681	0.725	0.725	0.522	1											
Abd	0.681	0.725	0.725	0.522	0.746	1										
Mah	0.522	0.522	0.522	0.713	0.522	0.522	1									
Ch	0.522	0.522	0.522	0.713	0.522	0.522	0.802	1								
Z	0.732	0.681	0.681	0.522	0.681	0.681	0.522	0.522	1							
M	0.681	0.725	0.725	0.522	0.821	0.746	0.522	0.522	0.681	1						
G	0.681	0.725	0.725	0.522	0.807	0.746	0.522	0.522	0.681	0.807	1					
B	0.698	0.681	0.681	0.522	0.681	0.681	0.522	0.522	0.698	0.681	0.681	1				
O	0.698	0.681	0.681	0.522	0.681	0.681	0.522	0.522	0.698	0.681	0.681	0.732	1			
N	0.681	0.691	0.691	0.522	0.691	0.691	0.522	0.522	0.681	0.691	0.691	0.681	0.681	1		
T	0.681	0.691	0.691	0.522	0.691	0.691	0.522	0.522	0.681	0.691	0.691	0.681	0.681	0.737	1	
Zg	0.698	0.681	0.681	0.522	0.681	0.681	0.522	0.522	0.698	0.681	0.681	0.706	0.706	0.681	0.681	1

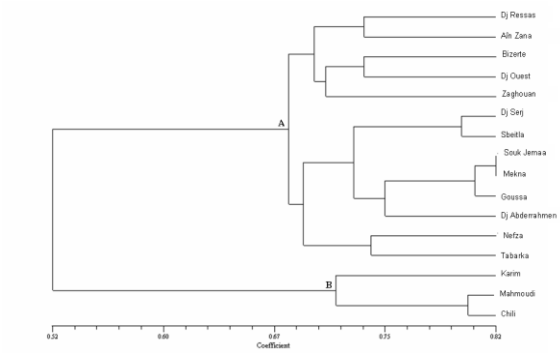

Fig. 4. Dendrogram constructed by UPGMA and based on RAPD similarity values showing relationships among the 13 *Ae. geniculata* populations and 3 durum wheat varities.

Discussion

Substantial variation was observed in our study for traits related to the adaptation of *Ae. geniculata* Roth. to salt stress. The variation among species and populations for morphological traits and gas exchange treatments was mainly explained by geographical origin, suggesting that those traits are mainly constitutive and result from natural selection pressure exerted by the climatic constraints. Although data were obtained from plants grown in pots, the result can be related to in situ performance. Sbeitla population, which is better adapted to naturally occurring salt stress, was more tolerant to experimentally imposed salt stress than Ain zana and Zaghouan populations. Ain zana was the most sensitive to the salt stress. Very high intra-and inter-specific variability existed for all morphological and physiological traits (Table 2), as already reported by Perrino *et al* (1993) for a collection of *Ae. geniculata* Roth from Southern Italy and Sicily. In fact, maturity occurs in *Ae. geniculata* Roth between March (in Libya) and August (in central Turkey) (Van Slageren 1994). In this species, morphological traits could represent an important trait favoring plant survival, reproduction, and maturity under salt stress.

Growth of the both species (wheat variety and the *Ae. geniculata* Roth populations) decreased with increasing salt stress, as indicated by plant height (PH), total tillers number, leaves number, leaves length, and plant weight. The results reported by Munns and Rawsan (1999) in wheat showed that saline stress generally appears by a weak growth, a reduction of the surface and the leaves number, an acceleration of senescence of the mature leaves. Also, Cramer and Quarrie (2002) in maize noted that salt stress reduced the development of the aerial parts by inhibition of the apparition of new leaves. These plants lost biomass continuously, this was a result of tillers survived, flowered and produced a few small but non-viable grains (Froster *et al.,* 1987).

Salinity may directly or indirectly inhibit cell division and enlargement in the plant's growing point. Reduced shoot growth caused by salinity originates in growing tissues; not in mature photosynthetic tissues. As a result, leaves and stems of the affected plants appear stunted. Chloride induces elongation of the palisade cells, which leads to leaves becoming succulent.

Reduction in plants growth with increasing saltines in this study reflects the increased metabolic energy cost and reduced carbon gain, which are associated with salt adaptation (Kao *et al.,* 2006). It also reflects salt on tissues, in photosynthetic rate per unit of leaf area (Netondo *et al.*, 2004b). Indeed, at reproduction stage, salinity stress markedly inhibited P_N, g'_s and g'_i on the flag leaf (Fig. 1a, b, d, e). Sbeitla population maintained the highest photosynthetic CO_2 (P_N) fixation rate under salt stress, and this was associated with higher g's (Fig. 3a, b) than Zaghouan population and wheat variety. Similar results were founded with *Triticum aestivum* and *Hordeum vulgare* (Sharma and Hall, 1991), also, in flag leaves of barely (Belkhodja *et al.,* 1999). At the three *Ae. geniculata* populations and wheat the decrease in P_N, L_s, and the strong P_N–g'_s relationship (Fig. 3a) may indicate that stomata were imposing a larger limitation on P_N under salt stress conditions. Possible reasons for this include stomatal closure, feedback inhibition due to reduced sink activity, decreased efficiency of Rubisco, displacement of essential cations from the endomembrane structure (leading to changes in permeability), and swelling and disorganization of the grana (Flowers and Yeo, 1981), or due to the direct

effects of salt on stomatal conductance via a reduction in guard cell and intercellular CO_2 partial pressure (Dionisio-Ses and Tobita, 2000). Many studies have reported that stomatal and non-stomatal components are responsible for decrease in P_N (Tezara *et al.,* 2003). In the present study, the increase of C_i attests that some non-stomatal limitation can influence the photosynthesis. Indeed, the variation of C_i is consequence of CO_2 flux from stomata and binding sites in the cytoplasm.

A slight increase of C_i and in favour of a slightly stronger internal limitation on P_N (Béjaoui, 2006). Similarly, the strong positive P_N–g'_i relationships (Fig. 1b) expresses that some non-stomatal factors were responsible for limiting P_N of the plants studies. In addition, the results showed that the internal concentration CO_2 reported depending on the intensity of the stress and population. Under 50 mM NaCl, it is rather the stomatal limitation acting on the photosynthesis of plants studied because there was not significant increase in C_i. This is in concordance with various studies indicating that under moderate salt stress stomatal closure often predominate (Cronic and Massacci, 1996). From 100 mM NaCl, salt induced the development of non-stomatal factors limiting photosynthesis evidenced by the average value of C_i than those of control and varying from 20 % at 100 mM and 25 % at 200 mM. Non-stomatal inhibition of photosynthesis by salinity has been reported in several species (Kao *et al.,* 2006; Abassi, 2009; Mguis, 2010). It can result from large malfunction in the chloroplast (Warran *et al.,* 2004) by inhibition photochemical process and/or incorporation of CO_2 (Terashima and Ono, 2002). It may also be due to reduce of chlorophyll concentration of the leaves of plants grown at NaCl concentrations higher than 100 mM and decrease of the concentrations of essential ions such as Ca^{2+} and Mg^{2+} In the mesophyll cells (Netondo *et al.,* 2004a). At reproductive stage, the plants studied showed the same behaviour at the limiting factors in P_N. Indeed, the three population of *Ae. geniculata* and wheat cultivar, the reduction of P_N was accompanied by reduction of g'_s and g'_i attesting to the simultaneous effect of stomatal and nonstomatal components on P_N. Similar results with salt stress have been reported in G. tabanica (Kao *et al.,* 2006). However, the similar correlation P_N–g'_s (Fig. 1a) and P_N–g'_i (Fig. 1b), translated stomatal and internal limitations to photosynthesis.

Genetic diversity of endangered species has always enthused evolutionary and conservation biologists. The ability of a species to adapt to environmental changes depends greatly on the genetic diversity in the species (Neel and Ellstrand, 2003, Anand *et al.*, 2004). Narrowing of gene pool and reduced genetic diversity pose challenges in the selection pressure brought in by environmental changes. The high level of polymorphism (86%) in *Aegilops geniculata* Roth and *Triticum durum* observed in this study corroborated with the results reported by Baghizadeh and Khosravi (2011) in *Aegilops* germplasm, and by Kadri *et al* (2010) in local Tunisian barley, but it is a little less than that obtained by do Amaral Júnior *et al* (2011**)**. High polymorphism revealed that RAPD could resolve genetic variation among crop germplasm, identification of cultivars and for estimating genetic relationship (Silva *et al.,* 2005a; 2005b). In this study, RAPD markers revealed 39.76% polymorphic fragments *in T. durum* species (Table 7) different from 25% polymorphic fragments in durum wheat (Eujayl *et al.,* 2002). These results suggested that the level of polymorphism detected by EST–SSRs was relatively low compared with RAPDs. However, the high level of polymorphism in *Ae.geniculata* was reported by Zaharieva *et al* (2001). It is important to confirm the genetic structure of population to understand its biology characteristics and explore the evolutional process and mechanisms. The genetic structure of population is mainly reflected by genetic differentiation within and between populations. The coefficient of genetic differentiation (G_{st}) as the most commonly used index expressed by the genetic variation between population accounts for the proportion in the total variation among populations. In our study, (H_t) was found to be 0.3195 and (H_s) was contributed 47.45%, indicating that the genetic diversity between the groups of populations

occupied the half of the total. Total gene diversity was attributable mostly to diversity within population, indicating that the groups were likely to differ genetically. In addition, the amount of genetic diversity present within population genetic diversity (H_s) value equalling 0.1516, which is not surprising since *Aegilops* and *Triticum* are a highly self-crossing plants. These results were also reported for other self-crossing *Poaceae* family plants. For example, 59% RAPD and 64% ISSR was found among 14 populations of *Oryza granulate* (Wu *et al.,* 2004).

In the present study, the genetic differentiation (G_{ST}=0.5255) was reasonable and considerable than the levels of differentiation detected among wheatgrass of *Thinopyrum junceum* based on isozymes (Nieto-Lopez *et al.,* 2003) and among *Titipyrum* lines using PCR-based molecular (Siahsar *et al.,* 2011). Gene flow, the genetic counterpart of dispersal, is an important content for study the genetic structure of species, which may lead to the 'genetic rescue' of genetically eroded populations (Richards, 2000; Ingvarsson, 2001). The indirect estimate of gene flow (N_m) between our populations was low (0.4515), implying that genetic drift could be the dominant evolutionary factor that shapes the populations structure of *Ae. geniculata* and *T. durum* according to Wright (1931). With a very low migration rate, genetic drift could have effectively isolated and differentiated the populations after a long period, consistent with the almost equal percentage of within and between the populations genetic variation.

RAPD and other discontinuous markers can serve as a means of genetic distances to establish phylogenetic relationships (Rabey *et al.,* 2002). Estimation of genetic differences and discrimination of genetic relationship between the two species are for utilization of plant genetic resources. In the present study, dendrogram indicated segregation of *Ae. geniculata* of populations and *T. durum* into two main clear pattern clusters (Fig. 4). Despite the relatively restricted geographical range covered by the investigation, studied populations exhibited a pronounced genetic divergence at different hierarchical levels. Kellogg and Mason-Gamer (1996) expressed their opinion that *Aegilops* and *Triticum* should be retained as two distinct genera. An inter-specific similarity was observed by a low genetic distance (52%) for the two closely related species. This result reflects the fact that they have divergence in their genetic characteristics, while hybridisation between them for wheat improvement has brought the two species closer genetically (Martin- Sanchez *et al.,* 2003. Li *et al.,* 2004)

Conclusion

The *Ae. geniculata* Roth. populations provides germplasm that has potential for crop improvement. Wild-related species have been considered until now much more as sources of resistance to diseases than as sources of diversity permitting deep modification of architecture and physiology of the cultivated species. There are indications that *Ae. geniculata* Roth. can contribute useful genes for salt tolerance and improvement of wheat (Monneveux *et al.*, 2000). In this study, a large variation for salt-stress tolerance was found within populations. Single population such as Sbeitla or Ain Zana or Zaghouan that exhibit rich morphologic, genotypic and photosynthesis diversity may provide valuable resources for traits of agronomic importance. The salt-stress tolerant identified in the *Ae. geniculata* Roth. populations and wheat variety 'Chili' may be combined with breeding lines exhibiting high yield potential. The establishment of genetic relatedness and molecular characterization of Tunisian *Aegilops* and wheat species is fundamental as an important informational basis for such programs. These techniques will also be useful for the organisation and conservation of genetic diversity of Tunisian species. In this case, we believe that the data presented here will be a tool for other wheat researchers

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