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Molecular and morphological characterization related to salt stress in natural populations of the *Medicago polymorpha* species

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Abstract

Legumes are important crops due to their nutritional benefits as well as their utility in agriculture rotation. The morphological and molecular markers can elucidate plant response to stresses. In the present study, we investigated indicators of molecular polymorphism related to salt stress tolerance in natural populations of *Medicago polymorpha* species. The plant samples were collected from different geographical sites in western Algeria. Morphological growth parameters such as root length stem length of plant and T/R ratio were examined under four NaCl concentration levels (0, 68, 102 and 137 mM). The molecular study was carried out using SSR molecular markers on all the studied populations. Significant differences were observed in mean squares of analysis of variance, indicating relation between geographical origins and populations. This analysis showed the existence of two contrasted populations (DZ221 and DZ312). DZ221 is a relatively salt tolerant, and DZ312 is sensitive. The Principal Component Analysis (PCA) was calculated using the PCA function, revealing a high correlation between the morphological traits and the geographical distribution of populations. It divided populations into four distinguished groups respecting their origin geographical sites. The molecular results of the polymorphism degree showed that the natural populations of *M. Polymorpha* species were strictly homozygous (100%). The PIC index of the three microsatellites was very informative (0.77). Additionally, the results showed that both microsatellites (FMT11 and MTIC297) revealed some alleles detected in the tolerant population (DZ221) suggesting that they can be used as indicators of saline stress tolerance adaptation.

Keywords: polymorphism; *Medicago polymorpha*; morphological character; salt stress; microsatellite.
Abbreviations: Dst_genetic diversity among the population; Fis_Fixation indice; Gst_genetic coefficient of differentiation; He_ Expected heterozygoty; Ho_observe heterozygosity; Hs_genetics population diversity; Ht_Total genetic diversity; N_Number of amplified sample; Na_Number of alleles per locus, PIC_Polymorphism information content, PCA_principal component analysis.

Introduction

Salinity is a major constraint for plant growth and crop productivity In the world (Charfedineet al., 2016). Salt stress reduces crop productivity in arid and semi-arid regions of the world (Ashraf and Fooled, 2007). Moreover, it is estimated that 800 million hectares of land and 32 million hectares of agricultural land are affected by abiotic salt stress in the global area (FAO, 2015). In North Africa and the Middle East, 15 million hectares of arable land is affected by salinity and this area is steadily increasing (Lahmar and Ruellan, 2007). Algeria is usually affected by a long period of drought leading to external signs of soil salinization (Belkhodja and Bidai, 2004). To resolve these constraints, the research on species that are relatively resistant to saline stress is a good alternative. So, the selection and the identification of salt-tolerant genotypes remains the best economic approach for the exploitation and rehabilitation of salt-affected regions. Annual species of the *Medicago* genus are important legumes for their forage quality and their capacity to improve the azotization of salty soils. Burr medic (*Medicago polymorpha* L.) (2n = 14) is an autogamous, hard-seeded annual legume originated from the Mediterranean Basin, adjacent to arid-semi-arid regions (Heyn, 1963; Lesins and Lesins, 1979). *Medicago polymorpha* could be a source of characters of agronomic interest such as resistance to biotic and abiotic stresses, which could be transferred to other forage plants like *Medicago sativa* (Scarpa et al., 1993). Genetic variability for salinity tolerance has been studied in many species (Pailles et al., 2019). For practical reasons, many explorations of this variability have been approached at the vegetative stage on very young plants. This approach is justified by the fact that the response of seedlings is sometimes strongly predictive for mature plants. In some cases, the difference between the tolerance at the seedling stage and that at the adult stage may justify the differences in the mechanisms involved from one stage of development to another. Several studies carried out on morphological traits such as stem, root or seedling growth have shown a negative effect with increasing severity of salinity stress (Divya and Viswanath, 2017; Zörbet al., 2019). Morphological characteristics are generally quantitative, with mono- or polygenic determinism. Understanding morphological traits facilitate the identification of desirable

traits and their genetic determinants. Molecular versus morphological markers offer a contemporary solution to improve the efficiency of the selection of complex traits such as salinity stress (Ashraf and Foolad, 2013). The use of molecular markers such as microsatellites or SSR markers have been widely used for the study of genetic diversity, genome mapping, variety identification, etc. and are now being used in many countries. The use of these markers for the study of genetic variation and the mapping of QTLs for salt tolerance in different cultivars has already been reported by some researchers (Forster et al., 2000). The objective of this study is to explore the possibilities of selection and identification of extreme genotypes for salt stress tolerance. The variability of salt tolerance by estimating the growth of morphological characters was evaluated in fourteen natural populations of *M. polymorpha* from different geographical sites in western Algeria. Then we used three microsatellite markers to find alleles that may reveal polymorphism of geographical adaptation to salinity tolerance.

Results

Adaptation of the different populations to salt stress

The morphological studies allowed us to identify tolerant populations of susceptible populations by analyzing the variability of salt stress tolerance, and by studying the growth of seedlings of different populations in saline and non-saline conditions. After nine days of treatment, the data indicated that the development rates of all studied parameters were decreased considerably with the increase of the salinity (Fig. 1, 2, 3 and 4). The two-way ANOVA test was highly significant for the four measured parameters (Table 1). These results indicated that salinity has an inhibitory effect on seedling growth and the effect varies according to the genotype. The tolerance indices (IT1, IT2, and IT3) of the seedling length parameter will make it possible to identify extreme populations (tolerant and sensitive). The medium concentration (102 mM) was chosen to identify both extreme populations. At this concentration plants can tolerate salt stress without effect on the different physiological and metabolic processes of their vegetative growth. The T2 ratio on T0 allowed us to identify both extreme populations (DZ221 and DZ312). These are considered tolerable and sensitive populations, respectively (Fig.5).

Total length of young seedlings under saline stress

Without stress (T0), the average length of seedlings is more developed for the DZ311 population (9.71 cm) than the DZ130 (5.05 cm). But the other populations have the least worth, including reference species. Under stress (T1, T2 and T3), we note a decrease in the growth of the total length of young seedlings as the concentration of NaCl increases. The DZ271 population recorded the highest growth values (8.01, 5.39 and 4.08 cm) for three treatments (T1, T2 and T3), respectively. However, the DZ313 population (1.63 and 0.14 cm) showed the highest growth reduction for both treatments (T1 and T3). Finally, the DZ221 population showed a 0.40 cm length under T2. Concerning the genetic variability of this parameter estimated by standard deviation measurement there is a very high genetic variability between the different studied populations in the absence of stress (T0). But in the presence of stress (T1, T2 and T3) the genetic variability is reduced proportionally with the severity of the saline concentration (Fig.1).

Stem length of young seedlings under saline stress

For the stem length parameter, we noted that in the absence of stress (T0), the DZ 311 population (1.63 cm) shows the higher growth, but the DZ130 population (0.73 cm) has the lowest one. In the presence of stress, the DZ270 population (1.07 and 0.79 cm) recorded the highest values for treatments (T1 and T2). The DZ 271 population had a 0.68 cm for treatment (T3). But the DZ313 population (0.29, 0.19 and 0.04 cm) showed the lowest growth rates for the three treatments (T1, T2 and T3), respectively (Fig.2).

Root length of young seedlings under saline stress

Under normal conditions (T0), the DZ 311 (8.10 cm) population has the highest average growth rate, while the DZ130 (4.57 cm) population showed the lowest growth rate among all the studied populations. Under stress (T1, T2 and T3), the DZ271 population recorded the highest growth values (7.12, 4.69 and 3.40 cm), respectively. However, the most pronounced growth drops in root length occurred in the DZ313 population (1.33 and 0.09 cm) at both treatments (T1 and T3), respectively. The DZ312 population showed a root length of (0.35 cm) for the treatment (T2). Examination of the standard deviation showed that a larger variance is dispersed among the different studied populations. This genetic variability decreases with increasing saline concentrations (Fig.3).

Stem to root ratio under salt stress

The results showed a low ratio in all studied populations for most treatments. In the absence of stress (T0), the DZ 313 (0.35) population had the highest ratio, while the DZ130 (0.17) population showed the lowest. In the presence of stress (T1, T2 and T3) the DZ291 population (0.36 and 0.30) showed the highest ratio for both treatments (T1 and T3) respectively. Under (T2), the DZ221 population (0.39) exhibited the highest ratio (Fig.4).

The tolerance indice of the seedling length of the different studied populations

The indice values show a significant decrease with increasing saline severity. By comparing the lengths of the treated plants (T1) with their controls (T0), it can be seen that the population DZ221 has the best tolerance indice (IT1). On the other hand, the population DZ 312 has the lowest tolerance indice. Comparison of the lengths of treated plants (T2 and T3) with their controls shows that the population DZ221 has the best tolerance indice for the treatment (T2) and the population DZ271 for the treatment (T3), while the population DZ312 has the lowest indice for both treatments. In the presence of the most discriminating degree of stress (T2), this allows the expression of the great variability among all treatments. The IT2 values will help to classify the two extreme populations from the most tolerant (DZ221) to the most sensitive (DZ312) (Fig.5).

PCA analysis under stress condition

An analysis of the main components was carried to study morphological characteristics and it revealed four factors. The two main factors F1 and F2 explain (95.64%) of the total variability (F1 with 67.83%, F2 with 27.82%). However, the two remaining factors F3 and F4 showed very low contribution to the total variability (F3 with 4.35%, F4 with

0.01%). The axes are considered according to the absolute criterion of "kaiser", whose eigenvalues are greater than 1. The parameters root length, stem length and total plant length are strongly negatively correlated with factor 1 (-0.95, -0.90 and -0.97) respectively, whereas the T/R ratio parameters is strongly and positively correlated with factor 2 (0.97) (Fig.6).

Analysis of global population variability

The projection of all studied populations, according to the two main components (PCA1 and PCA2) is shown in Figure 7. It can be seen that the populations occupy the entire area of the PCA plane and they are dispersed into four homogeneous groups. Both groups Cluster 1 (Jemalong, DZ311 and Poly tah), and Cluster 2 (*M.sativa*, DZ220, DZ270 and DZ271) are grouped on the negative side of Factor1, which means that the growth of the studied characteristics (root length, stem and total plant length) are the least affected by the increase in saline stress intensity. But the two remaining groups Cluster 3 (DZ310, DZ221, DZ290, DZ291 and DZ292), and Cluster 4 (DZ130, DZ312, DZ313, DZ460 and DZ461) occupied the entire area on the positive side of Factor 1. It is showing that the growth of the traits (root length, stem length and total plant length) is most affected by the increased salt stress. The T/R ratio is the main component of Factor 2. The projection of the populations of the two groups 2 and 3 on the positive side of Factor 2 shows that these populations were able to maintain a balance between root and stem growth despite the severity of the saline stress, but the populations of the two remaining groups 3 and 4 are on the negative side of Factor 2. It shows the difficulty to maintain the balance of stem and root growth with increasing severity of saline stress. The projection of the two main factors shows that the behaviour of all studied populations forms homogeneous groups, except for three populations (DZ220, DZ311 and DZ310), are distinguished to their groups of origin (Fig.7).

Estimation of molecular polymorphism by the SSR markers

Using the 3 SSR loci, a total of 36 individual alleles were detected in 225 individuals (Table 2). The number of alleles per locus ranges from 7 to 18 per locus. The FMT11 locus had the highest number of alleles (18), while the MTIC 564 and MTIC 297 showed the lowest number of alleles (12 and 7, respectively). The average value of the number of alleles per locus is 12 alleles per locus. This one is very important because it shows that the rate of alleles or allelic richness has an average of 12 alleles per microsatellite. This parameter reflects a high level of polymorphism for all the *M. polymorpha* populations. The calculated PIC values for these three microsatellites are presented in Table 4. In general, the PIC values are high. The average value for all primers was 0.77. The highest values (0.87, 0.83 and 0.63) are obtained by MTIC 297, FMT11 and MTIC 564 primers, respectively. This corresponds to a large number revealed by each primer (Fig. 8). Example of migration is shown in Fig S1.

Intra-population and inter-population diversity

Nei genetic diversity indices were estimated for all 14 populations. The total genetic diversity (Ht) calculated for each marker locus gives the following values: FMT11 (0.872), MTIC564 (0.738) and MTIC297 (0.654). The average of the three marker loci (0.799) was significant, revealing high genetic diversity in the different populations of the species *Medicago polymorpha*. The mean of the intra-population genetic diversity for the three loci marker revealed a low level of the genetic diversity with an average for all studied loci (0.134) as well for each marker locus: FMT11 (0.257), MTIC297 (0.130) and MTIC564 (0.017). There is a great genetic divergence between populations of the *Medicago polymorpha* species. Indeed, the inter-population genetic diversity contribution (Gst) for all loci to the total genetic diversity is an average of 84%. The observed (Ho) and expected (He) heterozygosity among the populations and for each locus are presented in Table 2. The averages (Ho) and (He) are ranged from 0 to 0.14, respectively.

Genetic differentiation

The population genetic differentiation was examined by fixation indices such as (Fis) and (Fst) for each of the three analyzed loci (Table 2). Calculations of three SSR loci showed that all populations have a fixation index (Fis = 1) that is due to their breeding system which is strictly autogamous. On the other hand, the differentiation index of population with the total (Fst) for each locus showed very important values. The obtained dendrogram make it possible to evaluate the genomic relationships between the different studied populations, revealing many of different groups (Fig. 6). The results show that the clustering of most populations has correlated with their geographic origins. The 17 genotypes of the genus *Medicago* used in this study were grouped into two main groups (GI and GII). The first group (GI) consists of a single cultivar (Orca) that has a tetraploid genotype. On the other hand, the second group (GII), which alone is subdivided into two subgroups (GII-1 and GII-2), contains only species of the genus *Medicago* diploid. The first sub-group (GII-1) was subdivided into five subgroups (GII-2-A and GII-2-B) that contained four populations in two localities near the Wilaya of Oran; (GII-2- C) consisted of a cultivar (Jemalong) and local control (Polytah), (GII-2-D and GII-2-E) consisted of three populations from the same region of the province of Mascara and a population of another population of the Wilaya of Ain temouchent that have a similar index. The second subgroup consisted of four subgroups; each subgroup is made up of different populations that are divided according to their index of similarity and their geographic sites. The (GII-2-A) is composed of a single population of the locality of the Wilaya of Tlemcen, for the two subgroups (GII-2-B and GII-2-C). The first is also constituted of a single population of the locality of Wilaya Ain-temouchent and the second included of two populations of the locality of the Wilaya of Mostagnem. The last subgroup (GII-2-D) is composed of two populations of the same locality of the Wilaya of Sidi belabes. The obtained results from the distribution of all studied populations of *Medicago polymorpha* species and the two other *Medicago truncatula* and *Sativa* species confirmed the output dendrogram. The similarity matrices values, as well as the distances, are shown in the Tables S1 (see the data supplementary section).

Association between microsatellites and the two contrasting population to salt stress tolerance

Resulting data in Table 3 allow us for determining the potential relationship between the three SSR markers and salt stress tolerance in both extreme populations DZ221 (tolerant) and DZ312 (sensitive).

Table 1. Two-way ANOVA analysis of the treatment effect, genotype and their interaction for the four parameters (Root length, Shoot length, Seedling length and Ratio t/r).

Source of variation	Def	Root length		Shoot length		Seedling length		Ratio t/r	
		MS	F	MS	F	MS	F	MS	F
Population	16	404.11	8.43***	7.94	4.06***	502.6***	7.81***	0.45	2.25***
Treatment	3	3997.59	83.41**	122.19	62.58**	5517.4***	85.73***	1.04	5.24***
Population x Treatment	48	47.92	4.95***	1.95	5.99***	64.35***	5.23***	0.19	2.46***
Error	3330								

* P ≤ 0,05 ; ** P ≤ 0,01 ; ***P ≤ 0,001 ; ns : non-significant ; F, coefficient of Fisher-Snedecor.

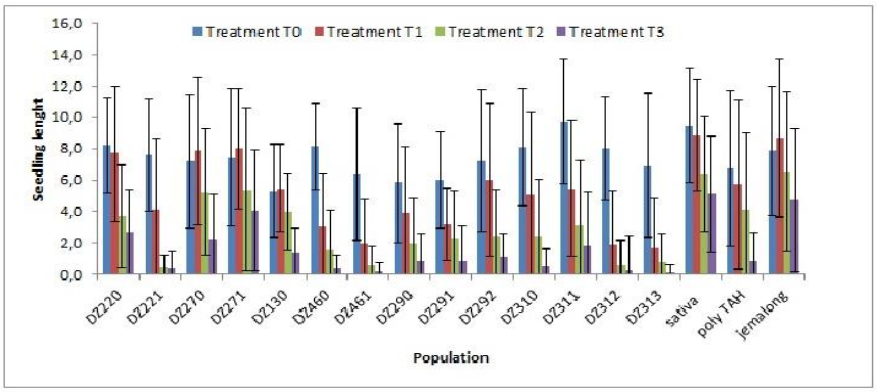


Fig 1: Average lengths analysis of young seedlings

Table 2. Details of SSR markers, number of alleles detected, diversity and polymorphism information content (PIC).

SSR Name	N	Na	Ho	He	Hs	Ht	Dst	Gst	Fis	Fst	PIC
FMT11	69	18	0	0.26	0.257	0.872	0.615	0.704	1	0.705	0.83
MTIC297	79	11	0	0.02	0.017	0.654	0.637	0.972	1	0.974	0.87
MTIC564	77	7	0	0.13	0.130	0.872	0.738	0.850	1	0.850	0.63
Means	75	12	0	0.14	0.134	0.799	0.663	0.842	1	0.836	0.77

N: Number of amplified sample, Na: Number of alleles per locus, Ho: observe heterozygosity He: Expected heterozygoty Hs: genetics population diversity Ht: Total genetic diversity Dst: genetic diversity among the population Gst: genetic coefficient of differentiation, Fis: Fixation indice PIC: Polymorphism information content.

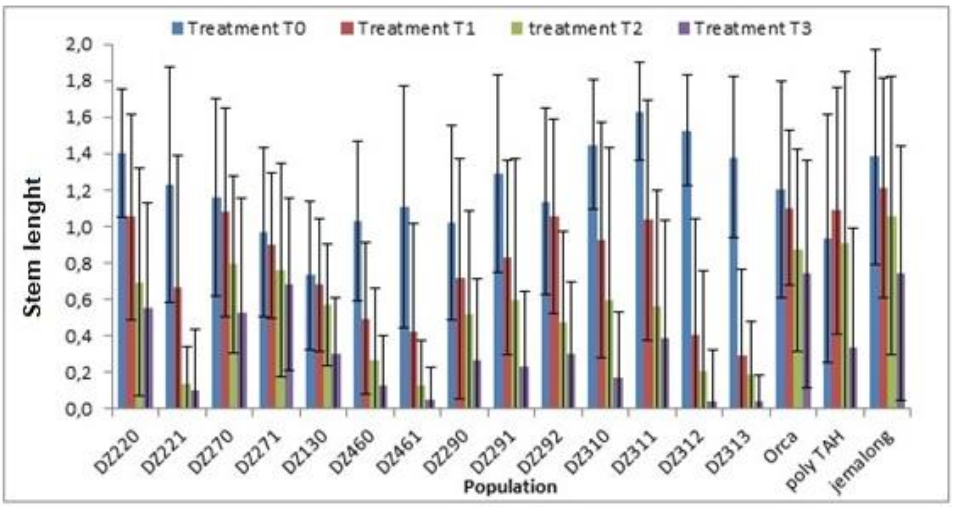


Fig 2. Stems average length analysis of young seedlings.

Table 3. Detected alleles of tolerant and sensible populations for marker loci MTIC564, MTIC297 and FMT11, in parenthesis (size in bp).

Populations	Marker Alleles						
	MTIC 564		MTIC 297		FMT11		
	145 bp	164 bp	170 bp	175 bp	168 bp	172 bp	185 bp
DZ 221 (T)	+	+	-	-	+	+	-
DZ312 (S)	+	-	+	+	-	-	+

T: salt tolerant; S: salt sensible; +: allele detected; allele non-detected, bp: base pair.

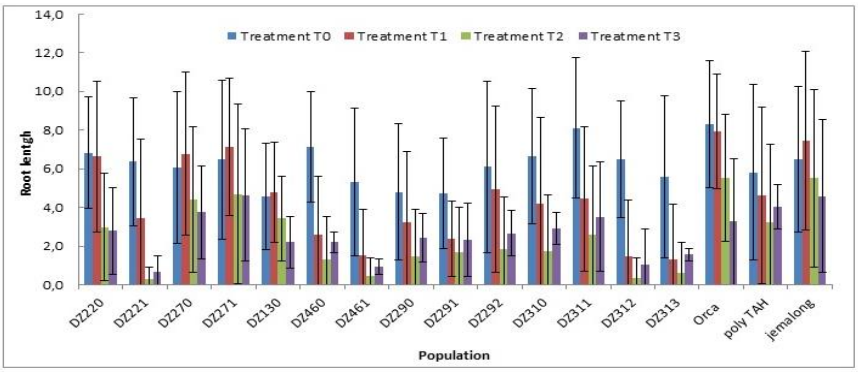


Fig 3. Root average length analysis of young seedlings.

Table 4. List of populations in the molecular characterization by the three SSR markers.

Experimental code and genotype name	Province and Origin	Latitude	Longitude	Altitude
*M.polymorphaDZ220**	Sidi bel-Abbes (Mekdera)	35.435594°	-0.445364°	470 m
*M.polymorphaDZ221**	Sidi bel-abbes (Ain elberde)	35.351636°	-0.518099°	469 m
*M.polymorphaDZ270**	Mostaganem (Oued chlef)	36.033809°	0.140448°	10 m
*M.polymorphaDZ271**	Mostaganem (Oued chlef)	36.022057°	0.131885°	10 m
*M.polymorphaDZ130**	Tlemcen			842 m
*M.polymorphaDZ460**	Ain temouchent (Hammam Bouhdjar)	35.396089°	-0.976915°	250m
*M.polymorphaDZ461**	Ain temouchent (hassighala)	35.477608°	-1.030114°	250m
*M.polymorphaDZ290**	Mascara 1	35.394905°	0.100433°	570 m
*M.polymorphaDZ291**	Mascara 2	35.397966°	0.111558°	570 m
M.polymorphaDZ292	Mascara hassine	35.467321°	-0.007124°	570 m
*M.polymorphaDZ310**	Oran Marsaelhadjaj	35.785180°	-0.152624°	115 m
*M.polymorphaDZ311**	Oran Boutlellis	35.566989°	-0.914932°	110 m
*M.polymorphaDZ312**	Oran (grand sebkha)	35.559981°	-0.907742°	84 m
*M.polymorphaDZ313**	Oran (grand sebkha)	35.559974°	-0.891277°	84 m
*M.polymorpha poly TAH***	Algérie (I.T.G.C)	--	--	--
*M.TruncatulaJemalong***	France (INRA Mpt)	--	--	--
*M.Sativa(CV.Orca)***	France (INRA Lusignan)	--	--	--

*Prospected population, ** Reference plant.

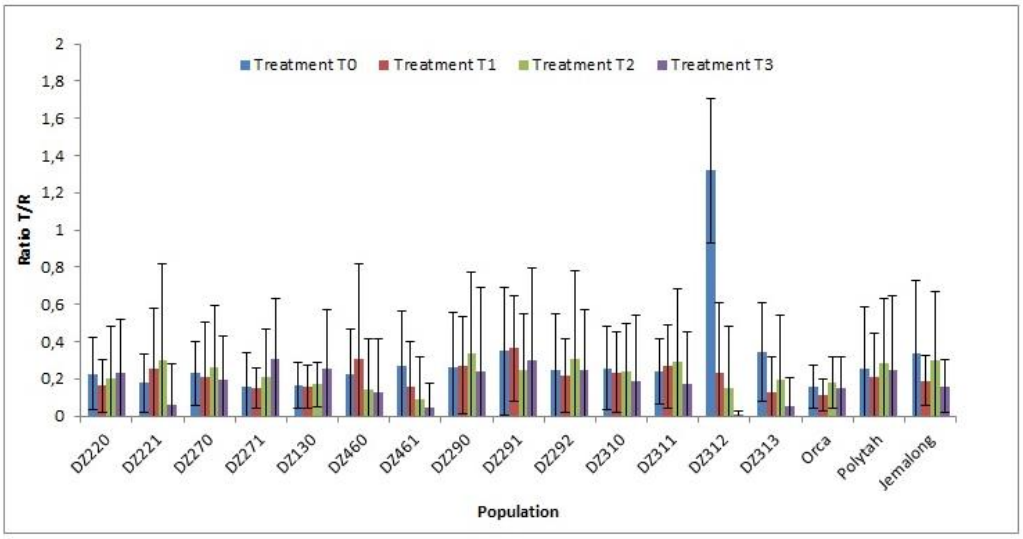


Fig 4. The T / R ratio of the control and the treated young seedlings.

Table 5. The tested primers for the three SSR markers.

Locus marker	Primer sequences (5'-3')	T(C°)	LG	Pattern of repetition	Expected size (pb)
FMT11*	L : GGCCCAACCACAATTTC	55C°	01	$(GA)_{16}$	100-300
MTIC 640	R : CATAACTTCCAATAACTGCCA				
MTIC 564	L : GCCGATGGTACTAATGTAGG	50C°	02	$(GA)_{13}$	100-300
MTIC 5641	R : AAATCTTGCTTGCTTCTCAG				
MTIC 297*	L : CTAAGCTTTGGCCATGTATC	50C°	04	$(TAC)_5$	115
MTIC 297	R : TGAAAATGAGTTTGACTGAGG				

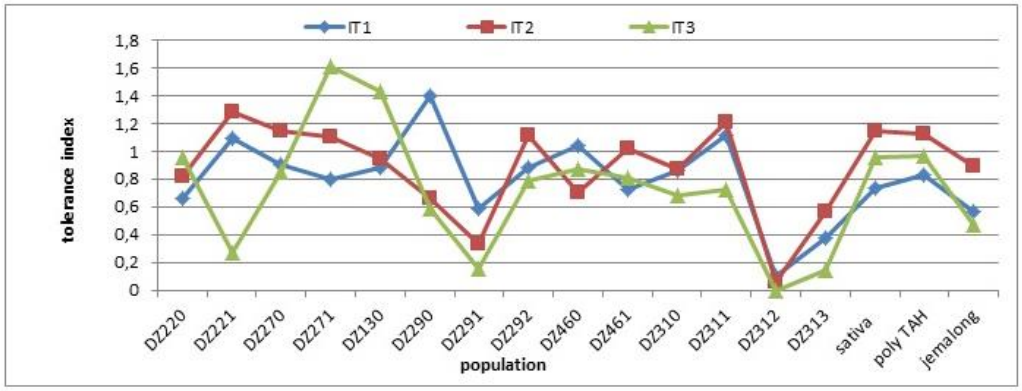


Fig 5. The tolerance index of young seedlings length under different salt concentrations in different populations.

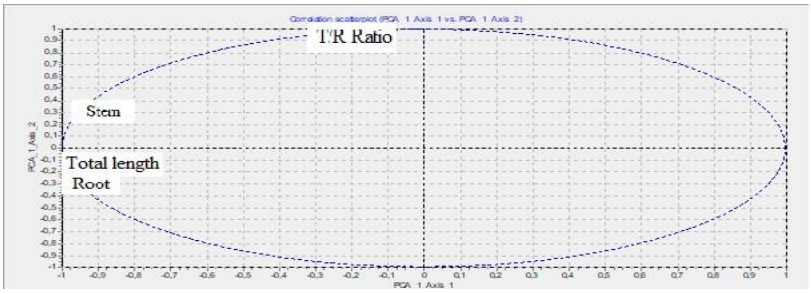
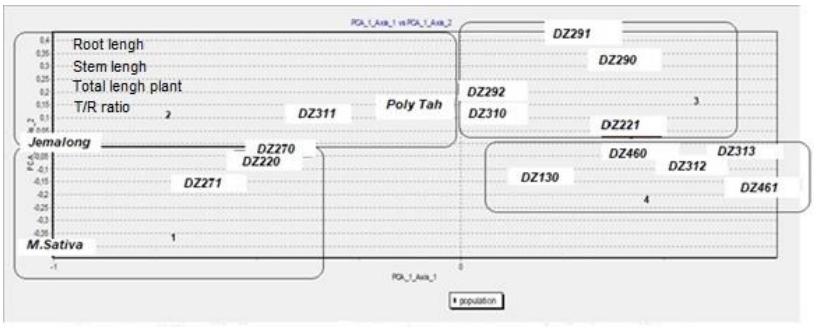


Fig 6. Influence of morphological characters on the two PCA factors (PCA1 and PCA2).



Cluster 1:*Medicago sativa*,DZ270,DZ271,DZ220; Cluster2 : Jemalong, DZ311, Poly Tah
Cluster 3: DZ290, DZ291,DZ292, DZ221,DZ310; Cluster4: DZ130, DZ312, DZ313,DZ460,DZ461

Fig 7. Graphical representation of all studied populations in plan 1-2 of a PCA.

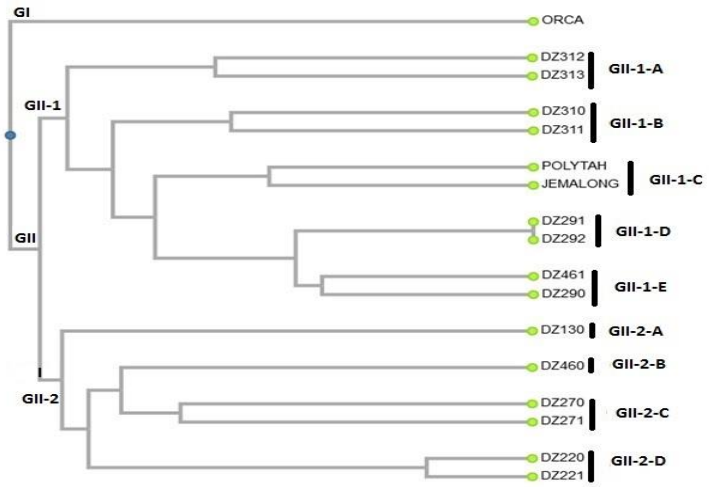


Fig 8. UPGMA dendrogram in different studied populations based on the three microsatellites.

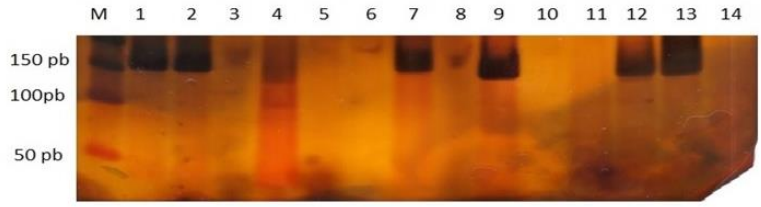


Fig 9. Example of SSR variation of the MTIC-564 locus in the two populations of *Medicago polymorpha* and in the four cultivars M. Marker leader (bp); 1-5: population DZ270 of five individuals, 6-10: population DZ271 of five individuals, 11: Polytah, 12: Poly serena, 13: Jemalong, 14: Orca.

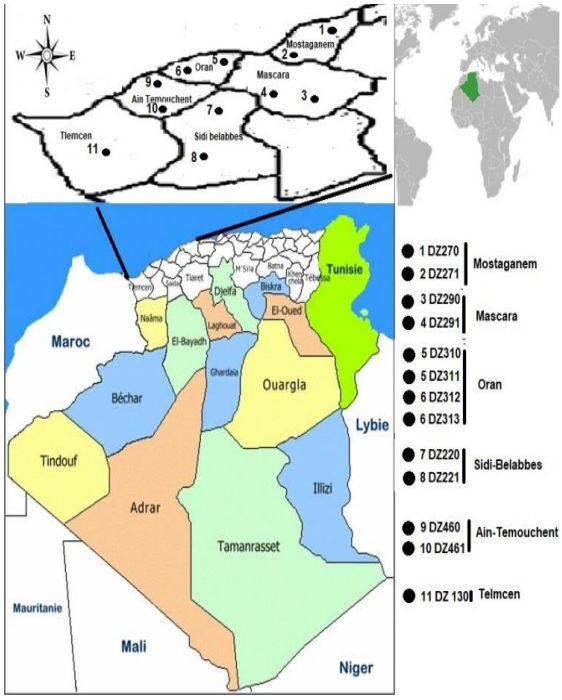


Fig 10. The location sites of the western Algerian collection of *Medicago polymorpha* species.

Among the detected alleles for the three microsatellites (FMT 11, MT564 and MC297), there are the presence and absence of certain alleles in the two extreme populations. The marker locus MTIC564 revealed the presence of a single allele (145pb) that is present in both the tolerant population and the sensitive population. In MTIC 297 locus, three alleles (164, 170 and 175pb) were detected, in which some were present in the tolerant population DZ221 (164pb). In the sensitive population DZ312, the presence of both alleles (170 and 175) is noted. On the other hand, in the third locus FMT11, three alleles (168, 172 and 185pb) were also detected in the two extreme populations. For the tolerant population, it presents two alleles (168 and 172pb). In the susceptible population, there is only one allele (185pb). In particular, the detected alleles in the tolerant population for both microsatellites MTIC297 (164pb) and FTM11 (168 and 172pb) can be considered as alleles-specific to salt stress tolerance.

Discussion

The results of this study show that saline stress exerts a depressive effect and a considerable reduction in the growth of the studied characters morphological for all populations of *Medicago polymorpha* species, as well as the two control species *M. Sativa* (Oraca) *and M. truncatula* (Jemalong). However, the rate of reduction differs according to the intensity of the exerted saline stress and to the degree of sensitivity or tolerance of each studied species. Some authors have demonstrated that both control species *M. Sativa* and *M. truncatula* are very tolerant to salinity, compared to the *M. polymorpha* (Friesen et al., 2014; Ammouri et al., 2016). Of all the morphological traits studied, stem length and root length appeared as most affected by the increase in salt stress. Comparable studies have demonstrated that application of salinity by increasing the concentration of NaCl results in a significant decrease in stem and root growth in different populations of *Medicago truncatula* (Arraouadi et al., 2012), *Medicago polymorpha* and *Ciliaris species* (Cherifi et al., 2016). The results also have demonstrated a low ratio for all studied populations for the majority of treatments. These data confirm that the part root is more resistant to salinity than the aerial part of the (DZ221) population. However, the stem/root ratio is increased in sensitive populations. The radicle part is more also sensitive than the stem in sensitive populations (DZ313). The tolerance indices (IT1, IT2 and IT3) and the discriminating stress level (T2), allow a high variability of expression, or the IT2 values make it possible to classify the populations of the *Medicago polymorpha* species from the most tolerant to the most sensitive to saline stress. Estimation of IT index in the determining extreme

populations has been used by previous studies under salt stress. For example, Ammouri (2015) studied two genotypes (Tru131 and Jemalong) of *Medicago truncatula* species. The ANOVA analysis revealed that the genotype and the treatment effect, as well as their interactions (genotype * treatment) are very significant, probably due to the genetic complexity of plant resistance to abiotic stress (salinity, drought and cold). The PCA applications revealed the confirmation that the traits (root, stem and plant length) are correlated with each other except for the T/R ratio trait, which depends on the tolerance of each genotype to salt stress. For the structuring of *Medicago polymorpha* species with the studied morphological characters, we can do the PCA analysis. It revealed four distributed homogeneous groups according to their geographical sites of origin except for a few populations which show marginal behaviour, both in *Medicago polymorpha* species and also in the two control species (*M. Truncatula and M. Sativa*). The application of correlation analysis between two or more morphological characters is an important indication of the improvement of the agronomic interest character such as salinity toleranceas it can cause simultaneous changes in other characteristics of the entire plant. Our results demonstrate that there are specific correlations between measured traits and both salt conditions (control T0 and treatment T1; T2; T3). This result is consistent with that reported by Arraouadi et al. (2012) and Badri et al. (2016) who demonstrated the existence of specific correlations between phenotypic parameters measured under saline stress. Although three microsatellites markers are considered to be very limited for the revelation and detection of a possible genetic diversity in the different populations of *Medicago polymorpha* species, but their use allowed us to detect very important polymorphism between the different genotypes of the populations *Medicago polymorpha* species. Also we have the ability to test and estimate the polymorphism index of each microsatellite. The different estimated indices showed a high level of polymorphism by using the three microsatellite markers, which revealed greater total genetic variability (Ht = 0.799) within the different studied populations of *Medicago polymorpha* species. The large contribution also noted high genetic diversity (Hs = 0.134) between populations. It could be explained by the high levels of autogamy in this species, and the index value (H0= 0) is much lower than the index value (He = 0.14). This result suggests the existence of a heterozygosity deficit that has also been observed in other autogamous species such as *Medicago truncatula* (Ellwood et al 2006). On the other hand, the contribution of interpopulation genetic diversity (DST = 0.842) to total genetic diversity, according to the calculated value of (Fst = 0.836) shows that the different studied populations present a very important differentiation according to the standards of Wright (1978). The calculated value of (Fis: 1) shows that all populations are incomplete fixation. The PIC values of the three examined microsatellites show a significant degree of polymorphism. The more its value tends towards 1, the more the microsatellite in question is polymorphic and conversational. Similar results using three microsatellites have already been reported for species of the same genus such as *Medicago sativa* which found a mean of 22.3 alleles and a very high PIC value with a mean of 0.90 (Cholastova and Dknotova, 2012). In the sense that the character of salinity tolerance is most often governed by a battery of genes (QTLs), we can suggest that these markers are probably linked to genes involved in salinity tolerance adaptation; and therefore, they can be used in the search for candidate genes linked to salinity tolerance QTLs. These can be used in breeding programs for salt-tolerant individuals. Besides, the molecular research allowed us to test the amplification transferability of the three microsatellites (MTIC564, MTIC 297 and MTIC 640) on different studied populations of the *Medicago polymorpha* species which were derived from the genome of the *Medicago truncatula* model species, and also for estimating their polymorphic informativity (PIC). The correlation of the biometric results with those molecular results shows a very important alternative for improving the agricultural lands which suffer from the problem of salinity. Lazerk et al. (2009) have demonstrated that these markers are linked to salinity resistance genes. Long-Xi et al. (2009) demonstrated the existence of a relationship between morphological markers and SSR markers for assessing genetic diversity in natural populations belonging to the species *Medicago sativa spp. Falcata*. Yahia et al. (2014) worked on the evaluation of adaptation to cold stress in a few ecotypes of different species of the Medicago genus using the SSR marker and were able to detect alleles that could be related to cold stress resistance QTLs.

Materials and Methods

Studied populations

A survey was conducted between September and October 2014 in ten different geographical sites in Western Algeria (Fig. 10). The pods of the harvested plants were identified using the IBPGR (1991) international standardized system for the genus *Medicago.* Their identification showed that the majority of them belonged to the species *Medicago polymorpha.*

Plant material, growth conditions and salt stress treatment

The first set consists of two annual species (*Medicago truncatula* and *Medicago polymorpha*) and one perennial species (*Medicago sativa*) belonging to a collection of plants from the Genetics and Plant Breeding Laboratory of the University of Oran, which are used as reference plants (Table 4). The second set represents 14 natural populations of the *Medicago polymorpha* species resulting from prospecting. All plants were tested to determine their behavior concerning four levels of saline treatment with a 0.8% agar solution as a control (T0=0, T1=68, T2=102 and T3=137mM) of sodium chloride (NaCl) (Ammouri and Fyad-Lamèche, 2012). Nine days after germination of the forty seeds for each population with five replicates for all treatments (10 seeds for each treatment). The experimental device is a block device, staggered randomly in time. The four parameters were measured, namely the length of the young plant, the length of the stem, the length of the root and the stem/root ratio. The degree of tolerance at different concentrations of NaCl is calculated relative to the control for all four characters. To determine the tolerance of one population relative to another, a tolerance index (IT) equal to the ratio of the value noted under stress on that off.

DNA extraction and agarose gel electrophoresis

Genomic DNA from five individuals per population was extracted from fresh young leaves (200 mg) using the standard CTAB extraction protocol (Doyle and Doyle 1990). The DNA concentration was determined using a 0.8%

agarose gel. The absence of smears at the freezing stage indicates the purity of the DNA.

Primers and SSR-PCR assays

The detection of population polymorphism has been performed using 3 primers. The details of SSR markers, their sequences and motif are given supplementary (Table 3). PCR reaction was performed in 20ul volume of PCR mixture, containing 100 ng genomic DNA, 1X *Taq* DNA polymerase buffer, 1,5 mM Mgcl2, 0,2mM dNTPs, 1U *Taq* DNA polymerase and 0,2 µM of each primer. The thermal profiling was set up with an initial denaturation temperature of 95°C for 05 min followed by the 35 cycles of denaturation (95°C for 60 s), annealing (55° or 50° for 45 s) and extension (72° for 60 s), ending with a final extension of 7 min at 72° and conserved at 4°C. PCR product for SSR primers screening was separated in 6.5 % polyacrylamide gels. The gels were stained for SSR band detection using the silver nitrate protocol according to Bassem et al. (1991).

Statistical analysis

The statistical study is carried out using the computer tool the statistical analysis System Statistica 6.1 version (Stat Soft, Inc France). Data were analysed by calculating mean and standard deviation values in different treatments. Differences between the treatment mean for each treatment were performed using two-way ANOVA. The relationship between the characters and the different studied populations with respect to saline stress was determined by Principal Component Analysis (PCA). Genetic diversity is estimated from the presence matrix (1) absence (0) of the polymorphic bands. Based on the similarity matrix, a dendrogram showing the genetic relations between the different populations was made using the Unweighted Pair Group Method using arithmetic average (UPGMA) and the Jaccard genetic similarity index (Garcia-Vallvé et al., 1999).

Analyzing data from the three microsatellites

The size of the amplified fragment was determined by comparing the migration distance of the amplified fragment relative to the molecular weight of the known size markers using the scale, 50 base pairs (bp) of DNA. Genetic analysis was conducted using Software Genetix4.05 (Belkhir et al., 1999). The Nei diversity (1987) indices were estimated for all populations studied (Table 4). Observed heterozygosity (Ho), Expected heterozygoty (He), Total genetic diversity (Ht), genetics population diversity (HS), genetic diversity among the population (DST) and genetic coefficient of differentiation (GST), were calculated from the allelic frequencies obtained at different loci. The indices F defined by Nei equivalent to those of Wright (1969) are computed from the allelic frequencies observed and under the assumptions of hardy-Wienberg using the following formulas: Fis = 1- Ho/Hs and Fst = 1- Hs/HT, with fixation indices (Fis), Inbreeding coefficient of an individual relative to the total population(Fit), Effect of sub populations compared to the total population (FST). The polymorphism information content (PIC) (Botstein et al., 1980; Anderson et al., 1993) is computed according to the formula: PIC = 1- ΣPij^2, where P_{ij} is the frequency of j^{th}allele of i^{th} locus, summed across all the alleles for the locus over all genotypes.

Conclusion

Generally, salt stress is a major factor limiting plant growth and productivity in many parts of the world. Researching and harnessing genetic diversity will help to solve this problem, but it would be much more effective if it has combined with the understanding of molecular tolerance mechanisms. We studied and evaluated the salt stress adaptation in the natural populations of the *Medicago polymorpha* species using four morphological characters (length of seedlings, stem, root and T/R ratio). Furthermore, various biometric parameter calculations of the four morphological characters allowed us to identify the two contrasting salt tolerant populations like DZ221 as tolerant and DZ312 as sensitive. We reveal that the three microsatellites (MTIC564, MTIC297 and FMT13) used in this study were highly polymorphic, and they are amplifiable by the genotype of all populations *Medicago polymorpha* species. In particular, the two microsatellites (FMT11 and MTIC297) revealed alleles in the tolerant population. They can be considered as indicators of genetic adaptation to salinity tolerance. As prospects, these results can be confirmed by the increase in the number of populations and in the number of SSR markers.

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